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Pathogenesis, pathology and chemotherapy of experimental Legionella pneumophila infection

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Pathogenesis, pathology and chemotherapy of experimental
Legionella pneumophila infection

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University of New Hampshire, 1989

PATHOGENESIS, PATHOLOGY AND CHEMOTHERAPY OF

EXPERIMENTAL LEGIONELLA PNEUMOPHILA INFECTION

BY

ARTHUR O. TZIANABOS

B.S. BOSTON COLLEGE, 1985

DISSERTATION

Submitted to the University of New Hampshire
in Partial Fulfillment of
the Requirements for the Degree of
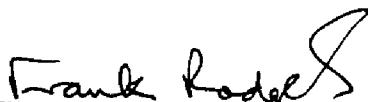
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in

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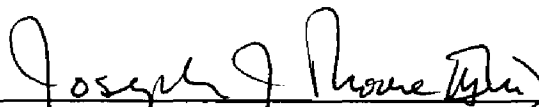This dissertation has been examined and approved.

Dissertation director, Dr. Frank G. Rodgers
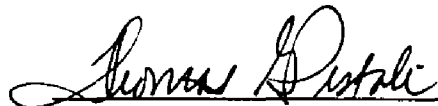Associate Professor of Microbiology

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TO MY PARENTS

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Abstract

PATHOGENESIS, PATHOLOGY AND CHEMOTHERAPY OF EXPERIMENTAL LEGIONELLA PNEUMOPHILA INFECTION

by

Arthur O. Tzianabos
University of New Hampshire, December, 1989

Legionella pneumophila is the causative agent of a severe, often fatal

pneumonic illness known as Legionnaires' disease. The mechanisms by which

L. pneumophila attaches to U937 (transformed human macrophage-like cells)

and to MRC-5 cells (semi-continuous human lung fibroblasts) were

investigated. Experimental parameters for adherence assays were established

prior to blocking studies designed to identify microbial adhesins and/or

eukaryotic receptors that mediate bacterial attachment to target cells. Results

from these studies indicated that a lectin-like component (s) associated with L.

pneumophila may be responsible, at least in part, for microbial adherence to

these eukaryotic host cells. Erythromycin is the drug of choice for the treatment

of clinical legionellosis; however, difficulties with this antibiotic have been

reported resulting in the need to seek alternative therapeutic regimens. In these

studies, the effect of clinically relevant antibiotics that inhibited bacterial cell

wall, protein and DNA synthesis of this pathogen was evaluated in vitro by

growth and viability studies as well as morphologically by negative stain,

scanning and thin-section electron microscopy. Of those tested, cefotaxime, an antibiotic of limited value in clinical trials, was most effective. The pathogenicity of L. pneumophila was assessed by LD_{50} and bacterial growth estimations in the chick embryo animal system in ovo. In addition, histopathological and electron microscopic examination of the cellular and sub-cellular pathology induced in the organs of embryos previously infected with 100 times the yolk sac (YS)LD_{50} of L. pneumophila was made as a prelude to chemotherapeutic treatment with a range of clinically putative antimicrobial agents. The promising new quinolone derivative, ciprofloxacin, was most effective in these trials. Results from these studies may be indicative of novel preventative and control measures for the therapy of human Legionnaires' disease.
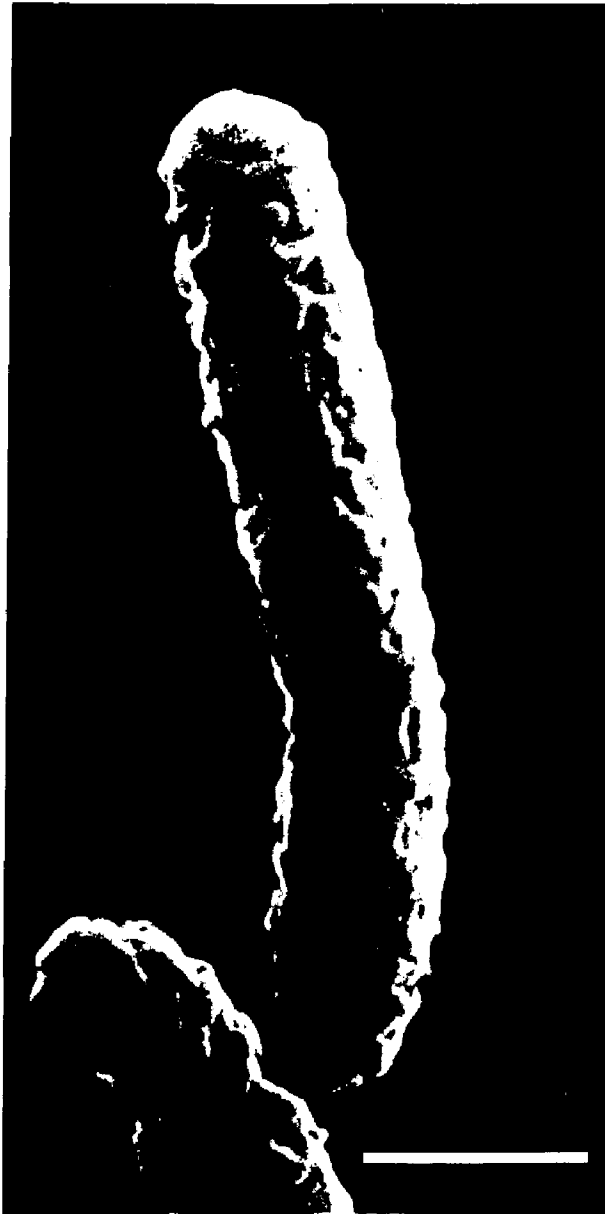
Legionella pneumophila, serogroup 1, strain Nottingham N_7. Scanning electron microscropy (SEM) preparation. Bar=0.5μm.

Section I

The Natural History of Legionnaires' Disease

1.1 Preface. Legionnaires' disease is a severe illness of humans which often occurs in spectacular outbreaks. Although an extensive data base concerning all aspects of legionellosis has been established since the outbreak in Philadelphia 13 years ago, the mechanisms by which the subsequently described causative agent, Legionella pneumophila, induces disease are not fully understood. This study examines the pathogenesis of this organism at the host, cellular and sub-cellular levels and defines the role of clinically relevant antibiotics in the disease process.

1.2 Historical perspective. The outbreak of a severe form of acute lobar pneumonia during an American Legion Convention held at the Bellevue Stratford Hotel in Philadelphia in July 1976 was the precipitating factor which led to the isolation and identification of this previously unrecognized human pathogen. During this epidemic, 29 of the 182 legionnaires who became ill, died. An additional 39 individuals who were in or passed by the hotel developed pneumonia. In all, 34 died from the disease or associated complications during this outbreak (49). The illness, eventually named Legionnaires' disease, took its name from the Philadelphia outbreak. Initially, a viral etiology was suggested due to the influenza-like symptoms manifested in

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these people. Indeed, influenza and other bacterial pneumonias are still common diagnostic considerations by clinicians presented with symptoms of *L. pneumophila* infection (99).

Failure to immediately isolate the causative agent following the Philadelphia epidemic caused the news media to heighten concerns about the presence of a new microbial killer. These embellished reports undoubtedly expedited the epidemiological and microbiological investigations following the outbreak and contributed to subsequent recognition of this important human pathogen. Although investigators at the Centers for Disease Control (CDC) in Atlanta announced the cause of the disease six months after the outbreak, they could not identify the mode of transmission but only pointed out that a common-source epidemic had occurred.

The inability to stain or grow the organism using conventional microbiological procedures proved the main problems in identifying the etiological agent of the disease. Dr. Joseph McDade at CDC finally isolated the organism using techniques established for the recovery and identification of rickettsiae. The yolk sacs of embryonated eggs were inoculated with lung samples from fatal cases and the bacteria were visualized by Gimenez stain which contains carbol fucshin rather than safranin. The latter fails to stain *Legionella* species and, as it is the usual component of the gram stain, offers explanation of the failure to visualize the agent initially (85). It was subsequently shown that the organism and the disease it caused were not new, and previously unresolved epidemics were retrospectively attributed to this

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organism. Among these was an outbreak of a febrile illness with a high attack rate that occurred in Pontiac, Mi. in 1968 and, due to its non-pneumonic form, was later called "Pontiac Fever" (59). The explanation for the variability of symptoms of Legionnaires' disease and Pontiac Fever is unknown. The spectrum of disease caused by this and related microorganisms is termed legionellosis.

After attempted cultivation on 17 commonly used bacteriological media, Feeley et al. (43) reported that Mueller-Hinton agar supplemented with 1% hemoglobin and 1% Isovitalex[R] supported sparse L. pneumophila growth. Feeley-Gorman agar, which contained added L-cysteine hydrochloride and soluble ferric pyrophosphate, was an improvement upon this medium (42). Finally, Edelstein (29) introduced a useful but expensive solid medium which contained α-ketoglutarate (BCYEα) that supported substantial bacterial growth after a 48-72 h incubation period at 37° C. This remains the medium of choice for the cultivation of legionellae in clinical and research laboratories.

Subsequent investigations have demonstrated that L. pneumophila is an environmental organism readily found in streams, lakes and potable water and when delivered in appropriate aerosolized form enters the respiratory tract of humans and replicates within alveolar macrophages of the lung (13, 111). Air conditioners, humidifiers, evaporative condensers, cooling towers and other water systems capable of generating aerosols are potential sources of disease. Human-to-human spread has never been recorded.

Experimental study of the pathogenic aspects of the organism has

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utilized a number of different laboratory animals, but to date none have fully

simulated all aspects of legionellosis. Guinea pigs, rats, mice, rabbits and

monkeys have been experimentally infected with L. pneumophila by a number

of different inoculation routes and these manifest some, but not all the

symptoms of clinical disease. Guinea pigs are used most extensively in such

studies, but restricted availability, high costs and legislative pressures have

forced researchers to seek alternative systems to study pathogenesis of the

organism.

1.3 The Organism:

1.3.1 Taxonomy and nomenclature. The classification and nomenclature

of this organism is difficult. Brenner et al. (15, 16) used DNA homology studies

as the primary tool to establish that the Legionnaires' disease bacterium of the

Philadelphia outbreak was a member of a new family (Legionellaceae) of the

genus Legionella and species pneumophila. Serological diversity within this

species is well established and numerous additional species, sub-species and

serogroups have been described since that time (Table 1.1).

The diversity of the genus has been noted (15) and attributed to the

failure of traditional biochemical, immunological and genotypic techniques to

yield corroborative results. Analysis of fatty acid and isoprenoid quinone

content have been useful, but do not accurately differentiate all species and are

not appropriate for use outside of a large research laboratory (15). The

determination of DNA relatedness has been and remains the main criterion for

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Table 1.1 Taxonomy and classification of the genus Legionella*

Species	Serogroup	Isolated From Humans	Environment
L. pneumophila[1]	1	Yes	Yes
	2	Yes	Yes
	3	Yes	Yes
	4	Yes	Yes
	5	Yes	Yes
	6	Yes	Yes
	7	Yes	Yes
	8	Yes	No
	9	Yes	Yes
	10	Yes	Yes
	11	Yes	No
	13	Yes	No
	14	Yes	No
L. micdadei[2]		Yes	Yes
L. bozemanii[3]	1	Yes	Yes
	2	Yes	No
L. dumoffii[3]		Yes	Yes
L. gormanii[3]		No	Yes
L. longbeachae	1	Yes	No
	2	Yes	No
L. jordanis		Yes	Yes
L. oakridgensis		No	Yes
L. wadsworthii		Yes	No
L. feelii	1	Yes	Yes
	2	Yes	No
L. sainthelensi		No	Yes
L. anisa		No	Yes
L. maceachernii		Yes	Yes
L. jamestowniensis		No	Yes
L. rubrilucens		No	Yes
L. erythra		No	Yes

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(continuation)

L. hackeliae	1	Yes	No
	2	Yes	No
L. spiritensis		No	Yes
L. parisiensis		No	Yes
L. cherrii		No	Yes
L. steigerwaltii		No	Yes
L. santicrucis		No	Yes
L. israelensis		No	Yes
L. birminghamensis		Yes	No
L. cincinnatiensis		Yes	No
L. moravica		No	Yes
L. brunensis		No	Yes
L. quinlivanii		No	Yes
L. tucsonensis		Yes	No

*Adapted from reference 136, Winn, W. C. Jr., Clin. Microbiol. Rev.

[1] L. pneumophila contains three subspecies: L. pneumophila subspecies pneumophila, L. pneumophila subspecies pascullei and L. pneumophila subspecies fraseri.

[2] Alternate genus name: Tatlockia.

[3] Alternate genus name: Fluoribacter.

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classification. However, practical identification of Legionella

species has depended on phenotypic characteristics which include gram

reaction as well as growth on BCYEα with added L-cysteine and the absence of

growth on blood agar or BCYEα without added L-cysteine.

The classification of Legionella species has been somewhat

controversial. Disagreement continues as to the degree of genetic divergence

that constitutes the establishment of a separate species as well as the disparity

with which researchers have applied criteria to make these distinctions. Brown

et al. (54) proposed alternate criteria for classification purposes in which two

new genera, Tatlockia and Fluoribacter, were created. These would contain

organisms otherwise called L. micdadei and the autofluorescing Legionella

species L. bozemanii, L. dumoffii and L. gormanii, respectively (Table 1.1).

However, this scheme has not found universal acceptance and most workers

accept the single genus terminology of Legionella within which all species are

located. It is clear that further work is necessary to establish uniform guidelines

for the classification of the legionellae and related species.

1.3.2 Morphological characteristics. Morphological properties of L.

pneumophila are shown in Table 1.2. Transmission electron microscopy of thin-

sectioned L. pneumophila cells has demonstrated the typical morphological

characteristics associated with gram-negative bacteria (105). L. pneumophila

organisms were shown to be pleomorphic gram-negative rods that measure 2

to 20 μm in length and 0.3 to 0.9 μm in width. Artificial media giving poor or

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Table 1.2 Morphological properties of L. pneumophila[1]

Short rods (from autopsied lung 0.6 x 1-2 μm, from agar 0.4 x 2-3 μm)

Tapered ends

Vacuolated surfaces, convoluted or smooth surfaces

Long forms (in vivo up to 20 μm in length, in vitro >50 μm)

Internal PBH granules-deformed in freezed fracture

Division by nonseptate binary fission

Flagella and fimbriae (pili) present

Blebs and granules on surface subunits, no evidence of acid polysaccharide capsule

F-1 antigen (serogroup specific) located at bacterial surfaces

Limiting cell envelope, "unit membrane", 10 nm thick

Peptidoglycan-like mucopeptide layer present after partial plasmolysis

Plasma membrane, "unit membrane", 10 nm thick

Rich in ribosomes, 25 nm diameter

[1]Adapted from reference 107, Rodgers, F.G., Legionellosis.

slow bacterial growth resulted in organisms with an elongated or filamentous form with tapered ends. Those rods visualized from autopsied lung were consistently shorter (21, 105).

The cytoplasm of each organism is enclosed within a double cell envelope of approximately 25 nm thickness with an electron-lucent zone or periplasm between the inner and outer membranes indicating, like the aquatic pseudomonads, an apparent absence of peptidoglycan (105). However, peptidoglycan and diaminopimelic acid have been demonstrated following partial plasmolysis and enzymatic digestion of the organism by electron microscopy and by other biochemical techniques (46, 105, 107, 108). The outer membranes of organisms contain substantial amounts of the bacterial lipopolysaccharide component, 2-keto-3-deoxyoctonate (46).

Cytoplasmic contents consist of evenly dispersed, electron-dense ribosomes, each of 25 nm diameter, arranged in small groups or polysomes and a fine skein of nuclear material (105, 110, 111). Electron-lucent vacuoles have been demonstrated as a regular feature of the cytoplasm of L. pneumophila whether present in human lung cells, guinea pig spleen, peritoneal macrophages or from bacteriological media (107). The reported size of these vacuoles varied from 30 to 200 nm (13) but larger forms up to 0.5 μm have been found (107). These structures have been confirmed as poly-β-hydroxybutyrate (PBH) granules by their appearance in freeze-fractured preparations (107, 108).

Negative-stain and scanning electron microscopy of L. pneumophila revealed bacterial cells with nonparallel sides, tapered ends and smooth undulating surfaces. Flagella and fimbriae (pili) have been demonstrated in vitro as surface appendages of L. pneumophila (110). The presence of small blebs and evaginations of the outer membrane have been noted on the surfaces of organisms by thin-section, scanning and freeze-fracture electron microscopy, but an extracellular mucopolysaccharide layer has not been detected. In addition, freeze-fracture electron microscopy of L. pneumophila demonstrated four short ridges enclosing the bacterial cytoplasm which corresponded to the double-unit membrane seen in thin-sectioned material (107, 108). Fractured aspects of the peptidoglycan were not evident in the intramembranous region of bacilli even after partial plasmolysis.

Poly-β-hydroxybutyrate granules were surrounded by a non-fracturable membrane evident as a single ridge in freeze-fracture preparations and confirmed reports of electron-lucent vacuoles in thin-sections. In keeping with the nature of PBH granules, contents of the granules in L. pneumophila underwent plastic deformation as an artifact of the fracture process and were evident as long, drawn out structures (107, 108).

1.3.3 Biochemical properties. L. pneumophila is a non-spore forming, aerobic, non-acid fast organism. All species of the genus demonstrate weak catalase and peroxidase activity. L. pneumophila also expresses gelatinase, phospholipase and β-lactamase as well as the ability to hydrolyze starch. Many

reports describe a number of exoproteases produced by the organism and these degrade a wide variety of proteins (10, 79). Keen and Hoffman (79) have isolated and partially characterized an exoprotease of L. pneumophila that exhibited cytotoxic and hemolytic activity.

L. pneumophila contains an unusually large proportion of branched-chain fatty acids located in the cell wall (15) but reports demonstrating the presence of an extracellular acid polysaccharide layer by ruthenium red staining have been contradictory (66, 105). The organism has been shown to possess a high molecular weight antigen or lipopolysaccharide (LPS), termed the F-1 antigen (34, 76) which had endotoxic activity, inhibited serological reactions and was localized on the surface of the bacterium. The LPS of L. pneumophila serogroup 1 was found to be tightly bound to the major outer membrane protein (MOMP), reacted specifically with the sera of patients with L. pneumophila serogroup 1 infection and was distinctly different from that of most enteric bacteria (136).

The MOMP of L. pneumophila has a molecular weight of 24 to 29 kDa (18, 53, 67, 76, 89) and is expressed on the surface of the bacterium (34). Reports have suggested that the MOMP may be associated with LPS (67) and peptidoglycan (52). Gabay and Horwitz (52) showed that this molecule formed ion-permeable channels similar in function to those found in Escherichia coli upon exposure to lipid membranes. Butler et al. (18) suggested that the MOMP is part of a larger, four sub-unit 95 kDa complex.

Investigations have shown that amino acids are the major source of

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energy for growth (55, 80), although the specific amino acid requirements are as yet undetermined. However, it is generally agreed that arginine, threonine, methionine, serine, isoleucine, valine, and cysteine are necessary. In addition, several groups have demonstrated that L. pneumophila can be grown in chemically defined media composed of amino acids (55, 94). While purines, pyrimidines, vitamins and other growth factors are not required, it is known that this organism has an unusually high requirement for L-cysteine.

The Krebs cycle is the primary route for carbon assimilation and energy production for L. pneumophila (55, 68, 122). These studies have shown that all the enzymes of this cycle were present but those of the glyoxylate shunt were marginal or absent in cell-free extracts of this bacterium.

Legionellae do not appreciably catabolize carbohydrates. Early reports showed negative sugar fermentation results but subsequent studies indicated that the Entner-Douderoff and pentose phosphate pathways but not the Embden-Meyerof pathway functioned to a limited degree in glucose catabolism (134). However, the gluconeogenic anabolic enzymes of the Embden-Meyerof pathway were responsible for sugar synthesis (55).

L. pneumophila growth is enhanced by trace metals, most notably ferric iron, but this may not be an absolute requirement (102, 121). Other important metals include Cu, Mg, Mn, Co, Ca, Mo, Ni, V, and Zn. In addition, Reeves et al. (103) have reported the absence of phenolate or hydroxamate ferric-binding compounds in Legionella species. The function of charcoal and α-ketoglutarate in the growth media of this organism has been questioned. Some investigators

1 2

believe that these compounds neutralize oleic acid produced in the media upon autoclave sterilization while others have suggested that they may serve to scavenge toxic oxygen radicals released from the yeast extract present in the medium upon exposure to fluorescent light (69). However, the precise nature of this requirement has not been elucidated.

The electron transport chain of L. pneumophila is composed of cytochromes c, b, a and d types but the organization of the respiratory chain remains undetermined (68). The concentrations of these heme-containing proteins were much lower in Legionella compared with those of other aerobes such as Pseudomonas or Campylobacter. Researchers have concluded that the respiratory chain is quite complex in L. pneumophila but is punctuated by at least three terminal oxidases (68). The biochemical properties of L. pneumophila are summarized in Table 1.3.

1.3.4 Physico-chemical properties. The legionellae have an optimum pH range for growth of 6.7 to 6.9 in BCYEα but may grow in other media with pH values as low as 6.0 (47, 55). Clinical isolates have an optimal temperature range of 35 to 37º C but environmental isolates prefer an optimum of 30º C. Isolation of the legionellae from water at 50 to 65º C has been reported (47).

1.3.5 Genetics. Genetic analysis of L. pneumophila has proved difficult. The fastidious nature of the organism and lack of conventional genetic transfer systems for the legionellae have limited our knowledge in this area. Plasmids . have been found in L. pneumophila but have not been shown to contribute to or detract from the virulent properties of the organism. Recent efforts to clone the

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Table 1.3 Biochemical properties of L. pneumophila

-non-spore forming
-aerobic
-non-acid fast
-motile (most strains)
-high proportion of branched chain fatty acids in cell wall
-amino acids as carbon and energy sources

-oxidase (+/-)
-catalase (+)
-peroxidase (+)
-nitrate reductase (-)
-urease (-)
-hippurate hydrolysis (-, most strains)
-gelatinase (-)
-B-lactamase (+)
-exoproteases (+)

genes for Legionella antigens in E. coli have been successful (27, 37, 38). In addition, Cianciotto et al. (23) were able to render a previously virulent strain of L. pneumophila non-infectious for cell cultures through the genetic deletion of a 24 kDa surface protein. Studies such as these may help further our knowledge concerning the pathogenic mechanisms of this organism.

1.3.6 Environmental aspects. The Legionellaceae are ubiquitous in natural aquatic habitats and man-made water systems. L. pneumophila has been isolated from lakes, streams, rivers, cooling water, hot water and potable water systems (48, 86). There is conclusive epidemiological evidence that the generation of aerosols from domestic and industrial air-conditioners, evaporative condensers, cooling, hot water and other aquatic systems which release micron-sized water droplets into the air serve as potential sources for legionellosis (86). An appropriate infectious dose of L. pneumophila may be delivered by aerosolized droplets 5 to 15 μm in diameter which can readily enter the nasopharynx and penetrate alveolar airspaces of the lung.

L. pneumophila has been primarily isolated from warm or thermally polluted freshwater systems. Although the organism has been found in a wide variety of aquatic niches, it is estimated to comprise a very small portion of the total bacterial population in the aquatic environment.

The association of Legionella with blue-green algae and freshwater protozoans including free-living amoebae Acanthamoeba, Tetrahymena and Cyclidium species has been demonstrated (3, 113, 124). Such interactions have been hypothesized to provide legionellae with an abundance of nutritional

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support and a host in which microbial numbers can be amplified. Evidence presented by Berendt (11) suggested that the survival of L. pneumophila in an aerosolized form was enhanced by its association with various species of the blue-green algae, Fischerella, and that dissemination away from the source of a generated aerosol may be aided by particular algal components. Results from other studies (127) indicated that legionellae may be carried in aerosolized form in cooling tower plumes 20 to 50 kilometers downwind and illustrated the potential danger posed by this pathogen.

The eradication of legionellae from water systems is rarely possible and studies should center on determining acceptable microbial densities for the organism. Effective biocidal treatments are being investigated for use as control and preventative measures. The most effective potable water treatments have been shown to be hyperchlorination, especially of hot water supplies and heating to above 50° C. Acceptable treatments for non-potable water included chlorination, use of quaternary ammonium compounds and halamines (118).

1.4 The Disease

1.4.1 Epidemiology. Legionellosis can exist in two forms: the severe pneumonic type with associated high fever known as Legionnaires' disease and the non-pneumonic form known as Pontiac Fever. Table 1.4 lists the symptoms for these conditions. The disease can occur as epidemics, sporadic cases, or nosocomial infections. Epidemic legionellosis has been documented in many countries including the United States, Great Britain, Spain, The Netherlands, Germany, Sweden, France, Canada and Italy and shows an

Table 1.4 Comparison of symptoms of patients with Legionnaires' disease or Pontiac Fever.

Symptom	Legionnaires' Disease	Pontiac Fever
Cough	Yes	Yes
Sputum production	Yes	No
Dyspnea	Yes	No
Hemoptysis	Yes	No
Myalgias	Yes	Yes
Upper respiratory symptoms	Yes	No
Headache	Yes	Yes
Confusion	Yes	Yes
Nausea or vomiting	Yes	Yes
Diarrhea	Yes	Yes
Abdominal pain	Yes	No
Fever	Yes	Yes

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increase in incidence during the summer months of July, August and

September (Figure 1.1). L. pneumophila serogroup 1 is responsible for 85% of

reported cases of legionellosis while L. micdadei causes approximately 6%

(136). A number of species of Legionella have been isolated from human and

environmental sources. The most recognized form of legionellosis,

Legionnaires' disease, has an incubation period of 2 to 10 days with an attack

rate of 1 to 5% and a case-fatality ratio of 0 to 40% (84, 139). It has been

estimated to be responsible for 1 to 29% of community acquired pneumonia.

Assessments of this nature have been difficult due to the environmental nature

of Legionella and the difficulty in establishing an etiology for many undiagnosed

pneumonias. Table 1.5 illustrates the clinical presentation of legionellosis.

The disease exhibits a predilection for immunocompromised individuals.

The risk of development of Legionnaires' disease increases in the elderly and

in those who excessively smoke and/or drink alcoholic beverages. In addition,

underlying illnesses such as diabetes mellitus, congestive heart failure,

malignancy, obstructive pulmonary disease or use of immunosuppressive

medication may increase susceptibility to this opportunistic pathogen.

It has been demonstrated that airborne transmission of aerosolized

organisms is the mechanism by which the organism and hence the disease

disseminates, but identification of pin-point sources of the infection in epidemic

legionellosis are often difficult. Investigation of the Philadelphia outbreak did

not conclusively demonstrate the source of the epidemic, but studies showed

that attendance in a 14th floor hospitality room of the Bellevue-Stratford Hotel
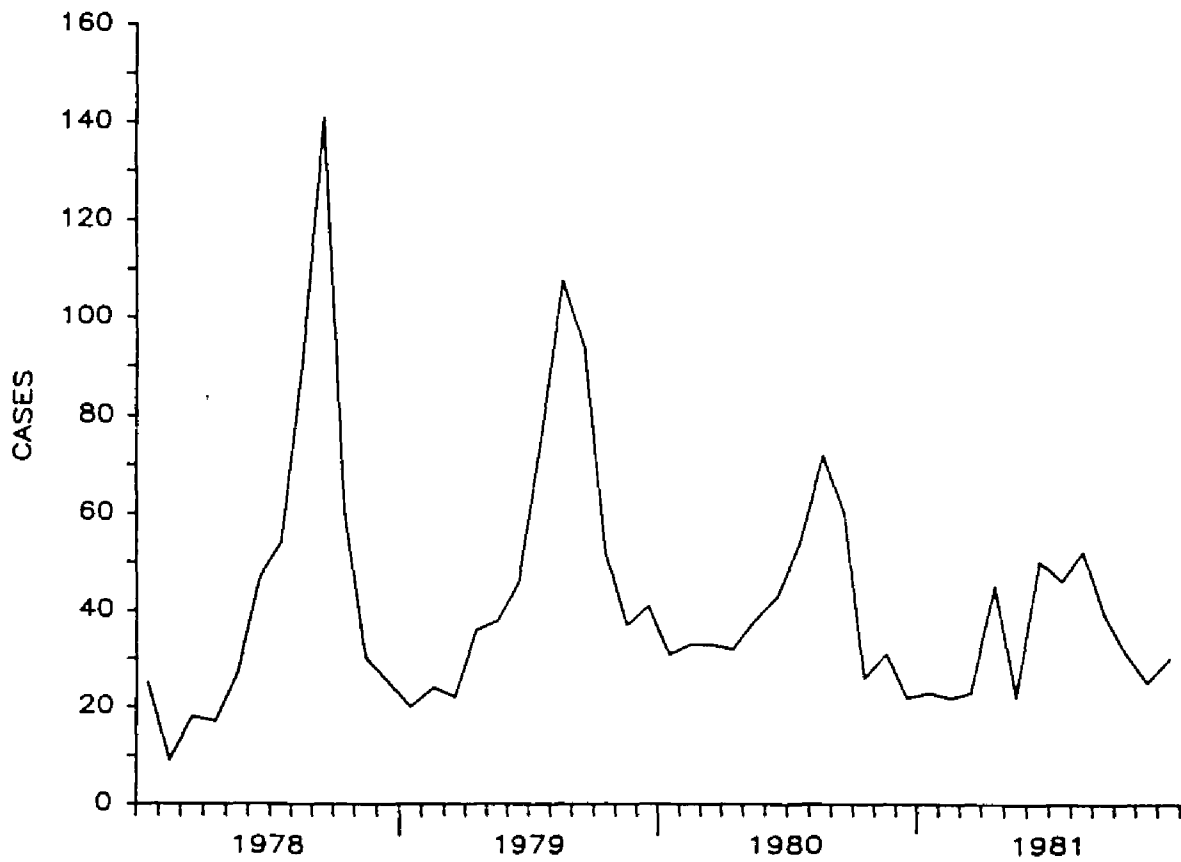
Figure 1.1 Average incidence of Legionnaires' disease cases reported to the CDC from 1978 to 1981. Note higher incidence during the summer months of each year. Reprinted with permission from Legionellosis, S. M. Katz (ed.), copyright CRC Press, Inc. Boca Raton, Fl.

Table 1.5   Clinical presentation of legionellosis

Two forms: Legionnaires' disease (LD) and Pontiac Fever (PF)

Genotypically and phenotypically identical

	LD	PF
Named for:	Philadelphia outbreak	Pontiac out break
Attack rate:	1-5%	95%
Incubation period	2-10 days	1-2 days
Symptoms:	Pneumonia	Non-pneumonic
Other organs affected:	Kidneys, G.I. tract, CNS	None
Case:Fatality rate	Variable 0-40%	0%

may have been a significant risk factor. However, the development of illness in people who never entered the hotel but were in close proximity was suggestive of airborne transmission. That evaporative condensers and air handling equipment have been identified as the source of other outbreaks further substantiated this hypothesis (84, 86).

The first reported case of Pontiac Fever was recognized retrospectively following the events of the Philadelphia epidemic. The Pontiac Fever outbreak occurred in July 1968 in a health department in Pontiac, Mi. and this episode provided significant evidence of airborne transmission of organisms from a evaporative condenser (59). Between the time that the first illness was reported until two days later, 89 of the facility's 100 employees and 15 visitors became sick. All cases had no common exposures outside the building and people working in all areas of the building were exposed. However, attack rates were higher in persons exposed during the morning than in people newly exposed in the afternoon suggesting increased risk after the air-conditioning system was first turned on each day. Subsequent investigation revealed that the central air-conditioning unit for the building was contaminated by an adjacent evaporative condenser.

Similar instances have been documented where burst exposure of individuals to aerosols or air handling equipment contaminated with L. pneumophila have led to illness. Most notably, a study of recurrent Legionnaires' disease in a hotel in Spain revealed that members of married couples who were the first to shower in the morning had contracted

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legionellosis in five of six cases (5). These data may indicate that inoculum size of L. pneumophila is a contributing factor to the initiation of disease.

Sporadic cases of legionellosis outnumber epidemic cases by a two to one ratio. This ratio may even be higher given the probability that only 0.8 to 2% of these cases are reported each year. According to the surveillance system of the CDC, from the period of 1978 to 1981, sporadic cases had been reported in every one of the states of the USA, occurred primarily in the summer months and had an incidence ranging from less than 0.25 to more than 1.0 cases per 100,000 people (Figure 1.2). Sixty-six percent of cases were in persons greater than 50 years old (84).

Tobin et al. (125) were the first to suggest that hospital water systems other than cooling units could act as reservoirs for L. pneumophila. Subsequent investigations have substantiated that potable water is a potential source of nosocomial or hospital-acquired legionellosis (1). Environmental sampling in several hospitals has led to the isolation of L. pneumophila and other species from a variety of sources including shower heads, tap water, medical drills and respiratory therapy equipment (1, 4, 17). Reports vary as to the incidence of hospital-acquired Legionella infections but these range from 0 to as high as 30%. In general, investigators actively searching for Legionella as a cause of pneumonia tend to find it to a greater frequency.

The incidence of nosocomial Legionnaires' disease can be attributed to the ubiquity of the organism in hospitals and the presence of a susceptible population. Haley et al. (63) have found that length of stay and compromised
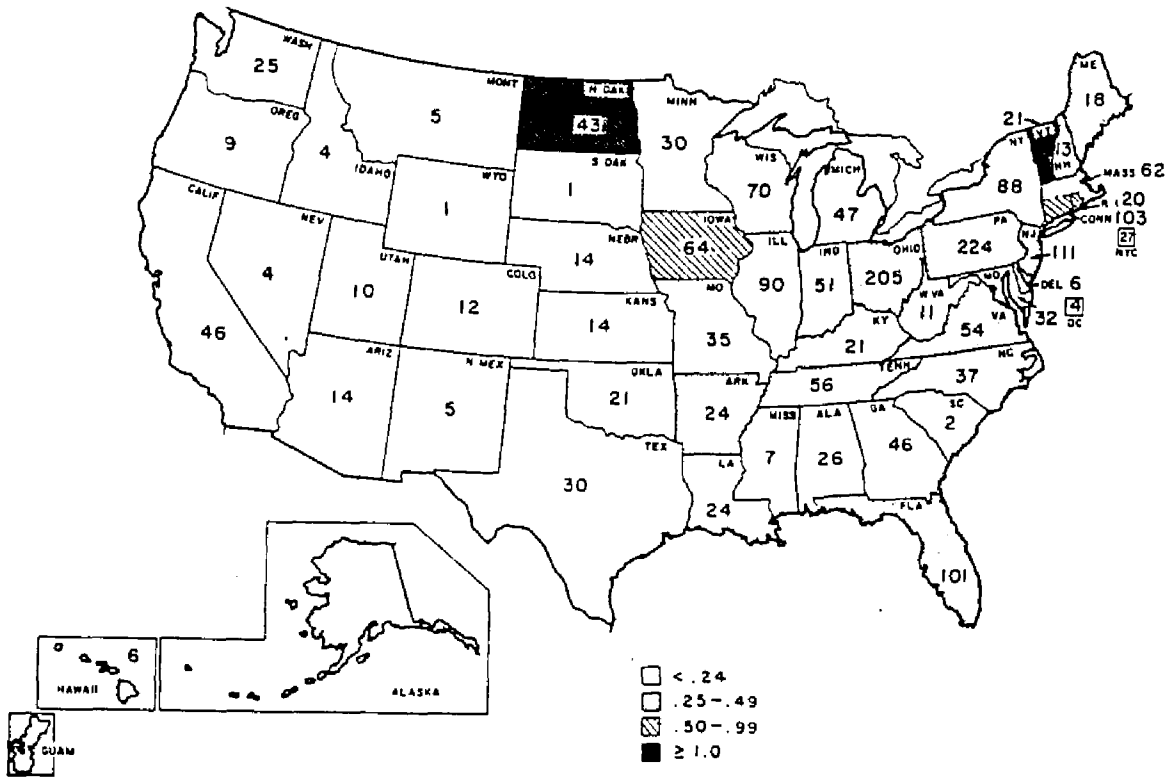
Figure 1.2 Reported sporadic cases of legionellosis to the CDC from 1978 to 1981 indicating average incidence per 100,000 individuals per state. Reprinted with permission from Legionellosis, S. M. Katz (ed.), copyright CRC Press, Inc. Boca Raton, Fl.

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immunity were the greatest risk factors for acquisition of disease by patients. Interestingly, the widespread presence of legionellae in water sources of hospitals does not correlate well with the incidence of disease. Explanations for this phenomenon include colonization of water lines with non-virulent legionellae, presence of sub-infective doses of organisms and variations in the susceptibility of patients. Further studies are needed to determine the pre-existing conditions within a hospital which are most likely responsible for the incidence of nosocomial Legionnaires' disease.

The most perplexing issues concerning the epidemiology of legionellosis is the manifestation of two separate illnesses, Legionnaires' disease and Pontiac Fever, from infection with the same organism. No differences can be attributed to the bacterium or to conditions surrounding outbreaks to explain discrepancies in symptoms, attack rate or incubation period. It is possible that either the infectious dose for each disease varies or alternatively that patient-related differences may be responsible. To date no evidence has accumulated to support either of these hypotheses. Further work is needed to determine whether this phenomenon is related to other pathogenic features exhibited by L. pneumophila.

1.4.2 Clinical features. Legionellosis is two to three times more common in men than in women and infections in children are rare. The disease presents as an atypical lobar pneumonia and has an incubation period of 2 to 10 days. The infection begins with acute fever, rigors, headache and myalgia. Upper respiratory tract symptoms are often absent and a non-productive dry cough

may appear three to four days later. On occasions when sputum is produced it is often non-purulent. Dyspnea and respiratory difficulties are common. Extrapulmonary symptoms such as gastroenteritis, fatigue and confusion are often reported and commonly lead to misdiagnoses by clinicians (see Tables 1.4 and 1.5). Legionellae are difficult to isolate from respiratory secretions but may be readily demonstrated in direct lung biopsy by immunofluorescence.

1.4.3 Pathology. X-ray radiographs of patients with Legionnaires' disease have been variable and do not uniquely identify _Legionella_ pneumonia. An average of three days occurs between the onset of symptoms and the appearance of radiographic pathology (40, 81). Deterioration of lung has been seen in two-thirds of cases and the most common features noted are the appearance of infiltrates and pleural effusions. Usually one lobe is affected but bilateral involvement has occurred in approximately one-quarter of reported cases (81). The resolution of radiographic problems has often taken longer than that of clinical recovery and may require an extended period of convalescence. Post-mortem lung samples have demonstrated the development of fibrino-purulent disease in one or both lungs with focal or lobar consolidation, edema, congestion and grey hepatization. In most cases, a fibrinous exudate is present in the pleural cavity. Macroscopic lesions are not always present but can include hemorrhagic areas and focal abscess formation (14).

Microscopic examination has revealed a severe inflammatory response within the distal airspaces of the lung. Terminal bronchioles and alveoli are

filled with proteinaceous material, fibrinous exudate and contain numerous macrophages and polymorphonuclear leukocytes (PMNLs) presenting in varying proportions. Proximal bronchi and bronchioles usually remain uninvolved throughout the disease process. Alveolar damage with hyaline membranes and desquamation of lining cells has been noted in lung biopsies (90, 136).

Shortly after the initial outbreak in 1976, Rodgers et al. (111) first demonstrated the presence of L. pneumophila within lung cells by electron microscopy and described ultrastructural degenerative changes associated with further dissemination of the organism into alveolar extracellular spaces. These findings were subsequently confirmed by other ultrastructural reports which described the intracellular nature of the organism (105, 106, 107).

Several investigators have demonstrated that patients with severe illness have developed extrathoracic pathology that involved the kidneys, heart, liver, spleen and brain. Instances of pyelonephritis, hepatic abscesses, cerebral myoglial reactions, pericarditis and endocarditis have been reported (4, 136). Degenerative changes have been found in these organs, but macroscopic lesions are uncommon.

Unusual complications such as dual infection with multiple species (26) or subtypes (78) of Legionella or other opportunistic pathogens have been reported. In addition, serologic confirmation of sub-clinical infection and diverse clinical presentation of disease following repeated exposure to the same source of L. pneumophila has been described (58, 136).

1.4.4 Pathogenesis. L. pneumophila is a facultative intracellular pathogen which replicates within alveolar macrophages of the lung. During the course of infection, the bacteria are actively phagocytosed by host cells and enter phagosomes associated with ribosomes and mitochondria. Phagocytosis of L. pneumophila by macrophages is independent of, but enhanced by, the presence of opsonizing antibody and complement. However, phagocytosis by PMNLs requires the presence of antibody, is not bactericidal and does not support the growth of L. pneumophila. Direct penetration of bacteria into eukaryotic cells has been suggested but uptake of L. pneumophila by guinea pig macrophages has been shown to occur by a conventional phagocytic process (36).

Studies have shown that macrophages exhibited an oxidative burst upon internalization of virulent L. pneumophila but subsequent events such as phagolysosomal fusion and acidification of the phagosome were inhibited (71, 73). Rapid intracellular multiplication of L. pneumophila was followed by cellular lysis with release of organisms and continued spread to adjacent host cells. Further spread of the organism via the bloodstream has been described and may explain the extrapulmonary pathology observed during advanced stages of the disease process (31, 105, 136).

Although no single microbial feature has been shown to confer virulence on L. pneumophila , a number of bacterial products have been implicated as potential factors which contribute to its pathogenicity. Release of toxins which neutralize the bactericidal pathways of phagocytes (50) and induce necrotizing

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inflammatory and cytolytic responses in human tissues (6, 10) may be the most important pathogenic aspects of the legionellae.

1.4.5 Diagnosis. Difficulties involved in the diagnosis of L. pneumophila infections are due primarily to the similarity between symptoms of Legionella infections with those of other bacterial and viral pneumonias and the non-pathognomic radiographic appearance of the disease. In addition, failure to stain the organism in clinical samples further complicates positive identification of legionellae as the causative agent. These problems make it necessary to rely on culture, serology, and detection of bacterial nucleic acid or antigen as the main methods for the diagnosis of Legionnaires' disease. Table 1.6 illustrates clinical and laboratory diagnostic indicators of legionellosis.

The mainstay of laboratory diagnosis remains culture on selective bacteriological media (31, 137). Growth on BCYEα with supplemented L-cysteine in the absence of growth on a blood agar media is indicative of L. pneumophila. Recovery of clinical specimens by culture will identify 50 to 80% of positive samples and this has been reported to have a specificity of 100% (137). Colonial morphology is distinct and requires two to five days for positive identification. Once isolated, confirmation of these tests should be made by a gram stain in which carbol fucshin is substituted for safranin as the counter-stain along with immunofluorescence. Figure 1.3 illustrates the rationale for determining the laboratory detection of L. pneumophila.

The development of a variety of serological tests including indirect immunofluorescence assays (IFA) has been useful in the identification of

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Table 1.6   Diagnosis of Legionnaires' Disease

Clinical diagnosis
Onset
fever
rigors
headaches
weakness
gastrointestinal abnormalities

Acute phase
unremitting fever
radiographic abnormalities
non-productive cough
nervous system manifestations
dyspnea

Laboratory diagnosis
Organism detection methods (culture, immunofluorescence, nucleic acid probes, etc.)
Slightly elevated white blood cell count
hyponatremia
proteinuria
hematuria

Identification Scheme for L. pneumophila

Growth on BCYEα of gram-negative bacillus

No ———————————————— Yes

Not L. pneumophila   Potential L. pneumophila

Growth on BCYEα without L-cysteine
at 37 ° C in 3 days

Yes ———————————— No

Probably not L. pneumophila   Possible L. pneumophila

Growth on blood containing agar

Not L. pneumophila

Immunofluorescence or Agglutination

Positive ———————— Negative

Presumptive L. pneumophila   Probable L. pneumophila

Biochemical Analysis for confirmation

Figure 1.3. Flow scheme for the identification of L. pneumophila.

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Legionella antibody for diagnostic purposes. Although, IFA has a sensitivity of approximately 75% and a specificity of almost 100%, problems have arisen in its use for diagnosis of sporadic cases of Legionnaires' disease. This is due to the incidence of cross-reactivity reported with the use of L. pneumophila antibody and the inherent difficulty involved in the subjective interpretation of the assay. Detection of seroconversion to a four-fold titer by serological methods is useful, but yields retrospective results and should be interpreted with caution (4, 64, 137).

Direct observation of L. pneumophila antigen in clinical specimens by IFA has provided relatively rapid diagnoses but with low sensitivity. Problems due to reagent cross-reactivity with other bacteria and the environmental presence of the legionellae have lead to false-positive results (4, 64). Commercial nucleic acid probes are now available and exhibit better sensitivity than IFA, and although they are gaining widespread acceptance, further examination is needed before they replace IFA completely. Radioimmunoassay, latex agglutination, microagglutination, counter immuno-electrophoresis, hemagglutination and enzyme immunoassay techniques have been used or are currently being investigated for application as rapid, sensitive and specific diagnostic tools (64, 114).

1.4.6 Immunity. The immune response to Legionnaires' disease is not well understood. However, epidemiological evidence has suggested that susceptibility and resistance to Legionella infection are directly related to risk factors which usually immunocompromise the host. Advanced age, underlying

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disease, excessive cigarette smoking and immunosuppressive therapy have been shown to influence attack and fatality rates of Legionnaires' disease. Experimental studies have demonstrated that the cellular arm of the immune system may have a significant role in resistance to infection with L. pneumophila. Freidman et al. (51) demonstrated that lymphokine activated macrophages did not support the growth of ingested legionellae while Horwitz et al. (72) showed that non-specific activation of these cells also inhibited intracellular replication of Legionella. That untreated alveolar macrophages of mice did not support intracellular growth of this organism (140) further suggested that aspects of the bacteria-macrophage interaction have a central function in the immune response to legionellosis.

The role of humoral immunity has not been well-defined. Theoretically, the introduction of anti-L. pneumophila antibodies would facilitate opsonization of bacteria with detrimental results to the host. However, animal studies have demonstrated that passive transfer of antibody against L. pneumophila is effective against subsequent challenge with whole organisms (104, 136).

It has been difficult to establish whether individuals who have contracted legionellosis are susceptible to re-infection. Researchers have shown that immunization of guinea pigs with killed antigen is protective in some cases, but not in others (7, 33). Winn and Davis (136) were able to protect guinea pigs for one month after infection by aerosol or intratracheal route with a sub-lethal inoculum of L. pneumophila.

Human defenses against legionellosis most likely involve synergistic action of the cellular and humoral immune system. Studies that have elucidated a

defense system in animals have demonstrated limited bacterial killing or bacteriostasis of L. pneumophila. Further work is needed to fully delineate the mechanisms of human resistance to Legionnaires' disease.

1.4.7 Antimicrobial Therapy. Erythromycin is the current clinical drug of choice for legionellosis. Studies have shown that the clinical reaction to this antibiotic may range from an immediate response to no therapeutic results at all. Relapse of infection and development of severe phlebitis with intravenous use of erythromycin have been reported (62). Rifampicin is generally considered the most effective drug in vitro and has been used effectively in combined clinical therapy with erythromycin, but can not be used alone due to the high frequency with which bacterial resistance is induced. Consequently, alternative antimicrobial therapeutic regimens are being sought for the treatment of clinical legionellosis.

L. pneumophila is sensitive to a wide variety of antibiotics in vitro (30, 60, 123). However, these sensitivity levels are not always reflected in clinical trials. Antimicrobial agents with an effective minimum inhibitory concentration (MIC) such as the aminoglycosides, cephalosporins and penicillins are reported as ineffective in the treatment of human Legionnaires' disease (82) while others such as erythromycin, rifampicin and the tetracyclines have an effective role in therapy (2). Discrepancy between experimental and clinical trials may be explained by the inability of various antibiotics to penetrate host cell membranes and reach intracellular bacteria. Indeed, it has been reported that ciprofloxacin, erythromycin and rifampicin (65, 130) inhibit intracellular growth of L. pneumophila in cell cultures, while the β-lactam antibiotics such as ampicillin, cefoxitin and cefotaxime do not (142).

Of the new antibiotics examined, the quinolones have shown great promise in vitro and in animal studies (44, 45, 60, 128, 129). Ciprofloxacin, the best studied compound of this group, is a broad spectrum antibiotic which offers exceptional oral bioavailability and achieves high tissue concentrations. It is well-tolerated by patients and has been used successfully in the clinical treatment of various urinary and respiratory tract infections. Results from animal studies indicated that the efficacy of ciprofloxacin is equal to or greater than that of erythromycin and rifampicin in treatment of experimental Legionella infections (44, 45, 128, 129). Further studies are in progress to develop practical in vivo screening methods for the evaluation of new antimicrobial agents for treatment of clinical Legionnaires' disease.

Despite the massive accumulation of data concerning the nature of L. pneumophila since its initial isolation and identification following the epidemic in Philadelphia, Legionnaires' disease remains a major cause of pneumonia in humans. Enhanced awareness of this syndrome and aggressive therapeutic regimens have helped in the maintenance and treatment of legionellosis, but further studies are needed to establish prophylactic or preventative measures especially for "at risk groups". Elucidation of the pathogenic mechanisms of the organism and the role chemotherapeutic agents play in the treatment of disease as well as the investigation of the mechanisms of attachment and penetration used by L. pneumophila to initiate the disease process may lead to new developments in the control of this important human disease.

2. Hypothesis and Specific aims.

L. pneumophila is the causative agent of a fatal form of pneumonia known as Legionnaires' disease. The organism is a facultative intracellular pathogen which attaches to, penetrates and replicates within alveolar macrophages of the lung. Continued growth and spread of L. pneumophila leads to the presentation of well-defined clinical features as well as to extensive pulmonary and extrapulmonary pathology. The mechanisms by which L. pneumophila recognizes and attaches to host cells are crucial to the initiation of disease in a susceptible host. The process of binding was investigated using human cells in culture in order to elucidate these earliest stages of microbial infection. It is postulated that determination of these adherence events will lead to better understanding of the nature of this infectious disease and to the development of more effective control measures. Of the present therapeutic regimens, erythromycin is the preferred drug for treatment of Legionnaires' disease. However, patients in the advanced stages of severe illness often respond poorly to such antimicrobial treatment. The in vitro activity of established antibiotics along with novel, potentially useful drugs were evaluated against the organism per se to define the kinetics and mode of action of these antimicrobial agents. Furthermore, the in vivo pathogenesis and pathology induced in experimental L. pneumophila infection in the chick embryo were

examined and the ability of clinically proven or potentially useful antibiotics to control or ameliorate the disease process was investigated in this animal system. From the results of these combined studies it is anticipated that novel preventative or control measures may be forthcoming for the treatment of Legionnaires' disease.

Specific aims of this project were:

1) to establish the experimental parameters leading to the characterization of Legionella pneumophila adherence factors by using the human cell lines U937 (macrophage-like) and MRC-5 (lung fibroblast) to partially define these adhesins;

2) to elucidate the in vitro activity of selected antibiotics on the growth and morphology of Legionella pneumophila;

3) to delineate the cellular and sub-cellular pathology induced in the chick embryo following experimental infection with Legionella pneumophila and to define the pathogenesis of infection in this system;

4) to examine the role of antimicrobial agents in the disease and recovery processes.

Section III

Partial Characterization of Legionella pneumophila Adherence to Host Cells

3.1 Abstract

The mechanisms by which the facultative intracellular pathogen, Legionella pneumophila, attached to macrophage-like (U937) and human embryo lung (MRC-5) cells in vitro was examined. Bacteria and host cell surfaces were modified or treated with degradative enzymes, detergents, fixatives, lectins, oxidizing chemicals, and sugars prior to adherence assays in order to elucidate putative bacterial adhesins and eukaryotic receptors responsible for mediating specific bacterial binding to these cell lines. Bacterial attachment to host cells was determined by viable count and indirect immunofluorescence assay methods. Results from these studies suggested that one or more lectin-like structures on the surface of L. pneumophila organisms may potentiate the specific recognition of eukaryotic host cells.

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3.2 Introduction

The specific recognition of host cells by pathogenic bacteria forms a necessary prelude to the initial stages of the disease state (8). Duguid (28) first coined the term "adhesin" in 1959 to describe the adhesive structures on bacteria which facilitate their ability to attach to susceptible eukaryotic cells. This work remained largely unnoticed until Gibbons (56) reported on the adherence of bacteria to specific areas of the oral cavity. Since that time, numerous and extensive investigations (9, 25, 39, 41, 92, 93, 95, 96, 97, 98, 100, 117, 119, 120, 126, 131, 132) concerning the interaction of pathogenic organisms such as Escherichia coli, Neisseria gonorrhoeae, Vibrio cholerae and Streptococcus pyogenes with host cells have increased our understanding of how bacteria initiate disease. These reports described experiments in which the in situ blocking, inhibition or enzyme degradation of putative bacterial adhesins and eukaryotic receptors were designed to identify these structures and characterize the nature of microbial adherence to host cell surfaces. Further studies demonstrating the inhibition of binding by these isolated bacterial components/adhesins or by adhesin/receptor specific antibody provided additional evidence that these molecules were responsible for bacterial attachment. Although a number of these structures have been implicated by these methods, pili are the best studied (98, 119) and have been shown for a number of bacterial species to act sequentially or concomitantly with other attachment factors (39, 131) during the binding process.

The elucidation of adhesins and their receptors has led to the

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development of novel strategies for the prevention and control of infectious diseases. Prophylactic or post-infection treatment with anti-adherence vaccines and purified adhesin/receptor or adhesin/receptor analogues used in a competitive fashion have yielded encouraging clinical results (8). In addition, studies have indicated that sublethal doses of antibiotics may alter the ability of some bacteria to adhere to host cell surfaces (22).

L. pneumophila is a facultative intracellular pathogen which replicates within alveolar macrophages in clinical disease but has been shown to multiply within a wide variety of eukaryotic cells in vitro (36, 74, 75, 91, 138). The preliminary steps involved in the attachment of this organism to potential host cells prior to invasion have not been elucidated. However, L. pneumophila has been shown to possess pili, fimbriae and flagella (108, 110) on their surface and these, as pointed out by Rodgers (106) and Figure 3.1, may potentiate binding by bridging the gap between the negatively charged surfaces of the invading pathogen and host target cells.

Oldham and Rodgers (91) studied the interaction of L. pneumophila with MRC-5 cells, a human embryonic lung cell line, both quantitatively (viable counts following eukaryotic cell lysis) as well as qualitatively (by electron microscopy). Attachment of the legionellae to cells was followed by a thickening of eukaryotic cell membranes and the proliferation of microvilli which led to bacterial endocytosis by a process which they termed "bacteriopexis". More recently, Cianciotto et al. (23, 37) used U937 cells, a macrophage-like, human histiocytic lymphoma cell line, as a cellular model of infection and

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suggested that a 24-kDa surface protein of L. pneumophila potentiated

infectivity and subsequent intracellular replication. In addition, because similar

results with explanted human alveolar macrophages were obtained, these

investigators have demonstrated the relevance of this transformed cell line in

the study of L. pneumophila pathogenesis in vitro.

Patients with systemic Legionnaires' disease often respond poorly to

antibiotic therapy. Vaccines prepared to stimulate antibody production against

whole L. pneumophila organisms lead to avid opsonophagocytosis by the

"preferred" host cells (macrophages) and may prove counterproductive to

therapy. The objective of this study was to conduct preliminary studies on the

attachment of L. pneumophila to U937 and MRC-5 cells as a prerequisite to

infection at the cellular level. Specific goals included the development and

establishment of the experimental parameters for adherence assays as well as

the partial characterization of bacterial "adhesins" and eukaryotic receptors

involved in the "recognition" process. Fixatives, lectins, detergents, oxidizing

chemicals, degradative enzymes and sugars were used to modify either

bacterial or eukaryotic cell surfaces. Following these treatments, L.

pneumophila was added to the test cells and adherence assay experiments

performed. Viable counts of organisms (following eukaryotic cell lysis) and

immunofluorescence assays (IFA) (for direct counts of labelled bacteria) were

done and the number of bound organisms following adherence was

determined. Percent inhibition of binding was ascertained by direct comparison

of results from treated and untreated control trials.

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Due to the complexity of the bacterial and host-cell membranes, data derived from studies of this nature are often hard to interpret and the experimental procedures often lead to contradictory results making definitive adherence criteria difficult to elucidate (8, 70, 120). For these reasons, researchers have based their conclusions upon well-controlled, stringently analyzed experiments. It has been generally accepted that only those experimental treatments that inhibited bacterial adherence at a level equal to or greater than 50% of untreated controls were significant. In the present study, similar inhibition levels were used to measure the experimental significance of treatments for the binding of L. pneumophila to U937 or MRC-5 cells. In addition, because quantitative measurements of bacteria binding are often difficult to assess accurately, two different types of assay methods were used, and the results cross-referenced, to evaulate bacterial binding to host cells.

The identification of the factors that lead to bacterial adherence will help characterize the initial stages of bacterial invasion of host cells and increase our understanding of the pathogenic properties of L. pneumophila. The results from these studies may suggest potential clinical regimens for blocking infection at the cellular level or the development of split vaccines leading to novel approaches in the prevention and control of Legionnaires' disease.

3.3 Materials and Methods

Reagent formulations and preparations along with detailed procedures are given in Appendices 1 and 4.

Bacterial strain and cultivation. L. pneumophila serogroup 1, strain

Nottingham N_7, was isolated from the sputum sample of a fatal case of Legionnaires' disease, subsequently passaged on bacteriological media, and maintained fully virulent in a frozen state in serum sorbitol at -70° C. Cultures were grown on buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYEα) (29) as previously described (128, 129) at 37°C for 48 h. To ensure the use of a consistent population of organisms in all experiments, L. pneumophila were harvested from plates, inoculated into buffered yeast extract broth supplemented with α-ketoglutarate (BYEα broth) (112) to give a initial concentration of approximately 10^5 organisms/ml and incubated in a shaking water bath for 24 h at 37°C until the culture had reached approximately 2-3 x 10^8 colony forming units (cfu)/ml as determined by subsequent viable counts. Bacteria were harvested by centrifugation at 3000 x g in a Beckman Microfuge 12 (Beckman Instruments, Palo Alto, Ca.). This procedure did not alter either the ability of organisms to adhere to eukaryotic cells or influence the LD_{50} for the fertile hen's egg (see Section 5). Organisms were washed in Hank's Balanced Salt Solution (HBSS) (Irvine Scientific, Santa Ana, Ca.) and diluted 1:10 in HBSS prior to adherence assays. The ratio of bacteria to cells was approximately 100:1 for both cell lines used in these trials. The rationale for the selection of this multiplicity of infection (MOI) as the inoculum for both cell lines was based on data derived from a) bacterial adherence experiments (see Adherence to U937 and MRC-5 cells, p. 44) and b) adherence studies on other microorganisms in which an inocula of 100 MOI was used.

Eukaryotic cells and growth conditions. U937 cells. U937 cells,

derived from a histiocytic lymphoma cell-line, were obtained from the ATCC

(Rockville, Md.) and maintained as replicative, non-adherent monocyte-like

cells in Minimal Essential Medium with Earle's salts (MEM) (Irvine Scientific,

Santa Ana, Ca.) supplemented with 10% bovine calf serum (Hyclone

Laboratories, Inc., Logan, Utah) and 3mM L-glutamine (Sigma Chemicals, St.

Louis, Mo.). The cells were grown in T-75 cm^2 flasks (Costar, Cambridge, Ma.)

and harvested for use in adherence trials at points when the culture reached

late exponential phase of growth (1-2 x 10^6 cells/ml). At this stage, the cells

were differentiated into a non-replicative, adherent macrophage-like cell line

with 12-O-tetra decanoylphorbol-13-acetate (TPA) (Sigma Chemicals, St. Louis,

Mo.), which was added at a final concentration of 10^{-8} M for 48 h. Cells were

removed from flasks with 0.2% EDTA, washed with media, and distributed into

24-well plastic plates (Costar, Cambridge, Ma.) at a cell density of 4-8 x 10^5

cells/well for use in viable count adherence assays or into similar 6-well plates

containing glass coverslips at a cell density of 5-8 x 10^5 cells/well for use in IFA

counts.

MRC-5 cells. MRC-5 cells, a semi-continuous, adherent line of human

embryonic lung fibroblasts, were obtained from Flow Laboraties (McLean, Va.)

and maintained in MEM supplemented with 10% bovine calf serum and 3 mM L-

glutamine. Cells were grown to a confluent state in 24-well or 6-well plates (on

glass coverslips) and used directly for viable count or IFA L. pneumophila

adherence assays. Confluent cell monolayers reached a density of

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approximately 1-2 x 10⁵ cells/well in 24 well plates and approximately 2-3 x 10⁵ cells/coverslip in 6 well plates.

Adherence to U937 and MRC-5 cells. To determine the maximum bacterial binding capacity to both cell types in these assays, L. pneumophila was grown in BYEα broth for 24 h as described. Cultures were pooled, centrifuged, serially diluted ten-fold and each dilution added to 6 and 24-well plates containing either U937 or MRC-5 cells for 1 h. The number of organisms bound to each cell type was assayed by viable count and IFA methods to determine the specificity of bacterial attachment to these cells.

Inhibition of adherence. Bacterial treatments. The role of L. pneumophila surface proteins and sugar moieties in the adherence process was examined. L. pneumophila was treated with degradative enzymes, detergents, fixative and oxidizing chemicals and adherence determined by the viable count and IFA methods described below (p. 45-47). The type, treatment time, concentrations and mode of action of the agents used to modify the surface of the bacteria are shown in Table 3.1. All agents, with the exception of glutaraldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.), were obtained from Sigma Chemicals (St. Louis, Mo.) and prepared at the concentration specified in HBSS prior to testing. Bacteria were treated with the test agent for the time indicated, washed three times in HBSS and used in the adherence assays (p. 45)

Eukaryotic treatments. The role of eukaryotic surface protein, glycoprotein, lipid, glycolipid or sugar was examined as possible adhesins in

the binding process. The type, treatment time, concentrations, and mode of action of the degradative enzymes, detergents, fixatives, oxidizing chemicals and lectins used to modify the surfaces of U-937 and MRC-5 cells are shown in Table 3.2. All agents were obtained from Sigma Chemicals (St. Louis, Mo.) with the exception of the glutaraldehyde and formaldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.) and prepared at the specified concentration in HBSS prior to testing. Cells were treated with agents for the time indicated and washed three times with HBSS prior to adherence assays.

Sugars. A number of sugars were examined as possible inhibitors of the attachment process by conducting adherence assays in the presence of these carbohydrates. Monosaccharides (Sigma Chemicals, St. Louis, Mo.) used in these studies are shown in Table 3.3. Sugars were prepared at the specified concentrations in HBSS, adjusted to pH 7.2 and added to cells 10 minutes prior to the addition of L. pneumophila for adherence experiments.

Adherence assays. U937 and MRC-5 cells were washed three times with HBSS prior to adherence experiments. Following either treatment of bacteria and/or eukaryotic cells with the agents to be tested or after competitive binding, cells were washed with HBSS and L. pneumophila added to wells of 6 and 24-well plates and allowed to adhere to eukaryotic cells for 1 h. Appropriate control organisms and cells were treated with HBSS. After the binding period, unattached organisms were removed by three-fold washings with HBSS and host-cell bound organisms assayed by viable count and IFA methods as follows:

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Viable counts. After removal of non-adherent L. pneumophila, eukaryotic

cells were lysed by the addition of 1 ml of sterile distilled water. This procedure

did not affect the viability of the bacteria as ascertained by viable count studies

of similarly treated organisms. Bacterial counts were made on lysates from

each of 10 separate wells by inoculating 10μl in duplicate of appropriate

dilutions onto BCYEα plates. Plates containing 30 to 300 colonies after an

incubation period of 72 h were used to calculate average viable counts of

lysates for each well as previously described (91). Ratios of cfu/eukaryotic cell

were determined by dividing average lysate values for each well by the number

of eukaryotic cells seeded per well. These values were compared to untreated

control values within each experiment for determination of the percent reduction

of adherence following each treatment used.

Non-specific binding of organisms to the plastic culture plates was

determined for each experimental trial. For every well, viable count estimations

of unbound organisms contained in washes made following the 1 h incubation

period for adherence assays were determined. This value was added to the

corresponding lysate value for the appropriate well and subtracted from the

inoculum. The resultant figure was used to estimate the presence of bacteria

non-specifically stuck to exposed plastic not covered by the cell monolayers. In

multiple experiments, this value was found to be a consistently small

percentage of the inoculum. The level of nonspecific binding was found to be

unaffected by bacterial or eukaryotic cell treatments and was not altered by the

addition of de-ionized water.

Immunofluorescence. Following bacterial or eukaryotic cell treatments and binding experiments, U937 and MRC-5 cells were fixed in 10% buffered formalin for 12 h, washed in HBSS, and treated in situ on the glass coverslips with a 1:100 dilution of rabbit anti-L. pneumophila antibody (see Appendix 4) for 1 h at 37o C. Unbound globulin was removed with three-fold washings of HBSS. Cells were then treated with a 1:200 dilution of goat anti-rabbit FITC conjugated antibody (Cooper Biomedical, Malvern, Pa.) for 1 h at 37o C. Unbound globulin was removed by repeated washings and cells counterstained with 1% (w/) propidium iodide for 5 min. Glass coverslips were mounted in 1% (w/v) 1,4- diazobicyclo (2.2.2.) octane (DABCO) (Sigma Chemicals, St. Louis, Mo.) in glycerin onto microscope slides and viewed with an Olympus BH-2 epifluorescence microscope using an excitation filter emitting a wavelength of 490 nm and a barrier filter blocking wavelengths above 515 nm. The number of fluorescing organisms bound to 200 eukaryotic cells was determined and expressed as adherent organisms/cell. These were compared to control values within each experiment to determine the percent reduction of adherence. Non-specific binding of labelled organisms to areas of glass not covered by eukaryotic cells was ascertained by IFA. Although this was noted to be insignificant in all experiments, these organisms were not counted in the IFA assay for adherence to host cells.

3.4 Results

Adherence to U937 and MRC-5. Bacterial binding to U937 and

MRC-5 cells is illustrated by IFA in plate 3.1 and binding curves for both cell lines are summarized in Figures 3.2-3.5. For each cell type, the use of approximately 5×10^8 cfu/ml as the inoculum gave adherence kinetics approaching saturation of the eukaryotic receptors. This was consistent for either U937 (Figures 3.2 and 3.3) or MRC-5 (Figures 3.4 and 3.5) cells as determined by both assay techniques. The saturation points corresponded to an average cfu/cell ratio of 2.5 and 11 for U-937 (Figure 3.2) and MRC-5 (Figure 3.4) cells, respectively, as assayed by viable counts. IFA equivalent ratios were 3.1 and 6.9 organisms/cell for U937 (Figure 3.3) and MRC-5 (Figure 3.5) cells, respectively. At this high inoculum level, marked non-specific binding to the plastic of culture dishes and glass coverslips were noted by both viable counts and IFA. However, the use of an inoculum with 10-fold fewer organisms (approximately 5×10^7 cfu/ml) gave non-specific binding levels that were dramatically reduced. This inoculum was equivalent to about 100 bacteria/ eukaryotic cell (or 100 MOI) and approached the saturation point of eukaryotic receptors of both cell lines.

Inhibition of adherence studies. Bacterial treatments. Figures 3.6-3.9 illustrate the percent reduction of adherence following modification of L. pneumophila with the agents listed in Table 3.1. Prior treatment of L. pneumophila with glutaraldehyde, β-galactosidase, chymotrypsin, lipase, protease, trypsin and sodium metaperiodate all effected a greater than 50% reduction in adherence to U937 cells as compared with controls in both viable count and immunofluorescence assays. For MRC-5 cells only bacterial

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treatments with glutaraldehyde, chymotrypsin, protease, trypsin and sodium metaperiodate effected a similar response; i.e., unlike for U937 cells, lipase and β-galactosidase treatment did not significantly influence bacterial binding to MRC-5 cells. Bacterial adherence or inhibition of binding to both U937 and MRC-5 cells was consistently similar whether assayed by viable count or IFA methods.

Eukaryotic treatment. Figures 3.10-3.13 illustrate the percent reduction in adherence following modification of U937 and MRC-5 cell surfaces with the agents listed in Table 3.2. Concanavalin A, wheat germ agglutinin, sodium metaperiodate, nonidet P40, glutaraldehyde and formaldehyde reduced binding of L. pneumophila to treated U937 cells by greater than 50% (Figures 3.10 and 3.11). Similar results were obtained by viable count and IFA generated data. It was noted that none of the enzymatic treatments reduced bacterial binding to U937 cells. In addition, modification of U937 cell surfaces with cytochalasin B, a eukaryotic microtubule formation inhibitor, did not result in differences between treated and untreated cells as measured by either assay technique. These results were difficult to interpret but suggested that bacterial uptake by the phagocytic U937 cells may be independent of attachment since the difference between the two assays was not great.

The percent reduction of adherence to MRC-5 cells following treatment with the modifiers listed in Table 3.2 are illustrated in Figure 3.12 and 3.13. Concanavalin A, wheat germ agglutinin, sodium metaperiodate, nonidet P40, glutaraldehyde and formaldehyde effected significant reductions in bacterial

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binding as measured by viable counts and IFA. However, viable counts but not IFA indicated that lipase and chymotrypsin treatment of cells reduced adherence by greater than 50%.

In all eukaryotic treatments with either cell line assayed by both techniques, concanavalin A, wheat germ agglutinin, glutaraldehyde and formaldehyde were the strongest inhibitors of bacterial binding.

Sugars. Co-incubation of L. pneumophila with either cell line in the presence of those sugars listed in Table 3.3 are illustrated in Figures 3.14-3.17. Of those tested, no monosaccharide was able to significantly reduce bacterial binding to U937 or MRC-5 host cells. Viable count and IFA techniques yielded similar results for all sugars tested.

3.5 Discussion.

The adherence of microorganisms to host cells as a prelude to the disease process has been well documented (8). A number of bacterial components, including pili and fimbriae, have been shown to mediate the specific attachment of microbes to eukaryotic cells (8, 70, 116), but evidence has suggested that these and other structures may act concomitantly or sequentially (39, 131) in this process. In addition, the role of van der Waals, thermodynamic forces and hydrophobic interactions in the initial attachment and eventual binding of bacteria to cell surfaces is still unclear. The parameters for both an effective bacterial binding assay and a rationale for determining adherence were established. These criteria were used to partially characterize the nature of "adhesins" involved in L. pneumophila adherence.

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The adherence curves indicated that a specific interaction between L. pneumophila and both cell types occurred. Bacterial binding to eukaryotic receptors approached apparent saturation levels when an inoculum of approximately 10^8 cfu/ml was used in adherence assays. However, this data required caution in interpretation as increasing quantities of non-specific binding to plastic wells were noted to exceed acceptable levels at this point.

Determination of adherence as measured by viable count and IFA techniques yielded consistent and corroborative results and provided evidence that a lectin-like adhesin(s) associated with the surface of L. pneumophila was in part, responsible for mediating attachment to U937 and MRC-5 cells. Degradation and immobilization of bacterial surface proteins with enzyme and fixative treatments as well as periodate oxidation of microbial sugars significantly reduced L. pneumophila adherence to both types of host cells. That lipase and β-galactosidase treatment of bacteria prior to adherence

experiments significantly reduced adherence to U937 cells, but not to MRC-5 implied that lipid and lactose moieties may also be important components in the bacterial binding process. Several gram-negative bacteria possess lectin-like molecules associated with pili, fimbriae, or flagella which enable them to adhere to host cell surfaces (8, 70, 120). L. pneumophila possesses analogous bacterial appendages (108 and Figure 3.1) which may facilitate bacterial binding by similar mechanisms. Isolation of these structures in pure form for use as potential blocking agents in similar adherence experiments will further elucidate their role as adhesins.

Treatment of eukaryotic cell surfaces with the lectins concanavalin A and wheat germ agglutinin substantially reduced bacterial binding and corroborated our results from the bacterial treatment trials. That oxidation of eukaryotic cell surface sugars with sodium metaperiodate inhibited bacterial binding further supported these findings. However, co-incubation of bacteria and cells in the presence of mannose or N-acetyl glucosamine (sugars specific for concanavalin A and wheat germ agglutinin) failed to appreciably inhibit attachment. This may be due to the presence of dissacharides or complex oligosaccharides on the surfaces of the U937 and MRC-5 cells that contain the appropriate sub-unit monosaccharides specific for concanavalin A and wheat germ agglutinin. Therefore, the addition of simple monosaccharides as potential blocking agents in these experiments may not be recognized by L. pneumophila lectin-like surface molecules and consequently failed to reduce adherence. Use of dissacharides or oligosaccharides in similar competitive blocking studies may elucidate putative receptors for L. pneumophila. However, determination of the nature of these "docking" sites by these methods are easily complicated by steric considerations such as the presence of disaccharide sugar receptors conjugated with proteins, lipids or other cell membrane moieties. In this study, efforts were focused on the bacterial component of L. pneumophila adherence to host cells.

That fixation of cell surfaces drastically reduced L. pneumophila binding to U937 and MRC-5 cells while treatment with proteolytic enzymes did not, may signify a requirement for mobile, complex proteins or glycoproteins to facilitate

the adherence of bacteria to these cells. In addition, oxidation of eukaryotic cell surface sugars with sodium metaperiodate actively inhibited bacterial binding. A possible role for these molecules could be selective steric masking or exposure of receptors specific for adhesins on the surface of L. pneumophila. In addition, treatment with nonidet P-40 blocked adherence of L. pneumophila to both cell lines whereas similar treatment with lipase was generally not effective (reducing adherence to MRC-5 cells by 53% as assayed by viable counts only). These results suggested membranous lipid moieties as possible components of eukaryotic receptors as well. It was clear from the compiled data that a simple and uniform receptor system for L. pneumophila may not exist on the surface of U937 and MRC-5 cells. Indeed, synergistic, sequential, steric and hydrophobic interactions between different membrane components of host cells may have a role in the recognition process and further complicate the elucidation of host cell receptors specific for L. pneumophila.

The cytochalasins inhibit the internalization processes of eukaryotic cells and have been used to determine whether pathogens enter host cells by means of active invasion or phagocytosis. Elliott and Winn (36) have shown that L. pneumophila fails to actively penetrate cytochalasin-treated guinea pig alveolar macrophages in vitro. However, the attachment of bacteria to phagocyte membranes was not inhibited in these trials. In the present studies, adherence experiments following treatment of the macrophage-like U937 cells with cytochalasin B resulted in similar binding capacities as assayed by viable counts and IFA when compared to untreated controls. Given that viable counts

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measure attached and internalized organisms while IFA staining facilitate the detection of only bound bacteria these experiments indicate that significant numbers of legionellae were not phagocytosed during the one hour binding assay and that a process of "uptake-independent-adherence" (UIA) occurred for L. pneumophila.

Caution should be exercised when extrapolating in vitro results derived from laboratory experimental conditions to the context of the clinical situation. Neither eukaryotic cell type used in this study were the natural host cell of L. pneumophila in human clinical disease. Although both are human cell lines, U937 cells are macrophage-like cells derived from a histiocytic lymphoma and may possess characteristics not resembling those of primary alveolar macrophages. MRC-5 cells are a semi-continuous fibroblast cell from the lung and exhibit few of the properties of alveolar macrophages. Other potential factors include environmental differences that exist between cells propagated in vitro as compared with those grown in vivo. Exposure to disparate nutritional sources, regulatory signals and other microorganisms could effect the expression of host target cell receptors. Similarly, varying nutritional conditions between the in vitro and clinical environment will most likely affect the growth of microorganisms and may alter expression of adhesins responsible for attachment to eukaryotic cells.

The data from these studies offer evidence that a lectin-like adhesin(s) mediates attachment to U937 and MRC-5 cells. The nature of this receptor(s) remains to be elucidated. Host cell receptors most likely consist of

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oligosaccharide and lipid structures that interact to form complex but as yet unresolved "docking site" for the organism. The finding that lectin-like adhesins occur on other gram-negative bacteria possessing appendages such as pili, fimbriae, or flagella in association with these adhesive molecules suggest that L. pneumophila may utilize similar attachment strategies. Studies using these and other fractionated bacterial structures as blocking agents along with wider classes of saccharides in adherence assays could further characterize or identify specific components responsible for mediating attachment. The elucidation of factors which contribute to the binding of L. pneumophila to host cells will increase our understanding of the pathogenic mechanisms used by this intracellular pathogen to initiate disease and may eventually influence the prevention and control of clinical legionellosis.
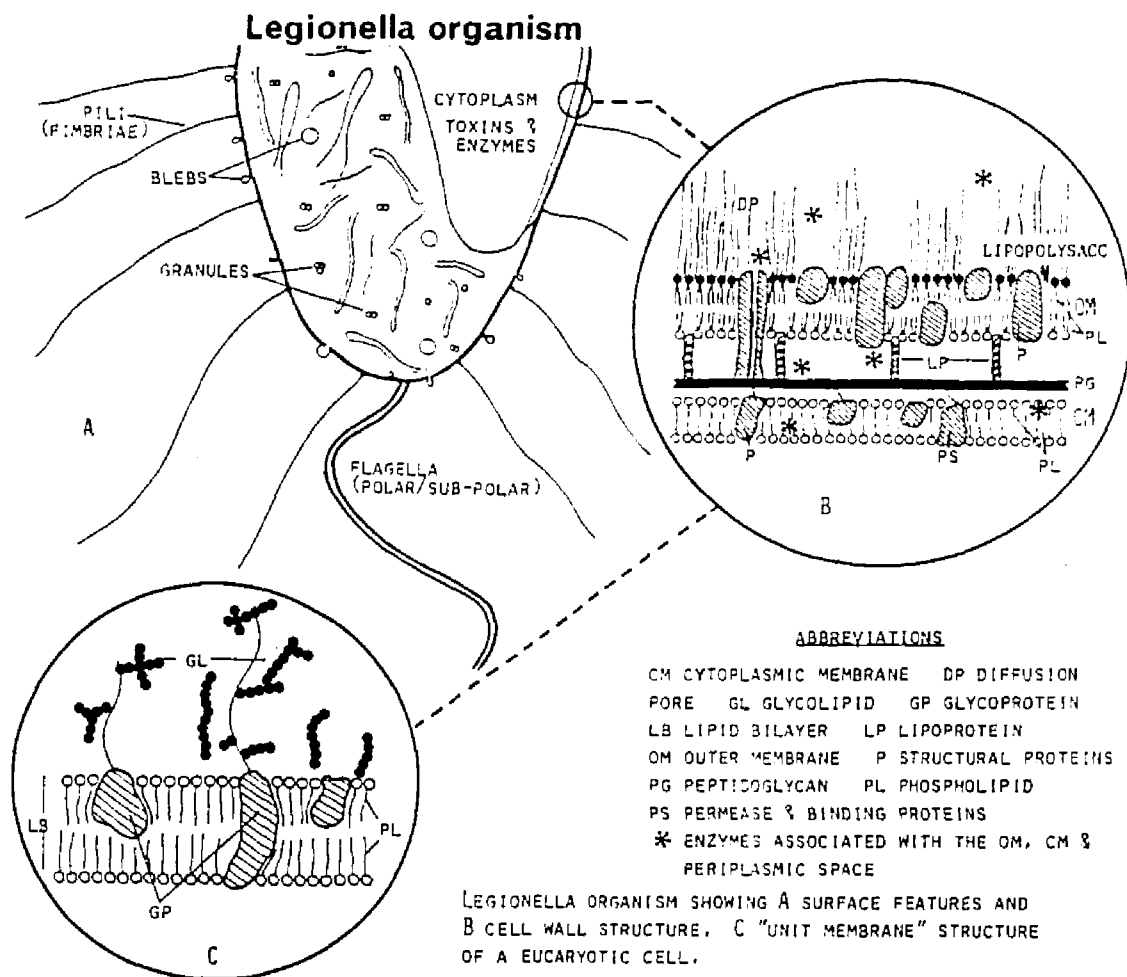
Figure 3.1. Legionella pneumophila. Schematic representation of potential "adhesins" and eukaryotic cell receptors.
(Reproduced with kind permission from reference 38, Oldham and Rodgers, Zbl. Bakt. I Abt. Orig. A.)

Table 3.1. Bacterial treatments prior to adherence assays

Agent	Concentration	Treatment Time (min)	Function
Enzymes	Activity (units/ml)	Treatment Time (min)	Function
B-galactosidase	100	60	Degradation of
chymotrypsin	250	60	protein/sugar-containing
lipase	100	60	adhesins on surface of
neuraminidase	20	60	L. pneumophila
protease	5	60	
trypsin	250	60	
Fixative	Concentration Time (min)	Treatment	Function
glutaraldehyde	0.1%	10	Immobilization of protein moieties
Oxidizing Agent	Concentration Time (min)	Treatment	Function
sodium metaperiodate	10mM	30	Oxidation of carbohydrate moieties

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Table 3.2. Eukaryotic cell treatments prior to adherence assays

Agent	Concentration Time (min)	Treatment	Function
Enzymes	Activity (units/ml)	Treatment Time (min)	Function
Chymotrypsin	0.1	30	Degradation of enzyme
Lipase	100	30	sensitive receptors
Neuraminidase	1.0	30	on host cells
Pepsin	100	30	
Protease	0.005	30	
Trypsin	50	30	
Agent	Concentration Time (min)	Treatment	Function
Glutaraldehyde	0.1%	10	Immobilization of protein-
Formaldehyde	1.0%	10	containing moieties
Nonidet P40	0.005%	60	Action on membrane lipids
Sodium metaperiodate	5mM	10	Oxidation of carbohydrate moieties
cytochalasin B	3ug/ml	60	Inhibition of microfilament formation/phagocytosis
Lectins	Concentration Time (min)	Treatment	Function
Concanavalin A	100ug/ml	60	Binds to mannose
Wheat Germ Agglutinin	100ug/ml	60	Binds to N-acetyl glucosamine

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Table 3.3   Competitive binding experiments

<u>Sugars</u>	<u>Concentration</u>	<u>Function</u>
1) N-acetyl-D-galactosamine	100mM	Competitive
2) N-acetylneuraminic acid	100mM	binding
3) N-acetylglucosamine	100mM	
4) α-D(-)-Fucose	100mM	
5) β-D(+)-glucose	100mM	
6) D(+)-mannose	100mM	
7) D(+)-galactose	100mM	
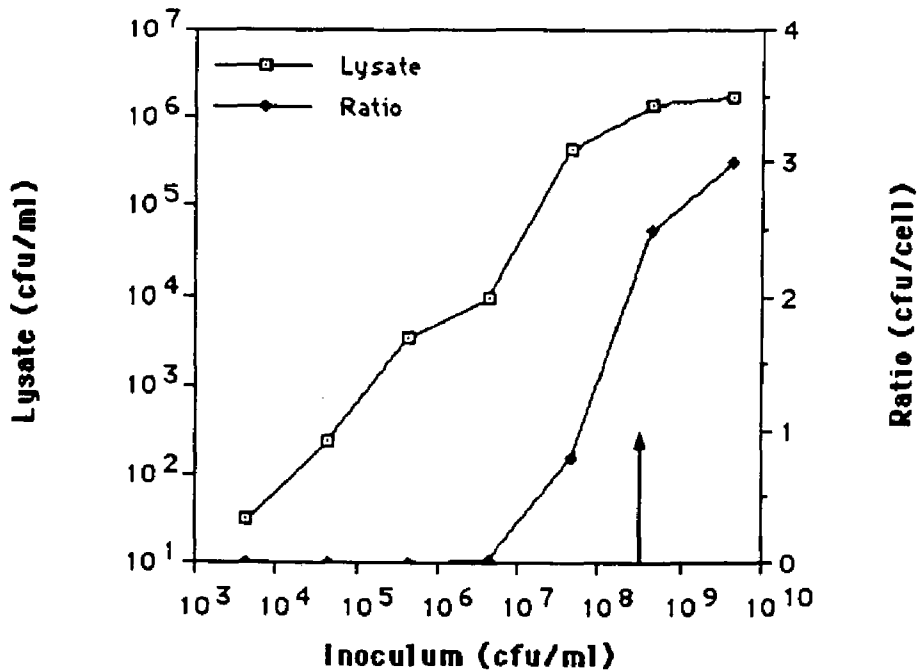
Figure 3.2 Bacterial binding of L. pneumophila to U937 cells as measured by viable counts of organisms released from lysed cells. Ratios of cfu per cell were calculated by dividing the average lysate value obtained from each inoculum by the average eukaryotic cell seed per well. For this experiment the average cell seed equaled 5.7 x 10^5 cells/well. Bacterial attachment to eukaryotic cell receptors approached saturation when an inoculum of approximately 5 x 10^8 cfu/ml was added to cell cultures (arrow). This corresponded to a ratio of 2.5 · cfu/eukaryotic cell. Each point represents an average of three separate trials.

60
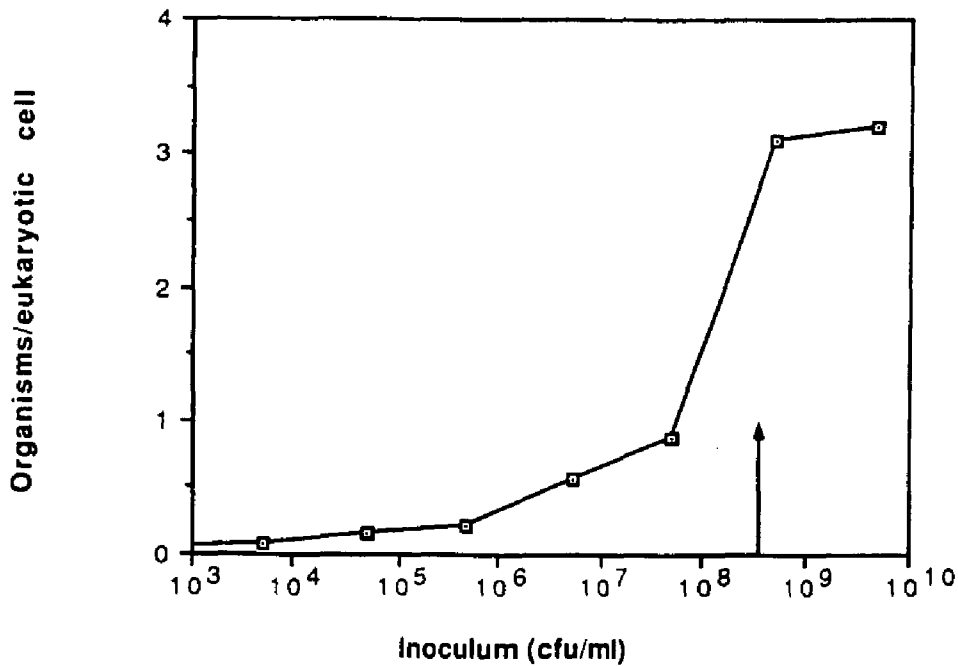
Figure 3.3 Bacterial binding of L. pneumophila to U937 cells as measured by IFA. The number of fluorescing organisms bound to approximately 200 eukaryotic cells is expressed as a ratio of organisms/eukaryotic cell for each inoculum used. An inoculum of about 5×10^8 approached saturation of eukaryotic receptors (arrow). This corresponded to a ratio of 3.1 organisms/eukaryotic cell.

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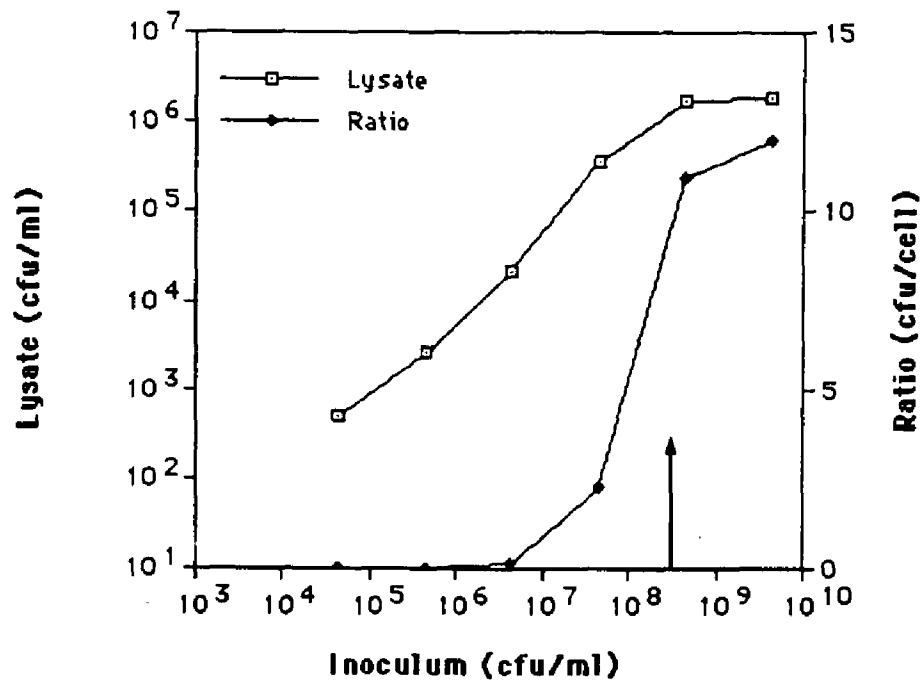
Figure 3.4 Bacterial binding of L. pneumophila to MRC-5 cells as measured by viable counts of organisms released from lysed cells. Ratios of cfu per cell were calculated by dividing the average lysate value obtained from each inoculum by the average eukaryotic cell seed per well. For this experiment the average cell seed equaled 1.6 X 10^5 cells/well. Bacterial attachment to eukaryotic cell receptors approached saturation when an inoculum of approximately 5 x 10^8 cfu/ml was added to cell cultures (arrow). This corresponded to a ratio of 11 cfu/eukaryotic cell. Each point represents an average of three separate trials.
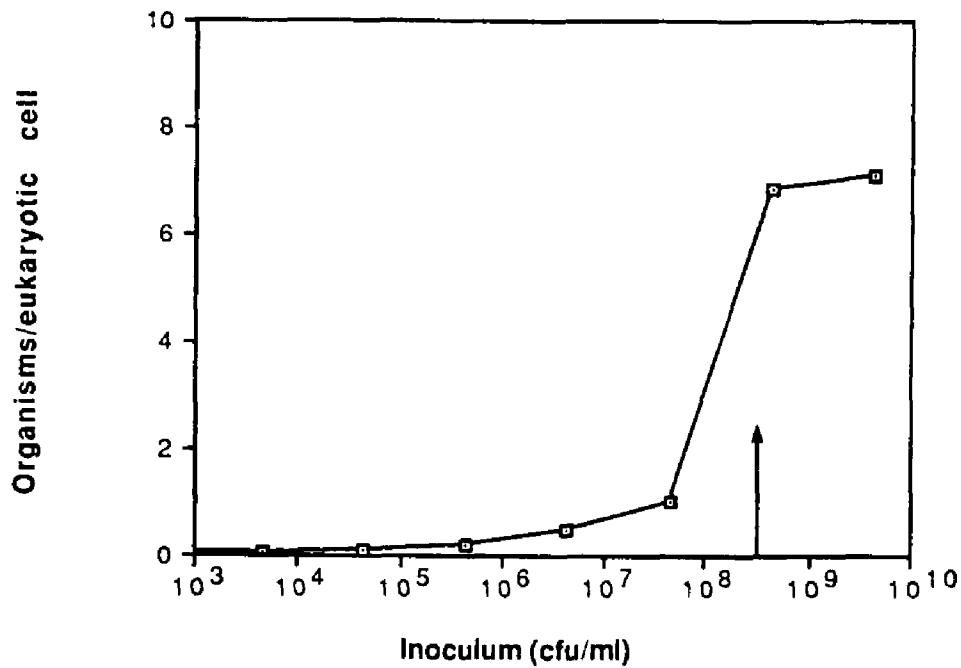
Figure 3.5 Bacterial binding of L. pneumophila to MRC-5 cells as measured by IFA. The number of fluorescing organisms bound to approximately 200 eukaryotic cells is expressed as a ratio of organisms/eukaryotic cell for each inoculum used. An inoculum of about 5×10^8 approached saturation of eukaryotic receptors (arrow). This corresponded to a ratio of 6.9 organisms/cell.
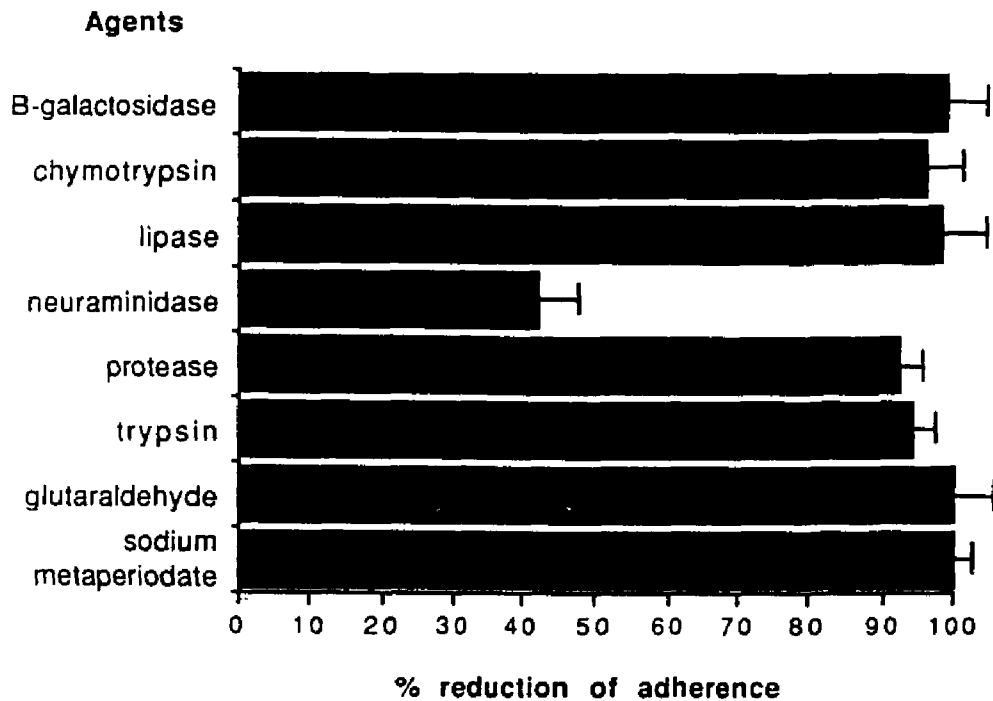
Agents

Figure 3.6 Bacterial treatments, U937 cells. Percent reduction of L. pneumophila binding to U937 cells as measured by viable count estimation of eukaryotic lysates following treatment of bacteria with the agents listed in Table 3.1. Each value represents the average of 10 separate trials. Error bars indicate standard error of the mean for each treatment.

Agents
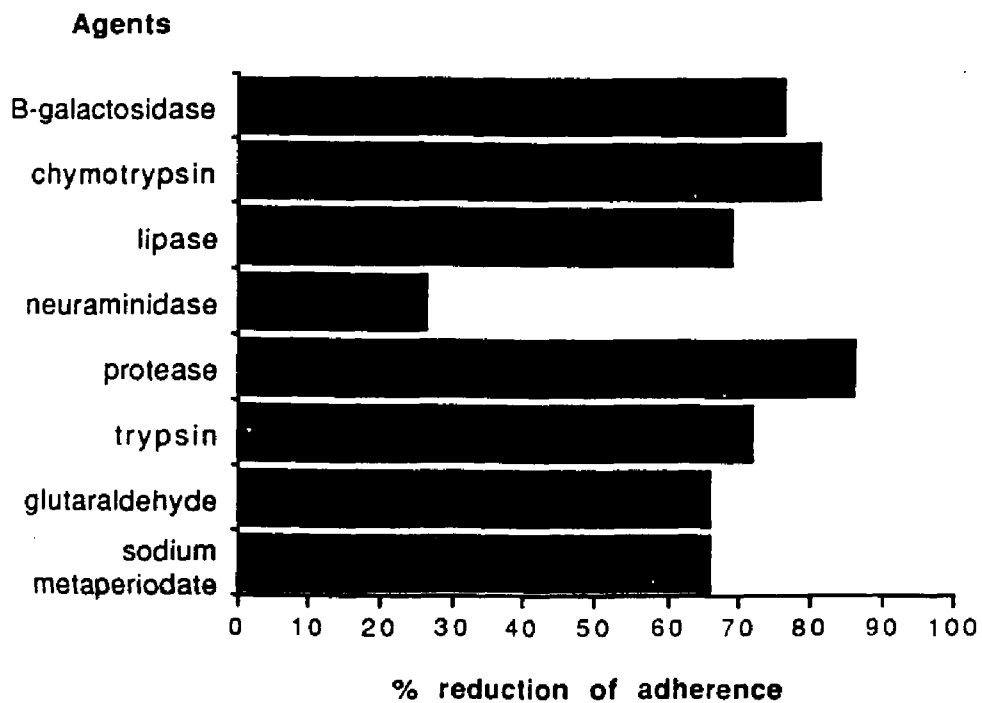
Figure 3.7 Bacterial treatments, U937 cells. Percent reduction of L. pneumophila binding to U937 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of bacteria with the agents listed in Table 3.1.
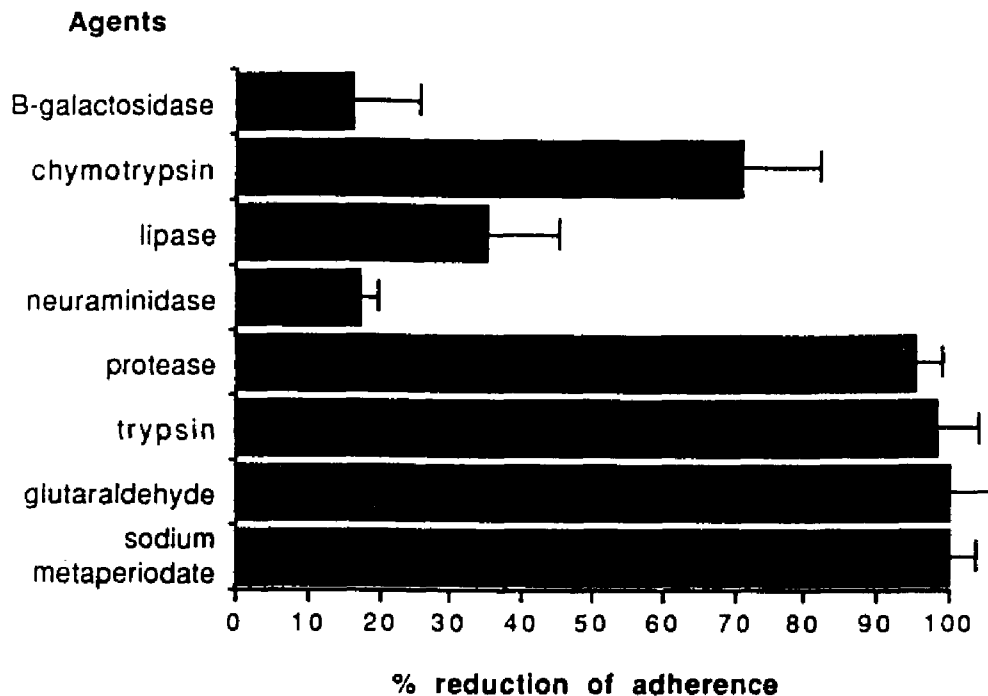
Agents

Figure 3.8 Bacterial treatments, MRC-5 cells. Percent reduction of L. pneumophila binding to MRC-5 cells as measured by viable count estimations of eukaryotic lysates following treatment of bacteria with the agents listed in Table 3.1. Each value represents the average of 10 separate trials as compared with untreated controls. The standard error of the mean is indicated for each treatment.

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Agents
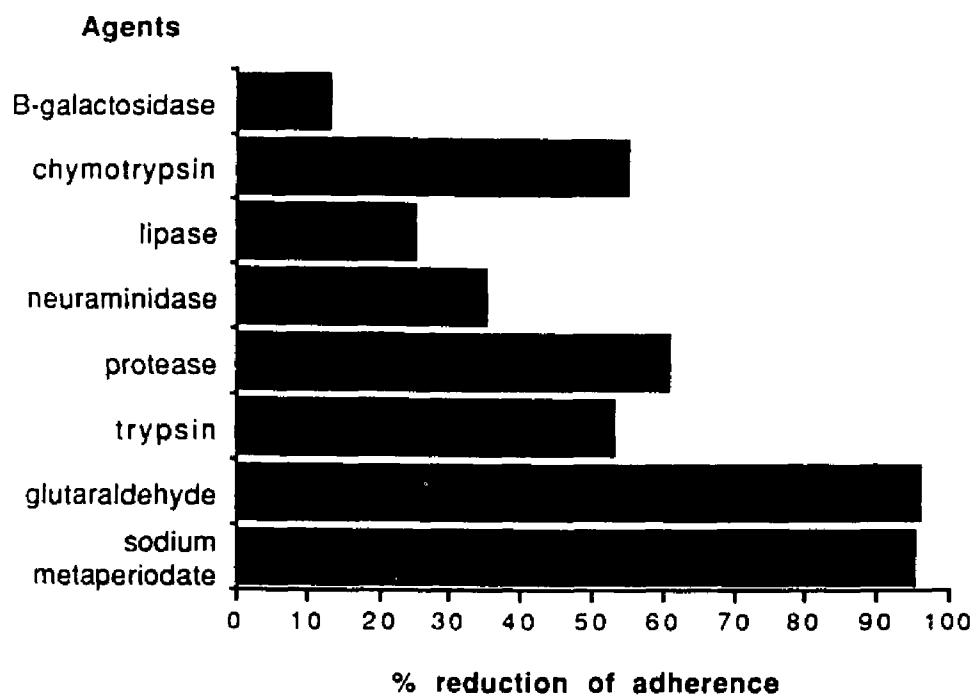
Figure 3.9 Bacterial treatments, MRC-5 cells. Percent reduction of L. pneumophila binding to MRC-5 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of bacteria with the agents listed in Table 3.1.

Agents

% reduction of adherence
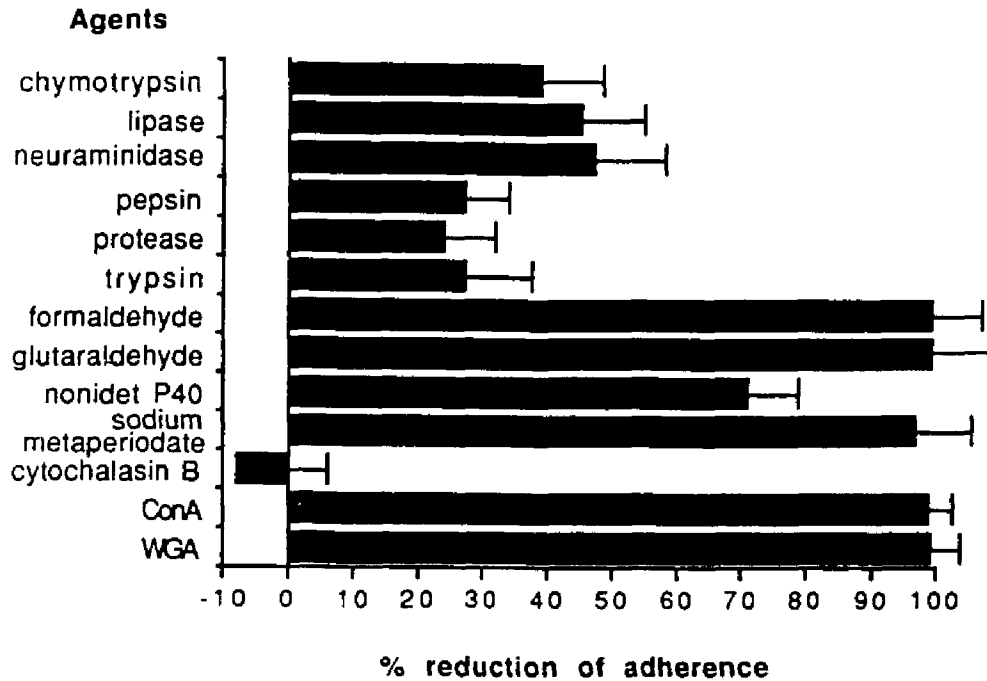
Figure 3.10 Eukaryotic cell treatments, U937 cells. Percent reduction of L. pneumophila binding to U937 cells as measured by viable count estimations of eukaryotic lysates following treatment of host cells with the agents listed in Table 3.2. Each value represents the average of 10 separate trials. Error bars indicate standard error of the mean for each treatment.

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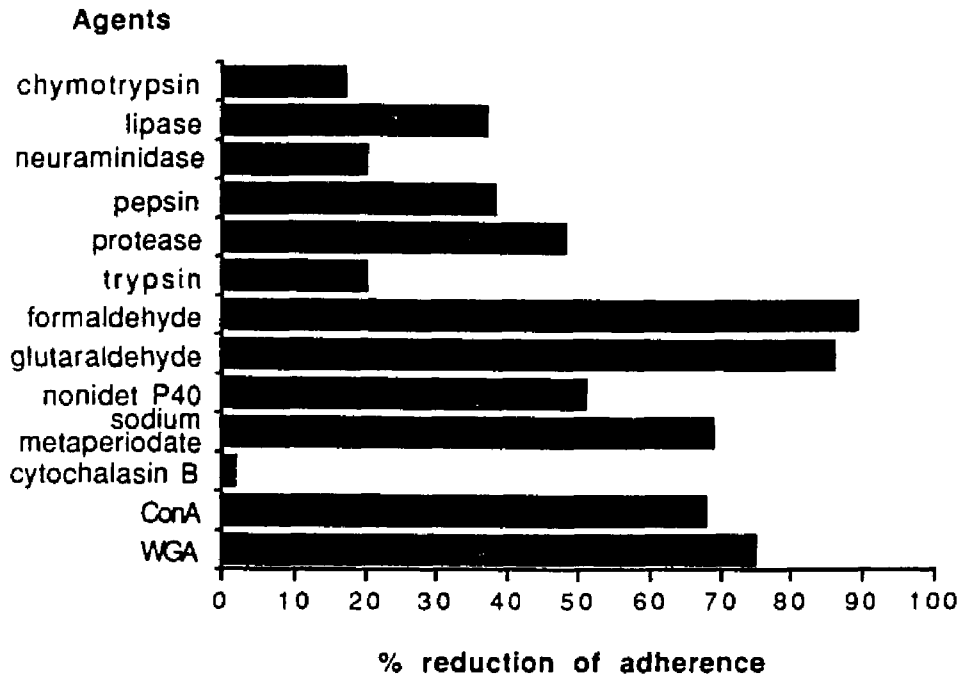
Figure 3.11 Eukaryotic cell treatments, U937 cells. Percent reduction of L. pneumophila binding to U937 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of host cells with the agents listed in Table 3.2.

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Agents

chymotrypsin
lipase
neuraminidase
pepsin
protease
trypsin
formaldehyde
glutaraldehyde
nonidet P40
sodium
metaperiodate
ConA
WGA

0 10 20 30 40 50 60 70 80 90 100

% reduction of adherence
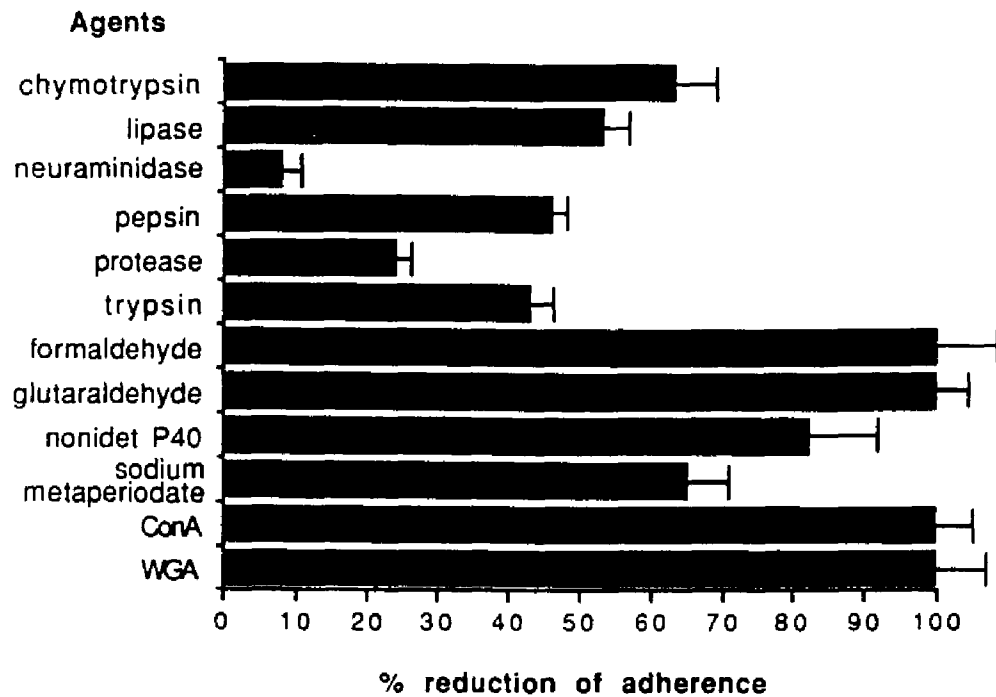
Figure 3.12 Eukaryotic cell treatments, MRC-5 cells. Percent reduction of L. pneumophila binding to MRC-5 cells as measured by viable count estimation of eukaryotic lysates following treatment of host cells with the agents listed in Table 3.2. Each value represents the average of 10 separate trials. Error bars indicate the standard error of the mean for each treatment.

Agents
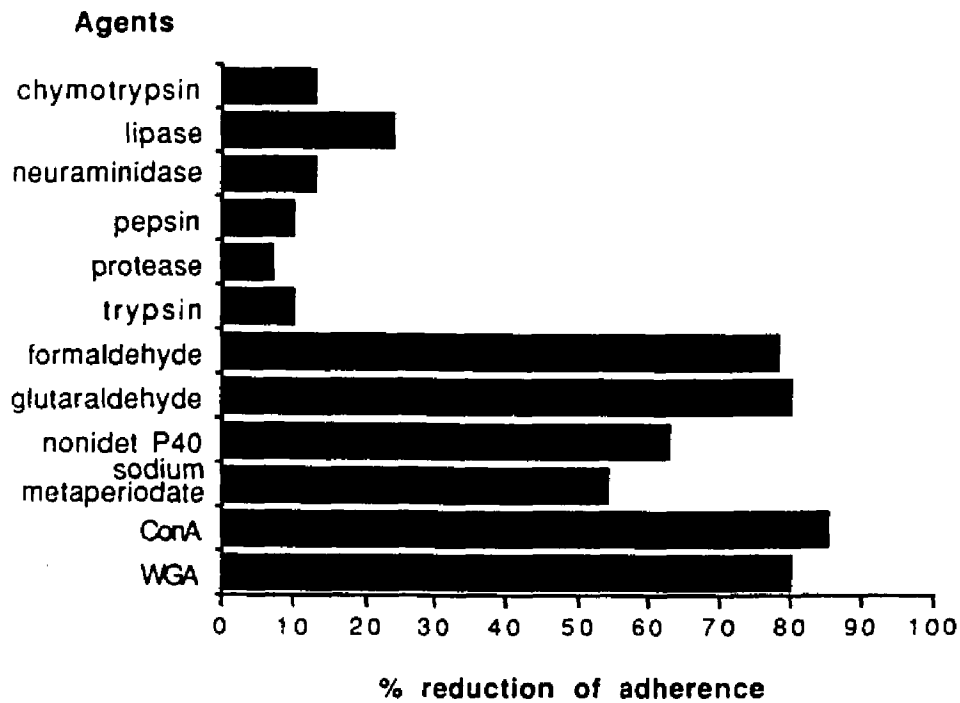
Figure 3.13 Eukaryotic cell treatments, MRC-5 cells. Percent reduction of L. pneumophila binding to MRC-5 cells as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells following treatment of host cells with the agents listed in Table 3.2.

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Sugar

% reduction of adherence
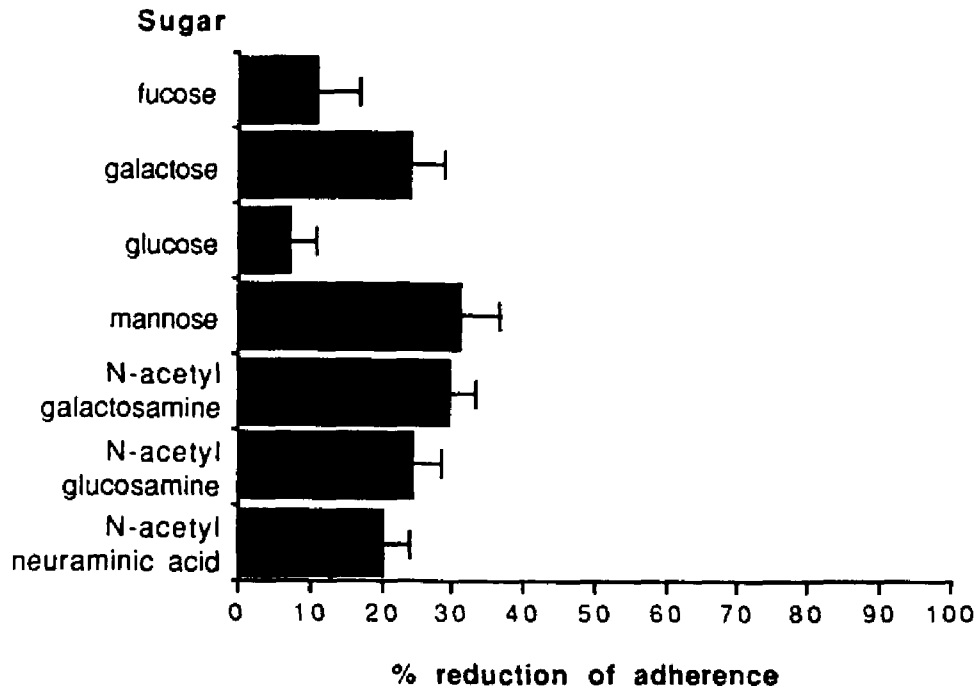
Figure 3.14 Competitive binding studies with sugars, U937 cells. Percent reduction of bacterial adherence following co-incubation of L. pneumophila and U937 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by viable counts of eukaryotic lysates. Concentration of added sugars was 100 mM. Each value represents the average of 10 separate trials. Standard error of the mean is indicated for each treatment. None of the sugars used in these experiments effected a significant reduction of adherence.

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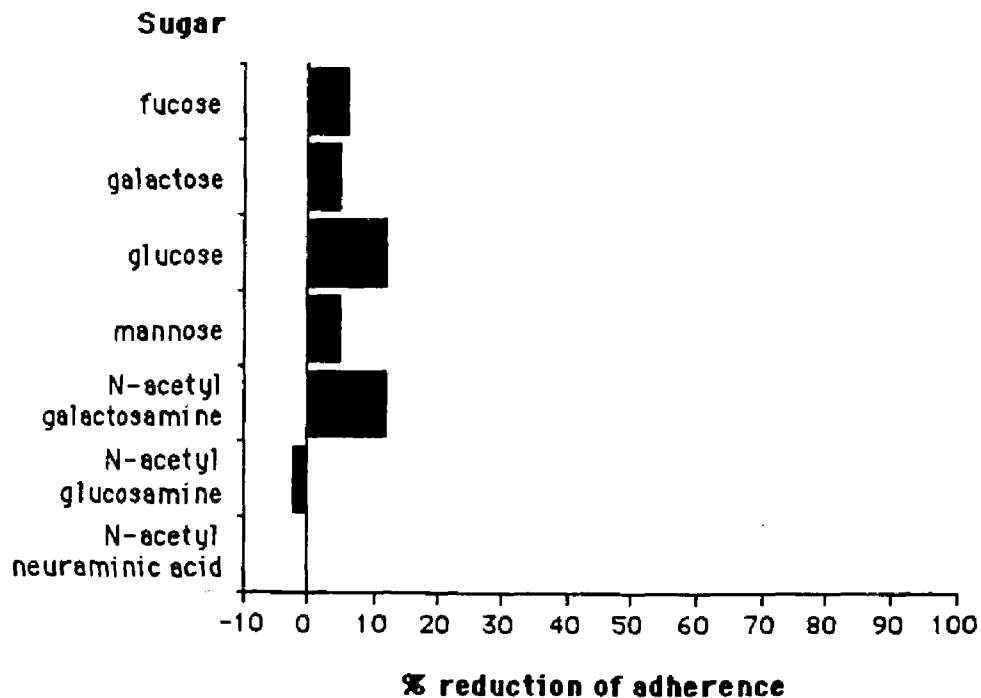
Sugar

Figure 3.15 Competitive binding studies with sugars, U937 cells. Percent reduction of bacterial adherence following co-incubation of L. pneumophila and U937 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells. None of the sugars used in these experiments effected a significant reduction of adherence. Concentration of added sugars was 100 mM.

Sugar

% reduction of adherence
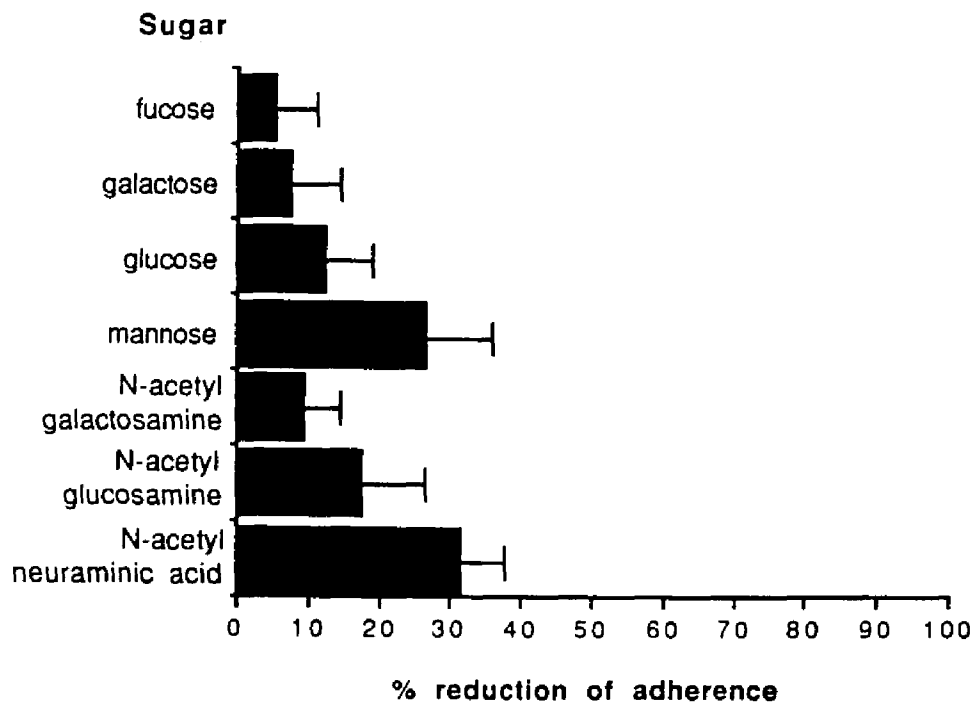
Figure 3.16 Competitive binding studies with sugars, MRC-5 cells. Percent reduction of bacterial adherence following co-incubation of L. pneumophila and MRC-5 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by viable counts of eukaryotic lysates. Concentration of added sugars was 100 mM.Each value represents the average of 10 separate trials. Standard error of the mean is indicated for each treatment. None of the sugars used in these experiments effected a significant reduction of adherence.

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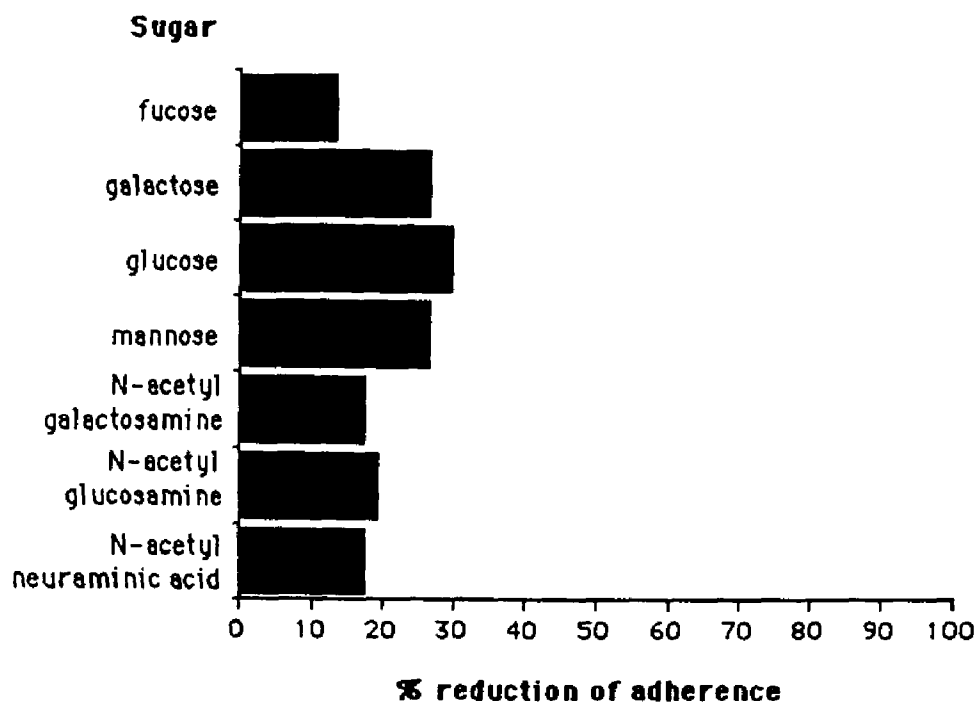
Figure 3.17 Competitive binding studies with sugars, MRC-5 cells. Percent reduction of bacterial adherence following co-incubation of L. pneumophila and MRC-5 cells in the presence of monosaccharide sugars listed in Table 3.3 as measured by counts of fluorescing organisms bound to approximately 200 eukaryotic cells. Concentration of added sugars was 100 mM.
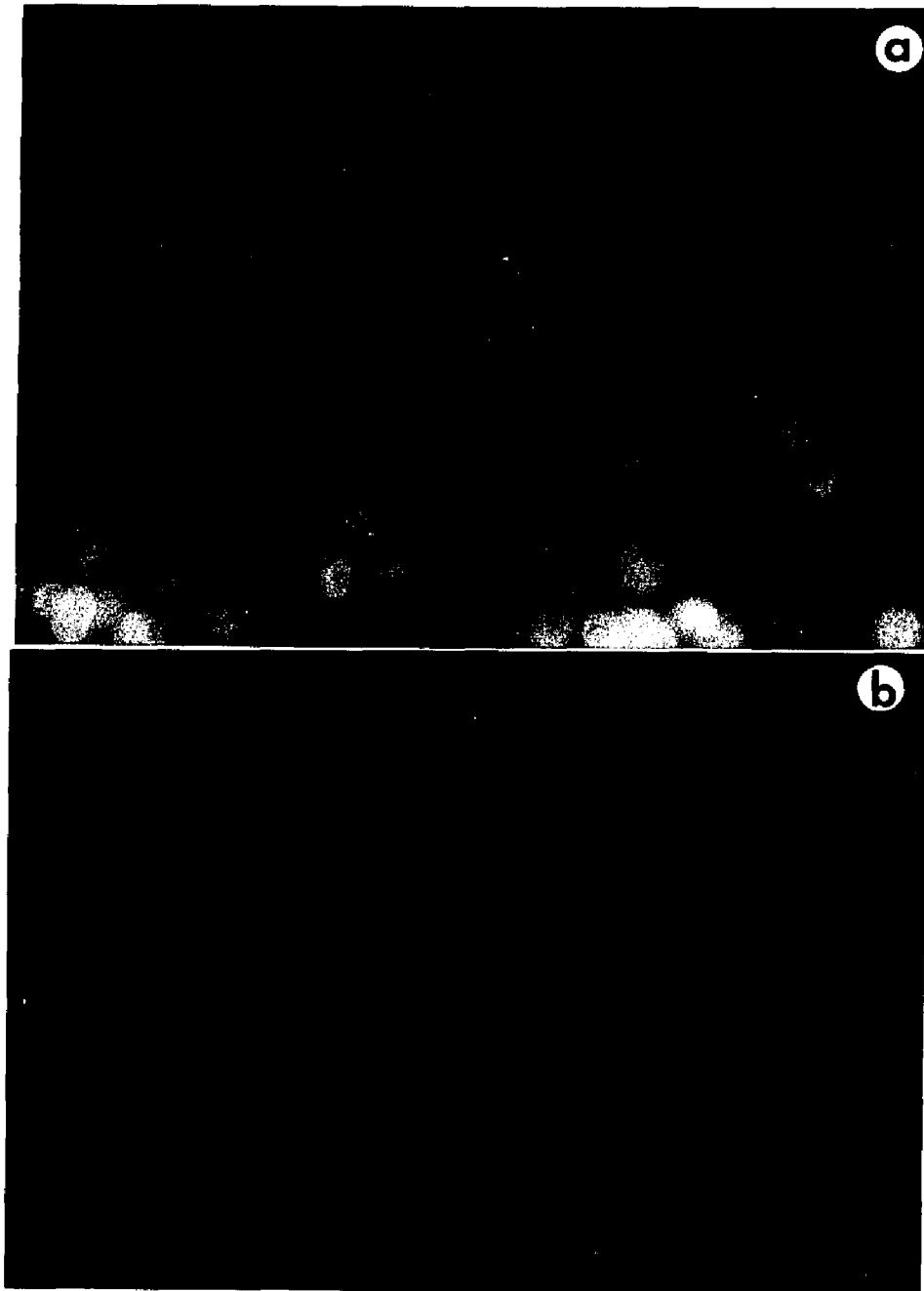
Plate 3.1 L. pneumophila binding to U937 and MRC-5 cells in an indirect immunofluorescence assay. Organisms are labelled with FITC (green) while eukaryotic cells are counterstained with propidium iodide (red).

a) L. pneumophila attachment to U937 cells. Note incidence of bacterial binding to host cell surfaces (X 660), b) higher magnification of L. pneumophila binding to MRC-5 cells. Note single organism attached along its length to the host cell surface (X 1,650).

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Section IV

The Effect of Antibiotics That Inhibit Cell Wall, Protein and DNA Synthesis on the Growth and Morphology of
Legionella pneumophila

4.1 Abstract

The response of L. pneumophila to cell wall, protein synthesis and DNA synthesis inhibitory antibiotics was examined by electron microscopy, MIC estimations and viable count assays. Cefotaxime, methicillin, rifampicin and ciprofloxacin, each used separately at 20 times their respective MIC values, showed activity against L. pneumophila in these studies. The cell wall inhibitors, cefotaxime and methicillin, effected the greatest bactericidal activity and induced the most extensive morphological changes. Organisms treated with these antibiotics lost cytoplasmic contents through membranous lesions induced in their cell walls. In terms of ultrastructural damage and loss of viability, the protein and DNA synthesis inhibitors were less efficacious than antibiotics that acted on the microbial cell wall. Rifampicin treated cells possessed irregular membranes and were partially or fully lysed while ciprofloxacin induced abnormally elongated organisms with intermittently lysed and detached inner membranes. These results illustrated the ability of clinically putative antibiotics with diverse modes of action to affect microbial cytology at · the ultrastructural level as well as the viability of L. pneumophila in vitro.

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4.2 Introduction

Legionella pneumophila, the causal agent of a fatal form of lobar

pneumonia known as Legionnaires' disease, has been studied by negative

stain, thin-section, freeze-etching, and scanning electron microscopy (105,

108). The response of this human pathogen to treatment with antimicrobial

chemotherapeutic agents has been investigated using various in vitro and in

vivo techniques (19, 30, 35, 44, 45, 57, 60, 65, 83, 107, 109, 112, 128, 129,

142). Although several antibiotics have excellent activity against L.

pneumophila in vitro as assessed in minimum inhibitory concentration (MIC)

studies, few show similar results in clinical trials.

Erythromycin is the current drug of choice for the treatment of

legionellosis; however, inherent problems arise in its use. These include

reports of relapse of infection, onset of phlebitis with intravenous antibiotic

administration and potential complications associated with the development of

bacterial resistance to therapy.   In addition, studies in which Elliott and Rodgers

(35) examined the morphological and growth response of L. pneumophila to

erythromycin and ampicillin illustrated that erythromycin had limited bactericidal

activity in vitro. Rifampicin has proven effective in clinical trials but is not used

as the sole therapeutic drug because of the high frequency with which

resistance is induced. For these reasons, a number of other antimicrobial

agents have been investigated as potential alternatives for clinical use in the

treatment of legionellosis. Of those examined, the quinolone antibiotics have

been the most promising. Ciprofloxacin, the best studied of this group, is a lipid

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soluble antimicrobial which inhibits bacterial DNA gyrase and has yielded encouraging results against experimental L. pneumophila infection in vivo (44, 45, 128, 129).

In the present study, the morphological characteristics and growth response of L. pneumophila was examined following exposure to antimicrobials derived from three groups of antibiotics (bacterial cell wall, protein synthesis and DNA inhibitors). The evaluation of the efficacy of these drugs was determined using MIC estimations, viable count data and ultrastructural investigations.

4.3 Materials and Methods.

Reagent formulations and preparation along with detailed procedures are given in the appendices 1 and 3.

Bacterial strain and growth conditions. L. pneumophila serogroup 1, strain Nottingham N_7 was isolated from a sputum sample of a fatal case of Legionnaires' disease, was subsequently passaged twice on bacteriological media and maintained fully virulent in a frozen state in serum sorbitol at -70° C. Cultures were grown on buffered charcoal yeast extract agar supplemented with a-ketoglutarate (BCYEα) (29) at 37° C for 48 h. Organisms were harvested from plates and inoculated into α-ketoglutarate enriched buffered yeast extract (BYEα) broth containing 10 g/l yeast extract (Difco), 10g/l ACES buffer (Sigma), 1.0g/l α-ketoglutarate (Sigma), 0.4 g/l L-cysteine (Sigma) and 0.25 g/l ferric pyrophosphate (Sigma). The broth was adjusted to pH 6.9

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with KOH and filter sterilized through a 0.2μm filter to give a final concentration

of approximately 10^5 colony forming units (cfu)/ml. Organisms were incubated

in static culture at 37°C and 5 ml aliquots removed at 4 to 6 h intervals to

develop growth curves for this organism in the broth to be used for subsequent

antibiotic activity studies.

Antibiotics. Dilutions of cefotaxime (sodium salt) (Hoechst-Roussel

Pharmaceuticals), methicillin (sodium salt) (Sigma Chemical Company),

rifampicin (Sigma Chemical Company), and ciprofloxacin (Miles

Pharmaceuticals) were prepared in BYEα broth. Each antibiotic was added in

the mid-exponential phase of organism growth to give a final concentration of

20 times the MIC of each antimicrobial agent. Aliquots of 5 ml were removed

after incubation for 6 and 24 h in the presence of antibiotics. Organisms were

harvested by centrifugation at 600 g for 10 min, washed twice in broth and

either serially diluted for viable counts or prepared for electronmicroscopy.

Control samples of untreated organisms were harvested and prepared in the

same manner.

Viable count technique. Bacterial counts were determined in

triplicate by inoculating 10 μl aliquots of serially diluted antibiotic-treated

cultures onto BCYEα agar. Resultant cfu/ml were counted from those plates

containing 30 to 100 colonies as previously described (91) to define the

influence of these antibiotics on bacterial viability.

Electron microscopy. Bacteria were fixed in 3% (v/v) glutaraldehyde

in 0.05M sodium cacodylate containing 10mM $MgSO_4$ (35). For negative stain

electron microscopy, 25 µl of each sample was mixed with an equal volume of

1% (w/v) phosphotungstic acid pH 6.7, and applied to 400 mesh formvar-carbon

coated copper electron microscope grids. For scanning electron microscopy,

samples were applied to specimen stubs, dehydrated in a graded ethanol

series, and treated with hexamethyldisilaxane (HMDS) (Polysciences, Inc.)

(Warrington, Pa.) (87). Prior to examination, samples were coated with 20 nm of

either gold/palladium with a target to specimen distance of 5 cm, at a current of

15 mA for 4 min in a Hummer V sputter coater. For thin-section electron

microscopy, all samples were post-fixed in 1% (w/v) osmium tetroxide,

dehydrated in an ethanol series and embedded in epon-araldite mixture.

Sections of approximately 60 nm thickness were cut on an LKB Ultratome III

with a diamond knife and stained with uranyl acetate 5% (w/v) for 1 min and

lead citrate 0.4% (w/v) for 20 sec (105, 108).

MIC determination. MICs were determined by the broth dilution

method (133). Briefly, serial two-fold dilutions of each antibiotic were prepared

in BYEα broth and inoculated with cultures in the mid-exponential phase of

growth to give a final concentration of 10^5 cfu/ml. The broths were incubated for

16 h and the lowest concentration of antibiotic which inhibited growth was taken

as the MIC (Table 4.1)

4.4 Results.

Viability studies. MIC values for all antibiotics are shown in Table 4.1.

The growth characteristics and effect on viability of L. pneumophila by the antibiotics investigated are illustrated in Figures 4.1-4.6 (data on ampicillin and erythromycin are from reference 35 with permission and are included for a complete comparative profile). L. pneumophila grown in BYEα broth, had a mean generation time of 1.5 h. The bacterial cell wall inhibitors, cefotaxime and methicillin, effected the most rapid decline in viable counts during the first 6 h of treatment, while the remaining antibiotics also showed excellent activity against L. pneumophila throughout the course of these experiments. Of those tested, cefotaxime most effectively reduced organism viability and induced the most dramatic morphological changes at the time intervals investigated.

Morphological studies. Untreated. Negative stain, scanning and thin-section electron microscopy studies of normal control organisms revealed the typical appearance of legionellae and confirmed the findings of others. Most cells were 2-10 μm long and 0.25-0.5 μm wide. The bacterial surface of normal cells was rugose and ruffled in appearance. Organisms showed a cell wall structure consistent with that of gram-negative bacteria. A lipid bilayer, or cytoplasmic membrane, enclosed the cytoplasm which consisted of ribosomes, a small number of intracellular vacuoles and a fine skein of nuclear elements. The latter were distributed evenly throughout the bacterial cytoplasm. The outer membrane enveloped the periplasm which showed little structural evidence of a peptidoglycan layer (plate 4.1).

Cell wall inhibitors. Cefotaxime treatment. Cefotaxime at 20 times

the MIC caused extreme damage to microorganisms. Incubation with this antibiotic for 6 h resulted in the formation of lesions and lytic points in the cell walls of a greater number of bacteria than any of the antibiotics studied at this time period. Extrusion of the bacterial cytoplasmic contents through such lytic points was the most common feature noted. Bulbous vesicles were observed on the surface of cells and many organisms possessed diffuse electron-lucent cytoplasmic contents. Extension of treatment with cefotaxime to 24 h resulted in an increase in damage to these cells in terms of the degree of membranous lesions and vesicles induced in the cell surfaces as well as the number of organisms affected. In addition, many bacteria lacked either cytoplasmic contents or prokaryotic structure. The formation of spheroplasts was not observed (plates 4.2 and 4.3).

Methicillin treatment. Organisms grown in the presence of 20 times the MIC of methicillin showed extensive morphological changes. Affected cells exhibited cell wall and membrane abnormalities and developed several lytic points through which the cytoplasmic contents extruded into the surrounding menstruum. Although many organisms appeared normal, spheroplast formation was evident at this stage. Incubation with methicillin for 24 h induced a greater degree of cell wall and membrane damage than the lesser incubation period. The outer membrane of the majority of organisms was found to have separated from the remaining cell wall and the development of small vesicles on the cell surfaces was evident. As a consequence of lysis of the inner and outer membranes, a loss of cytoplasmic material occurred (plates 4.4-4.8).

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Protein synthesis inhibitor. Rifampicin treatment. Exposure of L. pneumophila to 20 times the MIC of rifampicin induced marked damage to bacteria. Incubation for 6 h in the presence of this antimicrobial induced membrane damage to the organism but this appeared less pronounced than for the other antibiotics examined. Separation of outer membranes from the remaining cell wall and breakage points in the inner membranes were evident in affected microorganisms. In addition, the ribosomes of these cells were enlarged, much increased in electron density and associated with areas of cytoplasmic clearing. Extended treatment with rifampicin elicited a more pronounced effect in which lytic points in the membrane of cells and loss of intracellular contents led to eventual collapse (plates 4.9-4.12).

DNA inhibitor. Ciprofloxacin treatment. Organisms treated for 6 h with 20 times the MIC of ciprofloxacin exhibited marked morphological changes. The majority of cells were abnormally elongated forming filaments each of which possessed centrally located "pinched zones" suggestive of arrested division. The inner membranes of many legionellae were intermittently lysed and separated from the remaining cell wall material. In addition, the intracellular contents of cells were more densely packed due to an apparent overall increase in the size of individual ribosomes. Further exposure to this antibiotic induced damage to 98% of the Legionella organisms present. Twisted, convoluted bacteria were found with depressed, partially collapsed areas on their cell surface. In addition, some organisms at this time had lost their intracellular contents through breakage points in the cell wall while others

maintained the densely packed cytoplasmic appearance found in 6 h antibiotic treated organisms (plates 4.13-4.19).

Figure 4.7 shows the relative proportion of lysed and partially damaged cells compared with those of normal morphology following antibiotic treatment for both 6 and 24 h and Table 4.2 summarizes the morphological findings.

4.5 Discussion

Erythromycin is the preferred drug for the treatment of Legionnaires' disease. However, because patients with severe illness often respond poorly to antimicrobial therapy, new antibiotics are being studied for use as alternate therapeutic regimens. In vitro screening of these potential agents to determine the activity and mode of action of these antibiotics must be performed in order to evaluate subsequent in vivo and clinical trials. In the present study, the in vitro effect of clinically relevant antibiotics, including the new quinolone, ciprofloxacin, on the growth and morphology of L. pneumophila was examined.

Electron microscopy studies have been important for the delineation and understanding of antimicrobial activity against many pathogenic microorganisms (61, 77, 141). Elliott and Rodgers (35, 107, 109) evaluated the morphological and growth response of L. pneumophila following exposure to ampicillin and erythromycin for 6 and 24 hours using electron microscopy, MIC determinations and microbial viable count data. In that investigation, ampicillin exhibited greater bactericidal activity and induced more ultrastructural changes than erythromycin in vitro. Ampicillin treatment caused the appearance of vacuole-like lesions in the cell walls of organisms which led to extensive

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bacterial lysis and collapse of legionellae while exposure of L. pneumophila to erythromycin effected occasional breakage points in the cell wall along with cytoplasmic clearing, formation of membranous vesicles and some cell lysis.

In this study, all antibiotics tested were effective against L. pneumophila as assayed by MIC data, the reduction in bacterial viable counts and morphological response induced. Methicillin showed extreme bactericidal activity against multiplying organisms in broth culture. This was confirmed by the morphological studies in which many lysed and empty cells were evident after 6 and 24 h exposure periods. Formation of spheroplasts following treatment with 1280 µg/ml of this antibiotic was demonstrated and confirmed previous observations by Elliott and Rodgers of this phenomenon with another cell wall inhibitory antibiotic, ampicillin (35). These data suggested that the formation of minicell-like organisms and spheroplasts upon exposure to cell wall inhibitory antibiotics such as methicillin and ampicillin may constitute a mechanism by which osmotically protected legionellae survive within host cells and continue infection following reversion to vegetative forms upon discontinuation of therapy. Such findings may offer an explanation for the reported apparent "reactivation" infections due to L. pneumophila and may reflect ineffective killing of the organism in vivo in human lung.

Chan et al. (19) reported similar spheroplast formation for methicillin used at 100 µg/ml, but could not demonstrate this phenomenon with increased doses of up to 1000 µg/ml of methicillin. It is possible that the addition of

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magnesium salts to the fixatives and buffers used in the present study could have stabilized fragile bacterial membranes and may account for the differences in spheroplast detection.

Cefotaxime showed the greatest bactericidal activity and this resulted in the most rapid decline in numbers of viable organisms. Indeed, this third generation cephalosporin induced the greatest morphological changes in microbial cytology at either of the exposure times. These results conflicted with those of Chan et al. (19) who reported no morphological changes in response to treatment of L. pneumophila with different doses of this antibiotic. It was interesting to note that this drug failed to induce spheroplast formation by L. pneumophila although a similar finding was reported following treatment of other gram negative bacteria with increased levels of cephalothin (88).

Although most Legionella species produce β-lactamases, the β-lactam antibiotics are most effective against actively dividing L. pneumophila cells in vitro. In this study, antibiotics were added in the mid-exponential phase of growth, which for L. pneumophila using the broth selected and an inoculum of 10^5 cfu/ml was achieved 24 h into the growth cycle, to allow these agents to function at their full capacity. Failure of Chan et al. (19) to detect similar morphological changes due to this antibiotic may have reflected the non-replicative status of the organisms subjected to antimicrobial treatment.

Ciprofloxacin and rifampicin showed effective bactericidal activity in viable count estimations at all the times studied. Despite showing the greatest activity in MIC assays, these antibiotics induced only moderate structural

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damage after short term exposure. However, such changes increased with treatment time. That bacterial viability was lost prior to the induction of extensive ultrastructural damage probably reflected the mode of action of these antimicrobial agents against L. pneumophila cells in the exponential phase of growth.

Results from this and other studies (35, 107, 109) demonstrated that the cell wall inhibitory antibiotics were the most effective in terms of their ability to reduce L. pneumophila viability and induce morphological damage in vitro. Although all the antibiotics investigated in this study showed in vitro antimicrobial activity against L. pneumophila, it has been reported that the aminoglycosides, cephalosporins, and penicillins including ampicillin and methicillin were clinically ineffective in the treatment of legionellosis (82). Because of its intracellular nature, L. pneumophila can avoid the effects of antimicrobial agents that lack the ability to penetrate host cell membranes. Indeed, it has been reported that ciprofloxacin, erythromycin and rifampicin (65, 130) inhibit intracellular L. pneumophila growth while the β-lactam antibiotics such as ampicillin, cefoxitin and cefotaxime do not (142). This most likely explains the efficacy of erythromycin and rifampicin in the clinical treatment of human legionellosis and emphasizes the need for further in vivo testing of ciprofloxacin as a potential alternative to current clinical therapeutic regimens.

That bacteria with apparently normal morphology were found following extended incubation with all antibiotics examined in this study and in similar experiments performed by Elliott and Rodgers (35) with ampicillin and

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erythromycin may offer further explanation for failure of current antimicrobial therapeutic protocols for the treatment of clinical Legionnaires' disease. In addition, these results considered in combination with reports of increasing incidence of bacterial resistance to standard antibiotic regimens indicated a continued need for thorough in vitro investigation of the response of pathogenic agents to antimicrobial chemotherapy.

These data illustrate the in vitro activity and mode of action of clinically relevant antibiotics for the treatment of Legionnaires' disease. This information is necessary to the understanding and evaluation of subsequent in vivo trials investigating the activity of these agents for the treatment of experimentally infected animal systems and for potential therapy of clinical disease.

Table 4.1 Susceptibility of L. pneumophila to selected antibiotics

Antibiotic	MIC (μg/ml)[1]
Cell wall inhibitors	
cefotaxime	8.0
methicillin	64
(ampicillin	0.4)[2]
Protein synthesis inhibitor	
rifampicin	0.03
(erythromycin	0.5)[2]
DNA inhibitor	
ciprofloxacin	0.08

[1]For viability and ultrastructural studies antibiotics were added to broth cultures at a final concentration of 20 times the MIC, respectively.

[2]Data for ampicillin and erythromycin reproduced with permission (Elliott and Rodgers, J. Med. Microbiol., reference 35) and are included here to complete the comparative profile. Data were derived in a similar manner as for the other antibiotics.

Table 4.2   Morphological response of **L. pneumophila** following exposure to antibiotics for 6 and 24 hours.

Feature   **Antibiotic**

	Cef		Meth		Amp[1]		Ery[1]		Rif		Cipro	
	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h	6h	24h
formation of lesions in cell wall	+	+	+	+	+	+	+	+	+/-	+	+	+
cytoplasmic clearing	+	+	+	+	+	+	+	+	-	-	-/+	+
membrane vesicle formation	+	+	+	+	+	+	+	+	-	-	-	-
spheroplast formation	-	-	+	+	+	+	-	-	-	-	-	-
membrane abnormalities	+	+	+	+	+	+	+	+	+	+	+	+
enlargement of ribosomes	-	-	-	-	-	-	+	+	+	+	+	+
plasmolysed cells	+	+	+	+	+	+	+	+	+	+	+	+
convoluted organisms	+	+	+	+	-/+	+	+	+	+	+	+	+

[1]From reference 35, Elliott and Rodgers, **J. Med. Microbiol.**, with permission. Data included to complete comparative profile.

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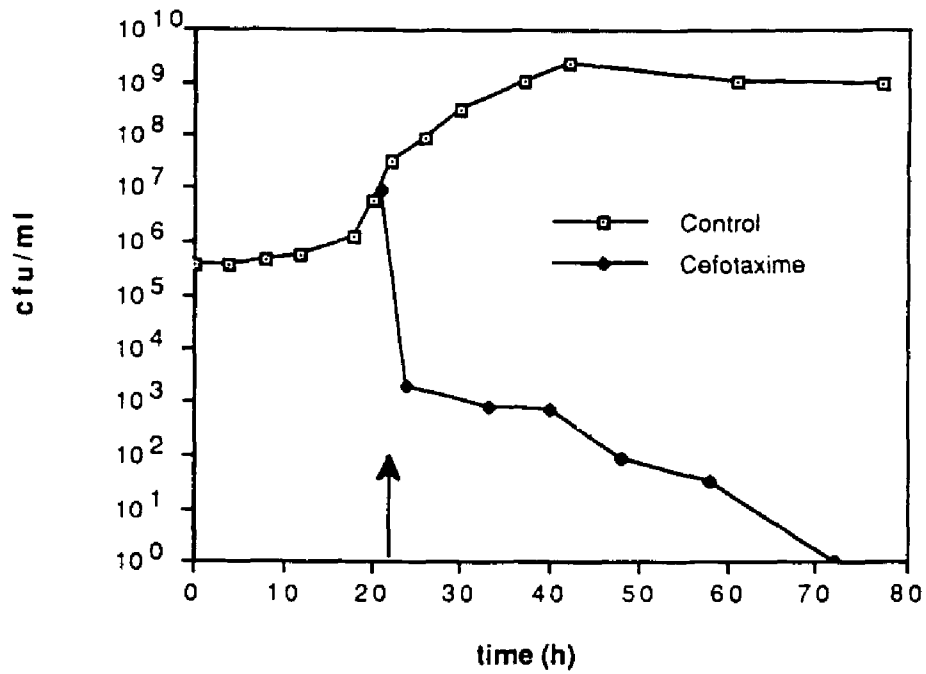
Figure 4.1 Growth of L. pneumophila in BYEα broth (■——■). The effect on bacterial viability of cefotaxime (◆——◆) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic was very effective at reducing bacterial viability.
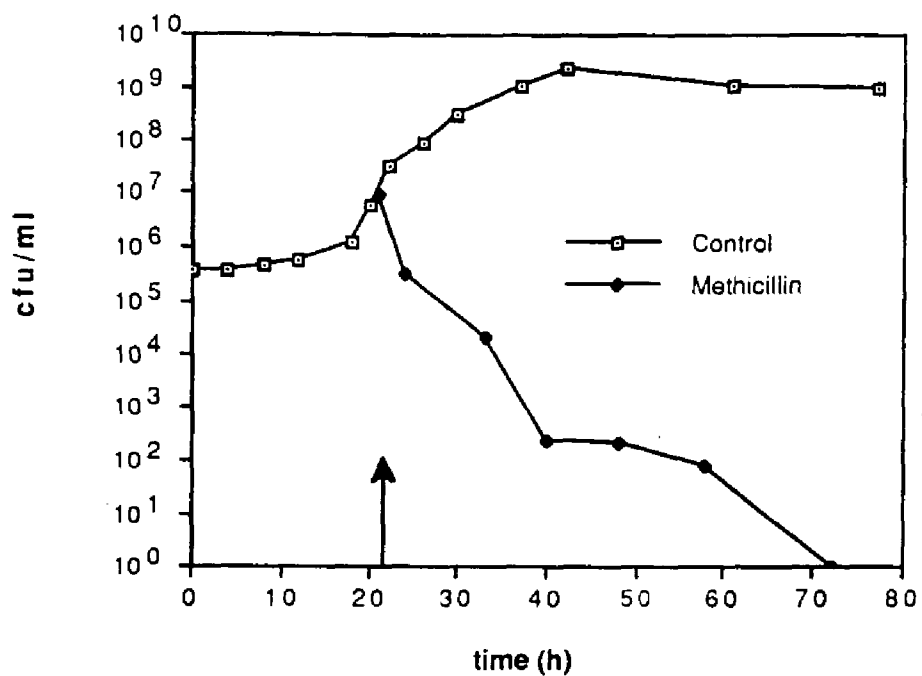
Figure 4.2 Growth of L. pneumophila in BYEα broth (■━━■). The effect on
bacterial viability of methicillin (◆━━◆) added during the mid-exponential
phase of growth (arrow) is shown. This antibiotic was very effective at reducing
bacterial viability.
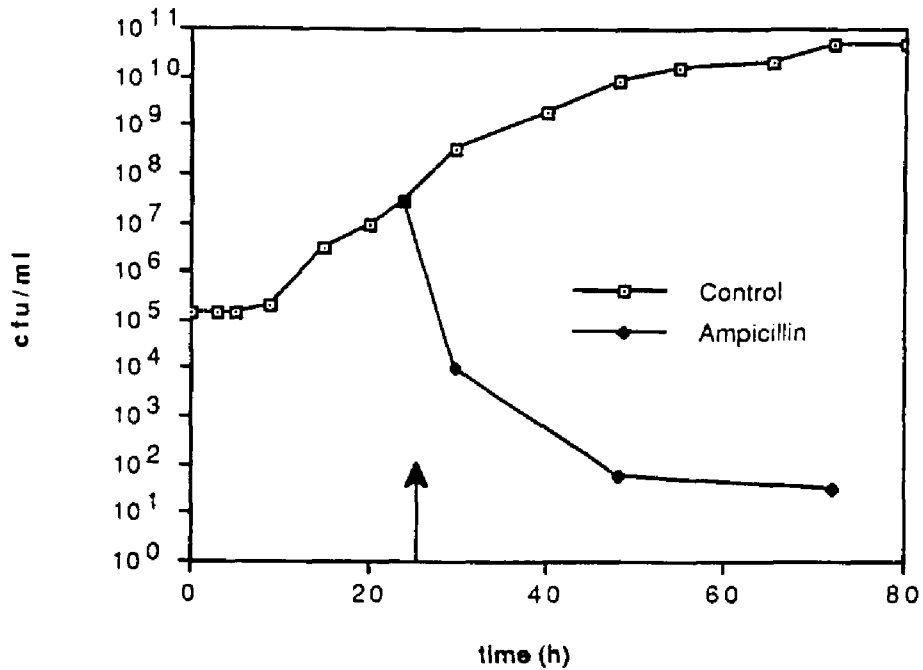
Figure 4.3 Growth of L. pneumophila in enriched blood broth. The effect on bacterial viability of ampicillin added during mid-exponential phase of growth (arrow) is shown. This antibiotic effected a rapid decline in viable counts but was not bactericidal for the entire bacterial population. Data reproduced with kind permission (Elliott and Rodgers, J. Med. Microbiol., reference 35) and included here to complete comparative analysis. Growth of L. pneumophila in this broth had a mean generation time of 2.0 h.
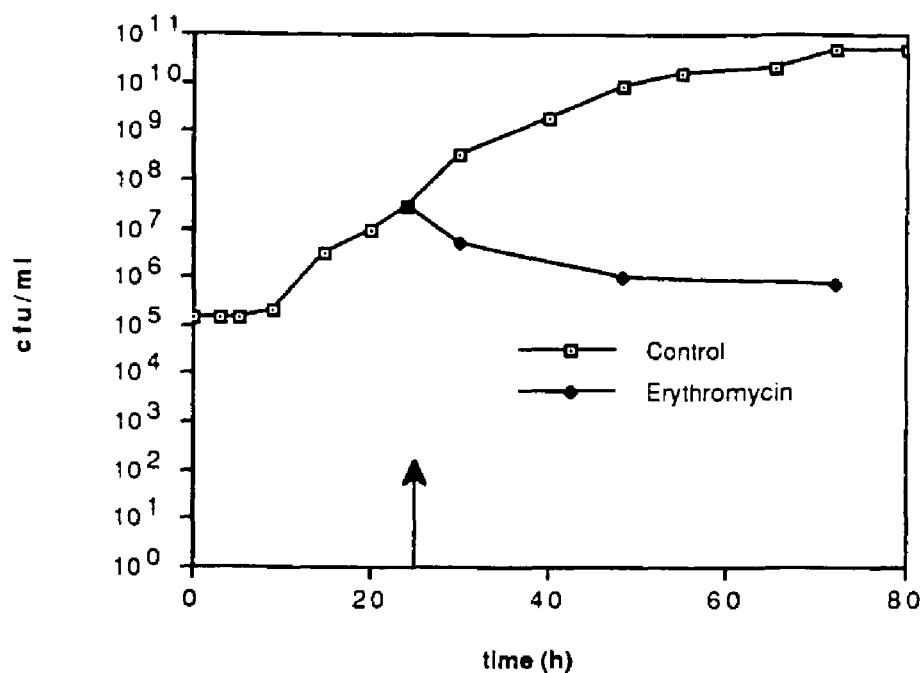
Figure 4.4 Growth of L. pneumophila in enriched blood broth. The effect on
bacterial viability of erythromycin added during mid-exponential phase of
growth (arrow) is shown. This antibiotic was the least effective in terms of
bactericidal activity. Data reproduced with kind permission (Elliott and Rodgers,
J. Med. Microbiol., reference 35) and included here to complete comparative
analysis. Growth of L. pneumophila in enriched blood broth had a mean
generation time of 2.0 h.

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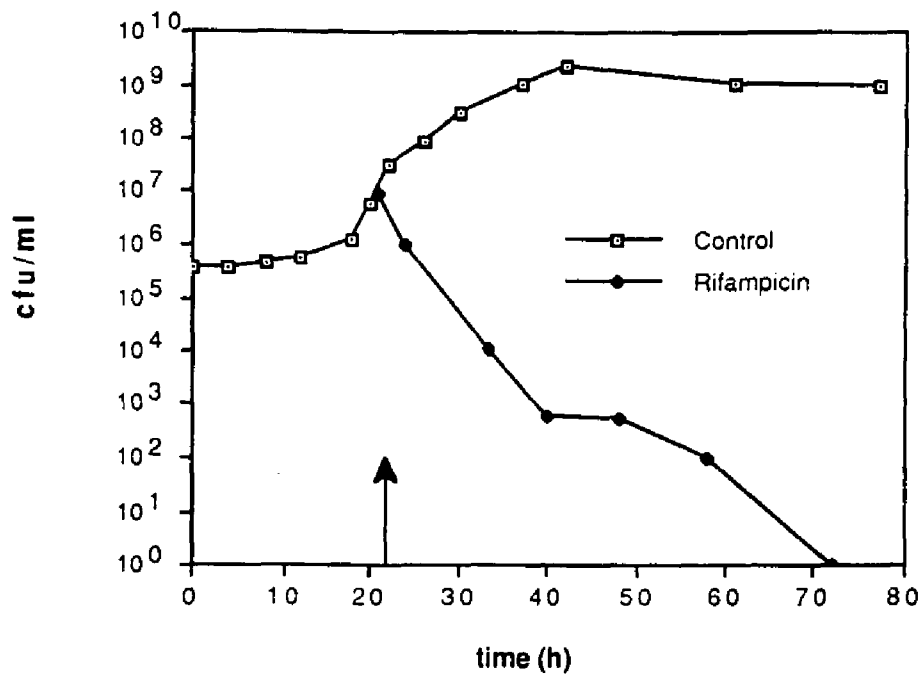
Figure 4.5 Growth of L. pneumophila in BYEα broth (■——■). The effect on bacterial viability of rifampicin (◆——◆) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic showed marked antibacterial activity.
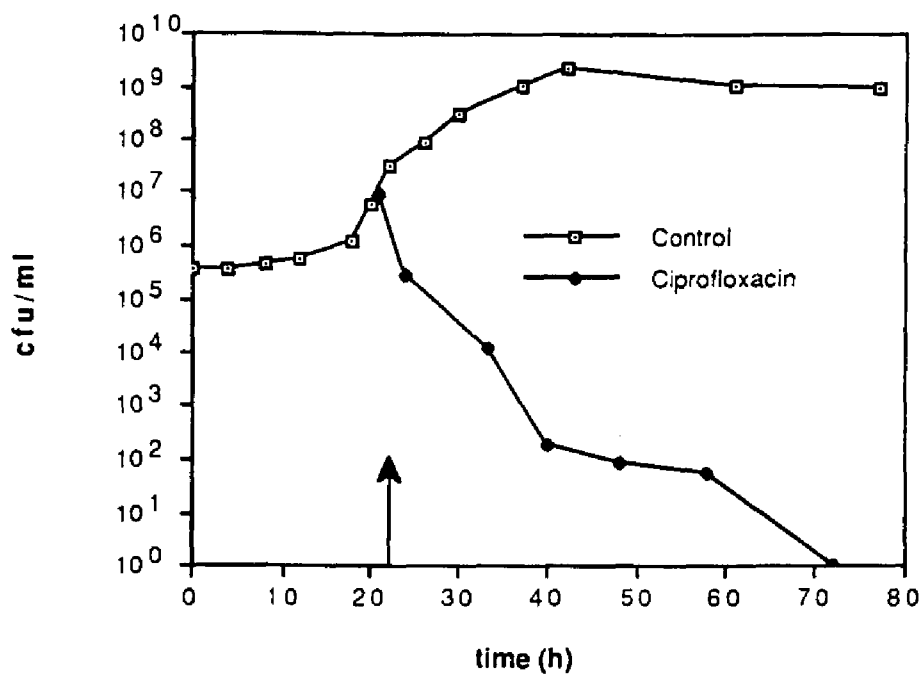
Figure 4.6 Growth of L. pneumophila in BYEα broth (■———■). The effect on bacterial viability of ciprofloxacin (◆———◆) added during the mid-exponential phase of growth (arrow) is shown. This antibiotic showed marked antibacterial activity.

6h   **CEFOTAXIME**   **24h**

| 92 | 8 | | 98 | 2 |

METHICILLIN

| 78 | 22 | | 95 | 5 |

AMPICILLIN

| 78 | 22 | | 95 | 5 |

ERYTHROMYCIN

| 64 | 36 | | 96 | 4 |

RIFAMPICIN

| 55 | 45 | | 96 | 4 |

CIPROFLOXACIN

| 60 | 40 | | 98 | 2 |
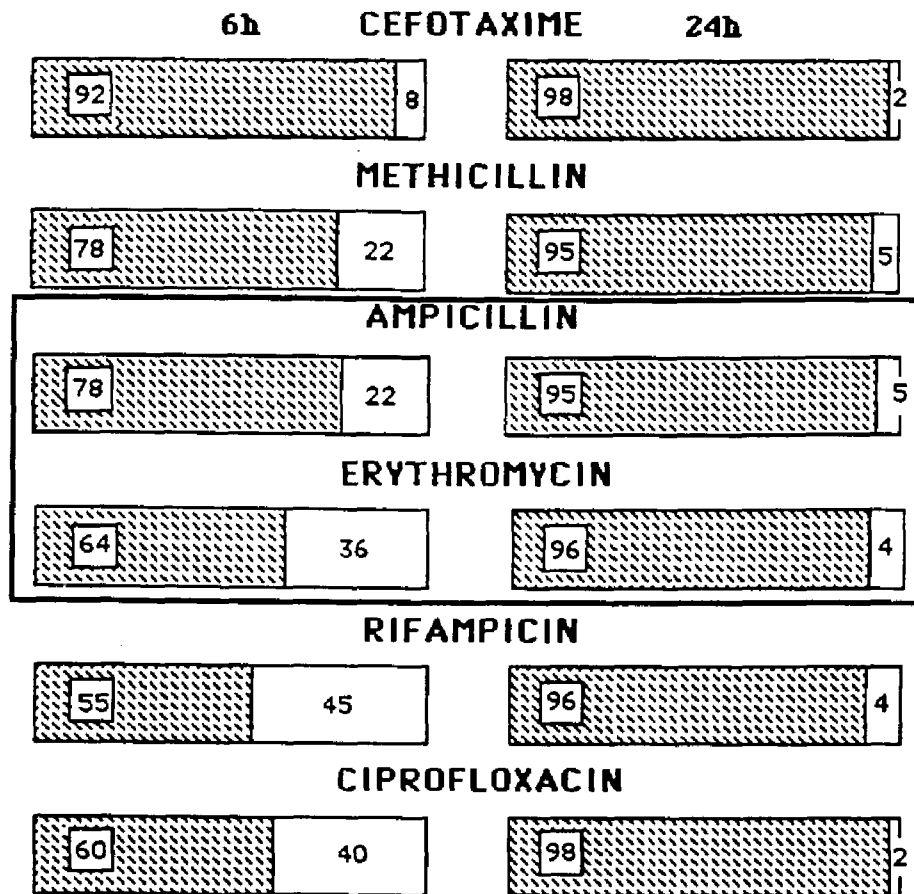
Figure 4.7 Proportion of bacterial cells showing normal morphology (▢) and those damaged (▨) after exposure for 6 or 24 h to the various antibiotics at 20 times their respective MIC values. Percentages were calculated from counts of 100 or more cells for each agent and for each treatment time. Data in boxed area is reproduced with permision (Elliott and Rodgers, J. Med. Microbiol., reference 35) and is included for the purposes of comparison.
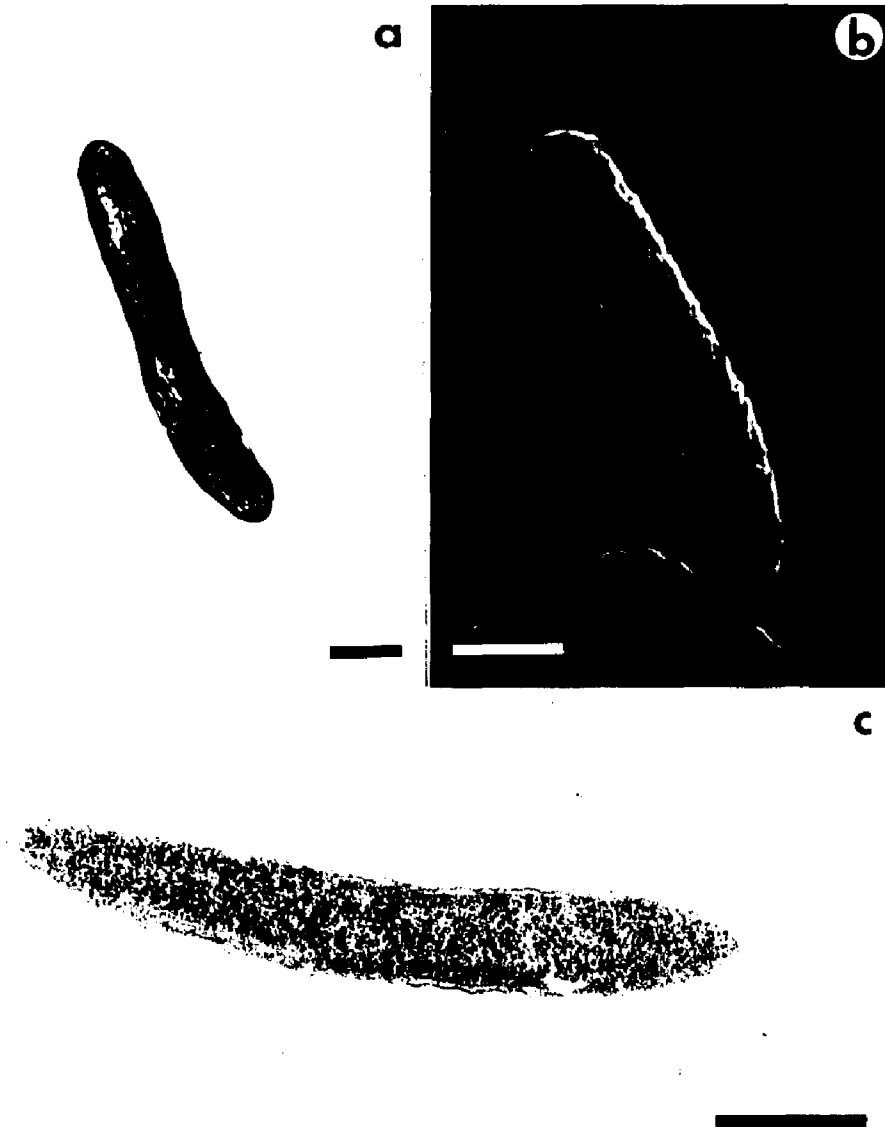
Plate 4.1 Untreated, control Legionella organisms showing normal morphology. The bacterial surface is ruffled and the cell wall structure is composed of a double track membrane with little visual evidence of a peptidoglycan.

a) negative stain (X 18,000), b) scanning (X 30,000), c) thin-section (X 40,000). Bars=0.5 μm.
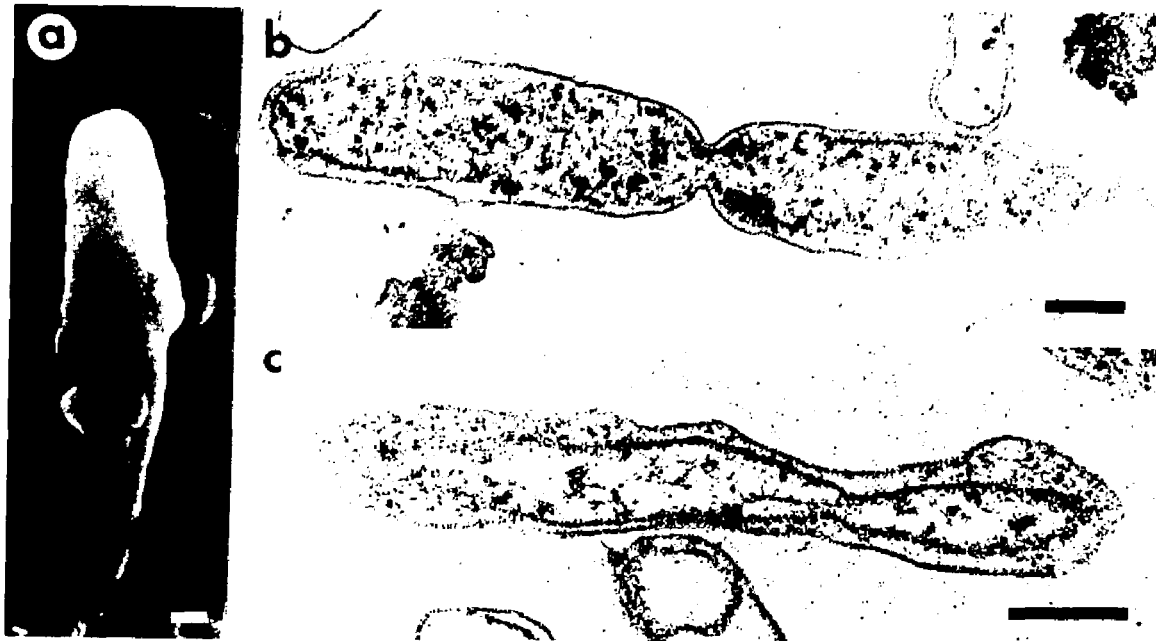
Plate 4.2 L. pneumophila organisms exposed to cefotaxime for 6 h.
a) note bulbous vesicles on the surface of the organism, scanning (X 20,000),
b) organism showing loss of intact membrane structure surrounding diffuse,
electron-lucent cytoplasmic contents, thin-section (X 40,000), c) bacterium with
advanced membrane damage and associated loss of cytoplasm, thin-section
(X 60,000). Bars=0.25 μm.
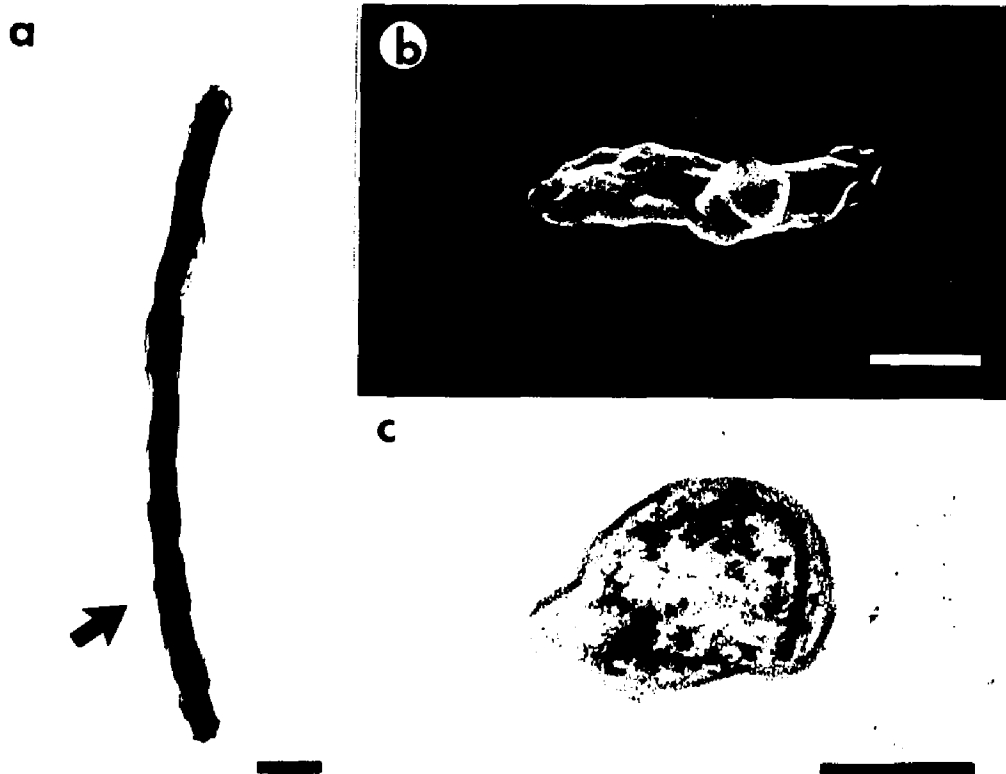
Plate 4.3. Legionellae exposed to cefotaxime for 24 h.
a) formation of lytic points (arrow) in the bacterial cell wall, negative stain (X16,000), b) collapsed organism with abnormal surface structure, scanning (X 30,000), c) plasmolyzed organism with loss of prokaryotic structure, (thin-section (X 80,000). a, b Bars=0.5 μm, c Bar=0.25 μm.

a

b

Plate 4.4 L. pneumophila exposed to methicillin for 6 h.
a) L. pneumophila organism with breakage point in cell wall (arrow), negative-
stain (X 16,000), b) advanced stage of lysis-cytoplasmic contents extruding
through lytic point in the cell wall of an organism, negative-stain (X 18,000).
Bars=0.5 μm.

Plate 4.5 Legionellae exposed to methicillin for 6 h.
a) damaged bacterial cells surrounding an apparently normal cell, thin-section (X 30,000), b) spheroplast formation is evident alongwith deformed and normal cells, thin-section (X 40,000). Bars=0.25 μm.

Plate 4.6 L. pneumophila exposed to methicillin for 6 h.
a) severely damaged cells with abnormal membrane distortion (arrow), thin-
section (X 40,000), b) plasmolyzed organism with pinching and ballooning of
membranes, thin-section (X 80,000). Bars=0.25 μm.

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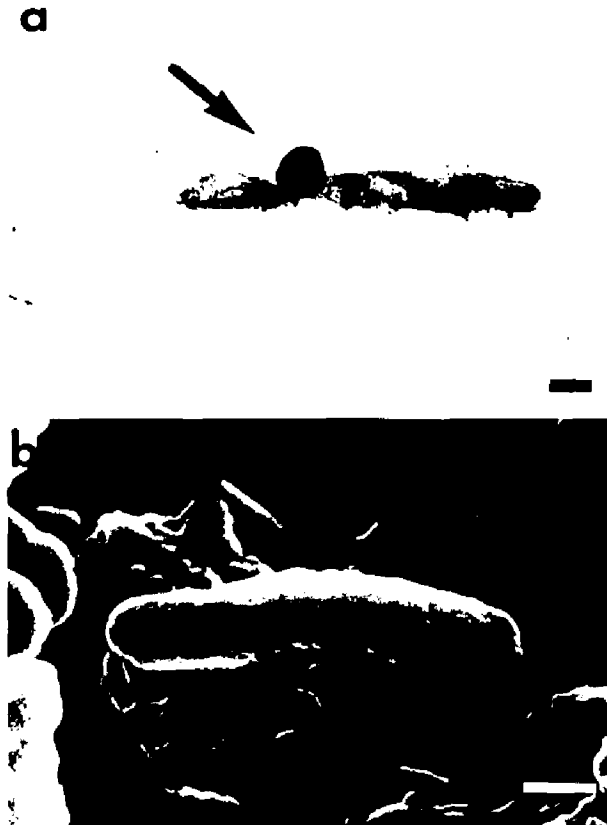
Plate 4.7 Legionella organisms exposed to methicillin for 24 h.
a) note loss of cytoplasmic contents through advanced lytic point in bacterial cell wall (arrow), negative-stain (X 12,000), b) induction of numerous blebs on the microbial surface, scanning (X 20,000). Bars=0.5 μm.

Plate 4.8 L. pneumophila exposed to methicillin for 24 h.
a) complete loss of bacterial cytoplasmic contents with extreme damage to the microbial cell walls, thin-section (X 40,000), b) organism devoid of cytoplasm with associated inner and outer membrane separation, thin-section (X 40,000). Bars=0.5 μm.

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Plate 4.9. Organisms exposed to rifampicin for 6 h.
a) note numerous breakage points in the cell wall and loss of cytoplasm from cell (arrows), negative-stain (X 16,000), b) extrusion of cytoplasm was evident (short arrow) as well as regions of bacterial collapse (long arrow), scanning (X 25,000). Bars=0.5 μm.
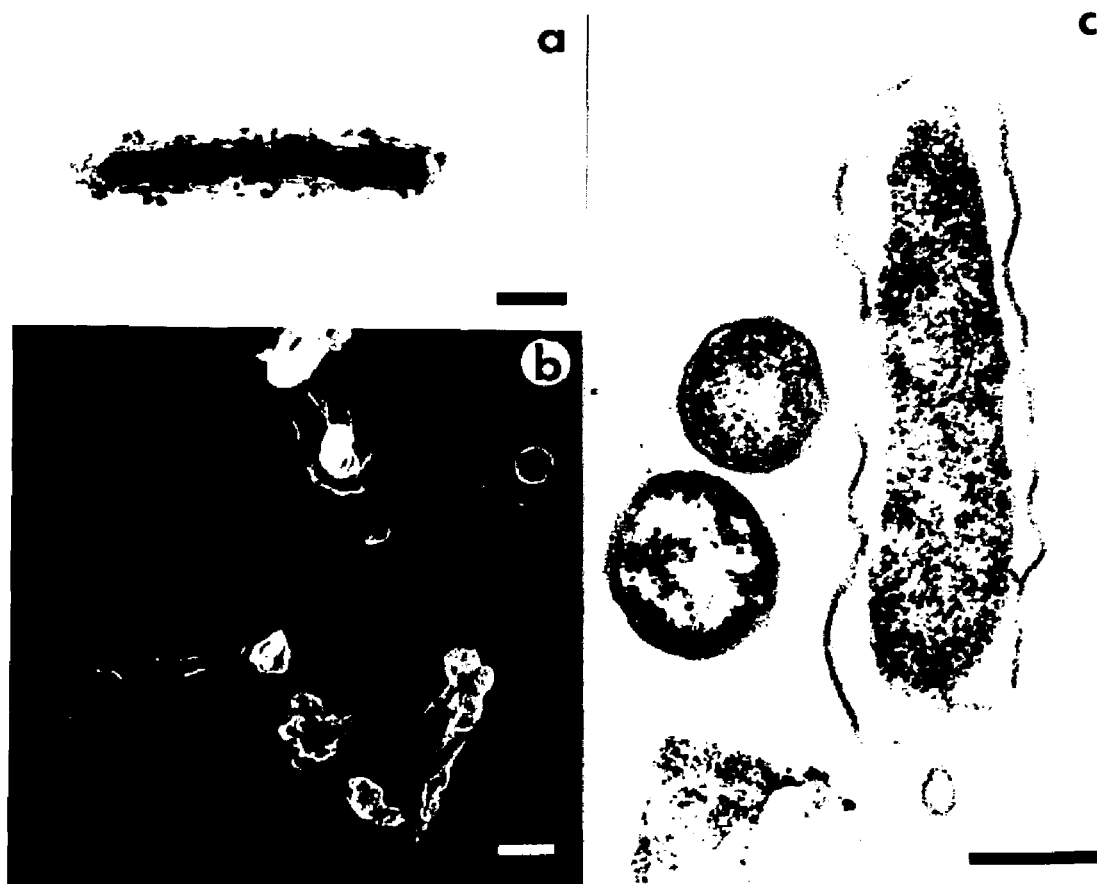
Plate 4.10 L. pneumophila exposed to rifampicin for 24 h.
a) darkly staining surface lesions and numerous small vesicles present,
negative-stain (X 16,000), b) distorted, partially collapsed organisms, scanning
(X 15,000), c) disruption of outer membrane and increased size and density of
ribosomal content of cells, thin-section (X 70,000).

a, b Bars=0.5 μm, c Bar=0.25 μm.

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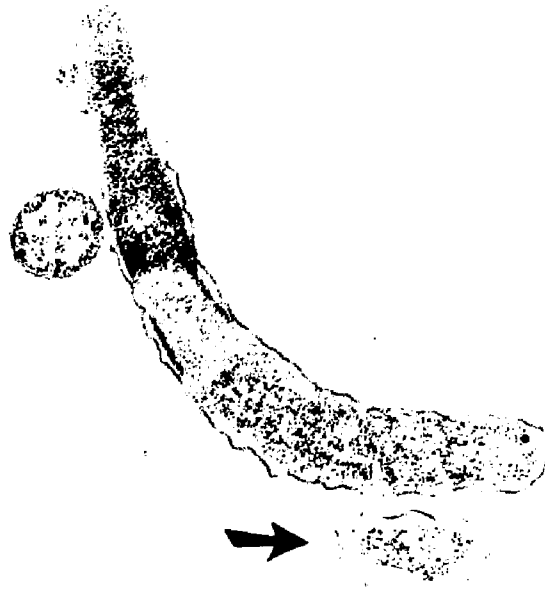
Plate 4.11 Organisms exposed to rifampicin for 24 h. Note localized zones of increased density and size of ribosomes within cytoplasm as well as distorted membrane structure of cells (arrow), thin-section (X 34,000). Bar=0.25 μm.

Plate 4.12 Organisms exposed to rifampicin for 24 h. Increased density of cytoplasmic contents is evident in addition to separation of cell walls from remaining cell contents, thin-section (X 34,000).

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Plate 4.13 L. pneumophila exposed to ciprofloxacin for 6 h.
a) darkly staining lesions in cell wall of a partially collapsed organism, negative-stain, (X 24,000), b) convoluted organism with centrally located pinched zone, scanning (X 20,000). Bars=0.5 μm.
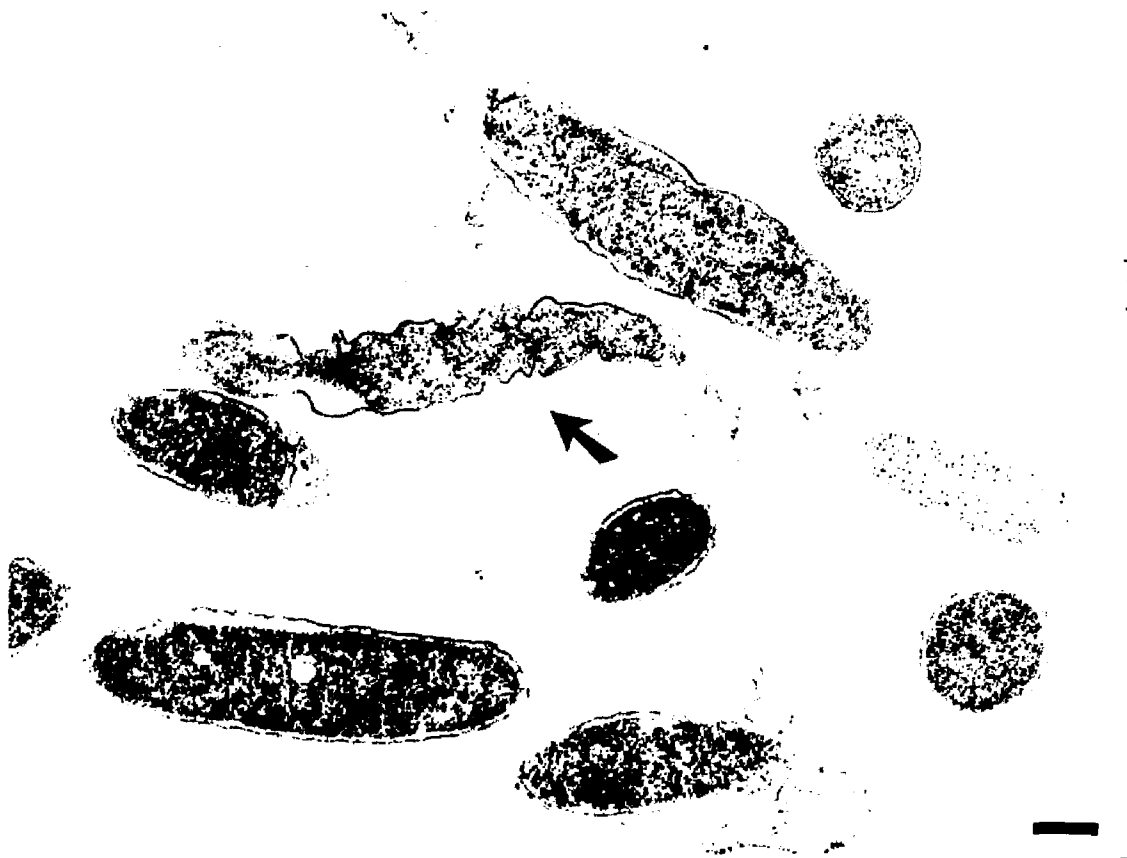
Plate 4.14 L. pneumophila exposed to ciprofloxacin for 6 h. Note severely deformed cell (arrow) with areas of cytoplasmic clearing and abnormal membrane structure, thin-section (X 34,000). Bar=0.25 µm.

Plate 4.15 L. pneumophila exposed to ciprofloxacin for 6 h. Abnormal distribution of ribosomes alongwith areas of localized clearing within the bacterial cytoplasm of cells, thin-section (X 40,000). Bar=0.25 µm.

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Plate 4.16 Legionellae exposed to ciprofloxacin for 6 h. Note vesiculation of bacterial cytoplasm (arrow) and increased size and density of ribosomes, thin-section (X 60,000). Bar=0.25 μm.
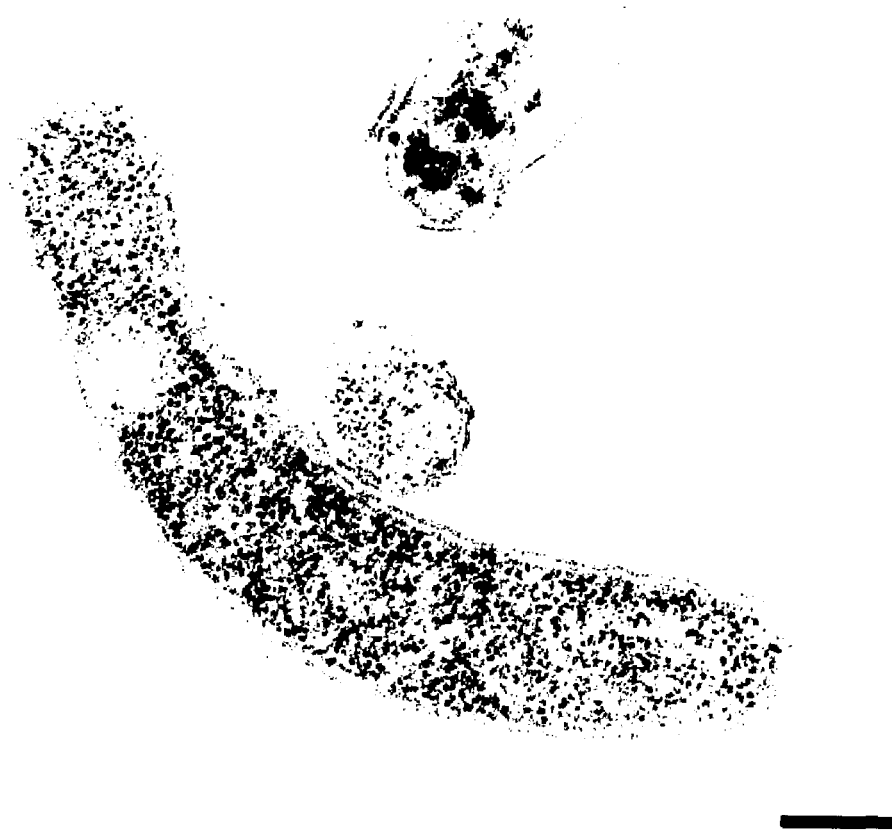
Plate 4.17 L. pneumophila exposed to ciprofloxacin for 6 h. Single cell showing very dense ribosomal contents within bacterial cytoplasm, thin-section (X 60,000). Bar=0.25 μm.
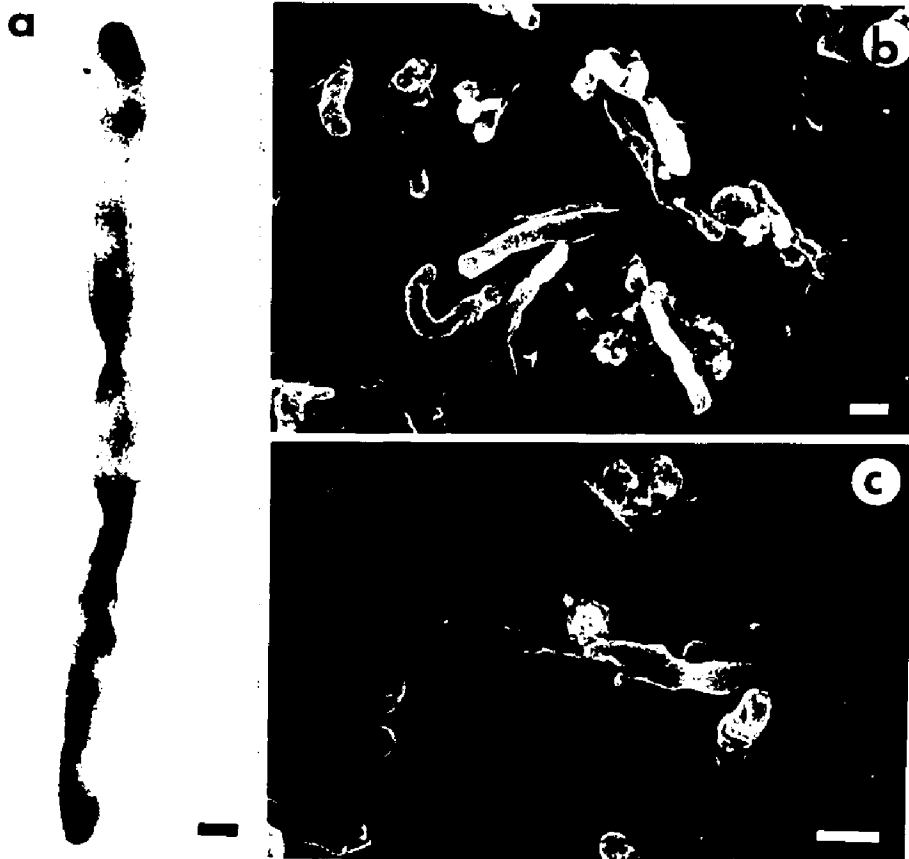
Plate 4.18 L. pneumophila exposed to ciprofloxacin for 24 h.
a) twisted, convoluted organism with multiple pinched zones, negative-stain (X 12,000), b) filamentous, twisted organisms are evident, scanning (X 10,000), c) partially collapsed organism with centrally located pinched zone, scanning (X 34,000). a, b Bars=0.5 μm, c Bars=0.25 μm.
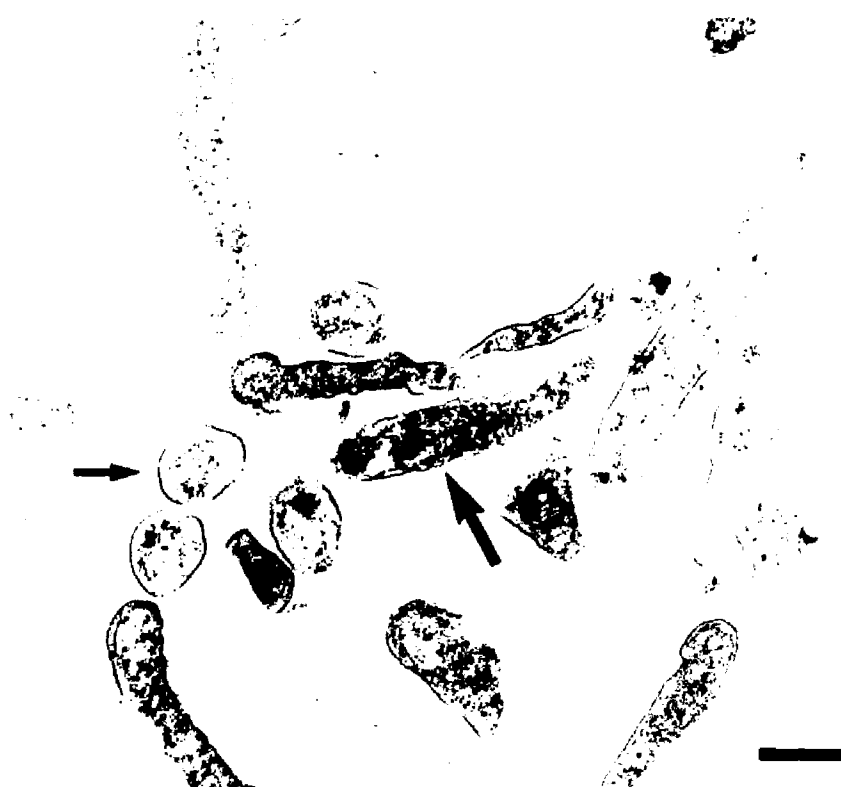
Plate 4.19 L. pneumophila exposed to ciprofloxacin for 24 h. Organisms showing various types of morphological response to treatment. Some cells have lost their cytoplasmic contents (short arrow) while others exhibit increased ribosomal density (long arrow). Note twisted, abnormally shaped cell morphologies, thin-section (X 24,000). Bar=0.5 μm.

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Section V

Pathogenesis, Pathology and Chemotherapy of Experimental Legionella pneumophila Infection in the Chick Embryo

5.1 Abstract

The pathogenicity of Legionella pneumophila, serogroup 1, strain Nottingham N_7, was assessed in terms of LD_{50} data and the ability of the organism to induce histopathological and ultrastructural lesions in the fertile hen's egg. Histological examination of embryo organs after inoculation with 1, 10, 100 and 1000 times the yolk sac LD_{50} revealed a disseminated infection. Systemic spread of the organism resulted in the appearance of pathological lesions and the generation of edema fluid. These were particularly severe in the liver, heart, spleen and kidney. Electron microscopy of the pathology induced in organs of chick embryos previously infected with 100 times the $YSLD_{50}$ of L. pneumophila confirmed these observations. Subtle pathological changes were noted three days post-inoculation while more extensive cellular and sub-cellular necrotic changes occurred at four days post-inoculation. The infection elicited an inflammatory cell response that consisted of polymorphonuclear leucocytes and lymphocytes. Selected antimicrobial agents were investigated in therapeutic studies for their capacity to ameliorate or control disease processes in this test system. Of those examined, ciprofloxacin was most effective at reducing or reversing the incidence of

lesions in these tissues and for prolonging embryo viability. Rifampicin, and to a lesser degree, erythromycin and doxycycline, also showed antimicrobial activity in these in vivo trials. These results illustrated the efficacy of the fertile hen's egg as a useful alternative in vivo assay system for the evaluation of clinically putative antimicrobial agents in the treatment of Legionnaires' disease. In addition, this study demonstrated the superior antimicrobial activity of ciprofloxacin in the treatment of experimentally induced Legionella infections.

5.2 Introduction

L. pneumophila is the etiological agent of legionellosis which in the severe pneumonic form presents as Legionnaires' disease (85). Despite the accumulation of considerable data describing the invasive nature of the microorganism, the pathogenic mechanisms by which this gram-negative rod causes infection are not well understood. In human disease and experimental animal infections, the legionellae are facultative intracellular pathogens which survive and replicate within alveolar macrophages.

Although primarily recognized as a pneumonic illness, Legionnaires' disease has been shown to produce a widespread bacteremia alongwith the appearance of soluble L. pneumophila antigen in clinical samples of serum and urine (12) in association with extrapulmonary pathology (13, 115). Multi-organ inflammatory lesions due to Legionella bacteremia are not common but have been described (24). Because Legionnaires' disease first presents as a classic pneumonic infection, the extra-thoracic aspects of the disease have been overlooked, but it is evident that the serious pathological sequelae that result from L. pneumophila infection may play a larger role in the overall disease process than was previously suspected.

Numerous antibiotics with different modes of action have shown activity against L. pneumophila in vitro (30, 35, 60, 109, 112, 123). However, many of these antibiotics with effective minimum inhibitory concentrations (MIC) are reported as ineffective in the treatment of Legionnaires' disease (82) while others such as erythromycin, rifampicin, and to a lesser extent, the tetracyclines

have been efficacious in therapy (2). Erythromycin is considered the drug of choice for Legionnaires' disease but variable clinical responses in patients to this antibiotic and problems associated with low dose or intravenous erythromycin therapy have been reported (62). Consequently, other therapeutic options are currently sought.

The quinolones are antimicrobial agents that effectively inhibit bacterial DNA gyrase activity. One member of this group, ciprofloxacin, is a promising new lipid soluble antibiotic with broad spectrum activity and the ability to penetrate eukaryotic cells. This antibiotic has few side-effects, offers oral bioavailability, achieves high tissue concentrations and has been used successfully in the treatment of various urinary and respiratory tract infections. Ciprofloxacin has been shown to be very active against L. pneumophila in vitro (60, 65) and in experimental Legionella infections of guinea pigs (44, 45, 135).

Because treatment of human legionellosis often commences well after onset of disease, controlled clinical studies exploring the use of alternative chemotherapeutic agents are difficult to perform and force researchers to depend on animal studies for data accumulation. Although in vivo testing of antibiotic efficacy in guinea pigs has thus far correlated well with clinical data, investigators using this animal system for these studies require specialized equipment, animal laboratory facilities, specialized expertise, and, according to current regulations, qualified staff, animal care and use permission, and in some countries animal licenses. In addition, the cost and availability of these animals limits the use of significant numbers in experiments and makes

interpretation of data difficult. It is clear that the ability to test alternative therapeutic regimens for the treatment of Legionnaires' disease has become increasingly important and necessitates the development of a simplified and reproducible in vivo antimicrobial assay system.

Lewis et al. (83) introduced the use of embryonate eggs to examine in vivo prophylactic and post-infection antibiotic therapy of experimental legionellosis following the initial outbreak of Legionnaires' disease in Philadelphia in 1976. This study evaluated antimicrobial therapy based on the ability of antibiotics to reduce mortality rates of infected embryos and yielded results in keeping with clinical findings. In the present study, the chick embryo was used to delineate the baseline histological and ultrastructural pathology associated with the progression of experimental infection in this animal with L. pneumophila in order to determine the therapeutic value of antimicrobial agents introduced into this animal following experimental infection. Results from these investigations were used to evaluate the chick embryo as an alternative in vivo assay system for the testing of new and/or putative antimicrobial agents in the treatment of human legionellosis. In addition, the quinolone antibiotic ciprofloxacin was examined for its chemotherapeutic activity and compared with clinically relevant antibiotics for its effectiveness at regulating infection in the chick embryo animal system. Elucidation of the mechanisms of pathogenesis and growth of L. pneumophila in this in vivo host system and the role of antimicrobial therapy in the disease process may have an impact on current procedures for the maintenance and care of patients with legionellosis.

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5.3 Materials and Methods.

Reagent formulations and preparations along with detailed procedures are given in Appendices 1, 2, 3, and 4.

Preparation of bacterial inocula. L. pneumophila, serogroup 1, strain Nottingham N_7, was isolated from the sputum of a fatal case of Legionnaires' disease, subsequently passaged twice on bacteriological media and maintained frozen at -70° C in 10% serum with added 1% sorbitol. Thawed aliquots were grown at 37°C on buffered charcoal yeast extract agar enriched with L-cysteine, ferric pyrophosphate and α-ketoglutarate (BCYEα) (29) and incubated aerobically for 48 hours. Organisms were harvested in 5 ml phosphate buffered saline pH 6.9 (PBS) to give a density equivalent to 10^8 colony forming units (cfu)/ml as determined by a Klett-Summerson photoelectric colorimeter. Organism mass, expressed in terms of viability, was determined in duplicate as cfu on BCYEα to confirm opacity data.

Egg type, incubation, inoculation route and determination of $LD_{50}s$. Antibiotic free, fertile White Leghorn hens' eggs (UNH Poultry Farm) were incubated at 36°C in a humid atmosphere. Organisms were introduced into eggs by four inoculation routes: allantoic and amniotic sacs (at 10 days of embryo incubation), chorioallantoic membrane (CAM) and yolk sac (at 7 days of embryo incubation) (Figures 5.1 and 5.2). Sixty embryonate eggs were used for each inoculation route. Serial 10-fold dilutions of L. pneumophila in PBS were made and those ranging from 10^1 to 10^6 cfu/ml were inoculated at a rate of 10

eggs per dilution. Inoculated eggs were incubated and candled twice daily to check for embryo viability. Mortality and protection experiments were performed three times and the LD_{50} for each inoculation route was determined from the averaged mortality data by the method of Reed and Muench (101).

Recovery of organisms and histopathology studies. Organism mass was calculated as described and the inocula adjusted to give inoculation doses equivalent to 1, 10, 100 and 1000 times the yolk sac LD_{50} ($YSLD_{50}$). Each multiple dose LD_{50} of L. pneumophila was inoculated into the yolk sacs of fertile eggs and these were harvested at 24 hour intervals from one to eight days post-inoculation. Organ samples including liver, heart, spleen and kidney were removed aseptically from embryos, rinsed thoroughly in PBS and weighed. Organs were homogenized in a Sorval Omni Mixer, serially diluted and aliquots inoculated onto BCYEα for viable count assay. Colony counts were expressed as cfu/gram of tissue and were calculated for each organ at each time interval in triplicate. Similarly inoculated and control eggs were harvested and whole embryos as well as samples of liver, heart, spleen and kidney were fixed in 10% buffered formalin and embedded in paraffin wax in a Lab Tek Tissue Processor. Four micron thick sections were cut, deparaffinized and routinely stained with hematoxylin and eosin for histopathological examination.

Immunofluorescence. Deparaffinized tissue sections and tissue homogenates were incubated with a 1 in 50 dilution of rabbit anti-L.

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pneumophila serum, followed by incubation with a 1 in 200 dilution of FITC-conjugated goat anti-rabbit serum (Cappel Laboratories, Malvern, Pa.). All labelling steps were followed by 3-fold washings in PBS to remove unbound globulins.

Electron microscopy studies. Eggs were inoculated with 100 times the $YSLD_{50}$ by the yolk sac route, and the embryo organs, liver, heart, spleen and kidney, harvested at 24 h intervals and prepared for electron microscopic examination. Tissue samples were rinsed in 0.1 M cacodylate buffer, pH 7.2 (CB) fixed in 3% (v/v) glutaraldehyde in CB for 24 hours, post-fixed in 1% (w/v) osmium tetroxide (OsO_4) in CB for two hours and dehydrated in a graded ethanol series. Samples were embedded in an epon-araldite resin mixture and polymerized for 24 hours at 60° C (105, 108). Thin sections, cut on an LKB Ultratome III with a diamond knife, were stained with 5% (w/v) uranyl acetate and 0.4% (w/v) lead citrate and examined by transmission electron microscopy in a Hitachi H600 electron microscope used at 80kV.

Antibiotic therapy studies. MIC values were calculated for ciprofloxacin (Miles Pharmaceuticals, West Haven, Ct.), erythromycin, doxycycline and rifampicin (Sigma Chemicals, St. Louis, Mo.) by a standard agar dilution technique (133) using BCYEα. For these experiments, eggs were infected by the yolk sac route with 100 times the $YSLD_{50}$ of L. pneumophila.

For histopathological studies, one or ten times the MIC of each of the four antimicrobial agents were administered intra-allantoically at one day post-

inoculation. Mortality data were calculated with time for each antibiotic and bacterial viable counts determined from embryo organ homogenates. Similar samples were harvested and prepared for histological examination to assess the ability of each antibiotic to inhibit either an inflammatory cell response or the development of pathological lesions in embryo organs.

Antibiotic therapy of chick embryos was commenced at three days post-infection for electron microscopic investigations. This schedule was selected because electron microscopic studies of untreated control samples showed that this time interval allowed for the earliest expression of ultrastructural pathological lesions to develop after the introduction of L. pneumophila into the chick embryo. In such treatments, 10 times the MIC levels of each of the four antibiotics were administered intra-allantoically and organ samples of liver and kidney from viable embryos were harvested for electron microscopy at four and eight days post-inoculation (one and five days post-therapy, respectively).

5.4 Results

Pathogenesis. The LD_{50} values for L. pneumophila introduced into the embryonate egg were determined and are illustrated in Table 5.1. The inocula required to induce embryo death ranged from 1.4×10^2 cfu/ml for the amniotic sac to 5.5×10^4 cfu/ml for the allantoic sac. The LD_{50} studies demonstrated that those inoculation routes which accessed the immediate environment or the nutritional source of the embryo required lower organism numbers to induce fatal infections compared with more peripheral routes.

Inoculation of multiple doses of the $YSLD_{50}$ into fertile hens' eggs

resulted in dissemination and growth of Legionella organisms in the liver, heart, spleen and kidney. A tissue response and dose effect was observed in that viable counts on harvested organs of embryos infected with 1 or 10 times the $YSLD_{50}$ revealed that exponential bacterial growth began at four days post-inoculation in the liver and heart but after five days in the spleen and kidney (Figures 5.3 and 5.4). In addition, bacterial assays of organs from embryos inoculated with 100 and 1000 times the $YSLD_{50}$ showed that organism growth began at four days post-inoculation in all examined organs (Figures 5.5 and 5.6). Microbial assay of tissues at eight days post-inoculation with 1000 $YSLD_{50}$ yielded counts ranging from 3.3 X 10^4 to 9.9 X 10^5 cfu/gram in the kidney and liver, respectively (Figure 5.6). Organism concentration in organs was highest in the liver and lowest in the kidney irrespective of the multiple dose used as inoculum.

Histopathology. Gross examination of the organs of embryos infected by various routes of inoculation with multiple doses of the $YSLD_{50}$ showed no ostensible lesions. Inoculated CAMs failed to demonstrate visible pocks or pathological changes. Following yolk sac inoculation, histopathological examination on organs of embryos infected with 1, 10, 100 and 1000 times the $YSLD_{50}$ revealed pathological changes in the liver, heart, spleen and kidney and were most evident in regions rich in blood vessels (plates 5.1-5.5). With inocula of 1, 10, or 100 times the $YSLD_{50}$ an inflammatory cell response appeared simultaneously at five days post-inoculation in all organs examined

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but at four days following administration of 1000 times the $YSLD_{50}$. At this stage, congestion of the blood vessels within tissues were most evident (plates 5.1-5.2). Heart tissue showed marked vascular congestion with evidence of an edematous response (plate 5.2). In the kidney, pathological changes in the glomeruli, with an associated cell exudate, were evident as compared with the relatively undamaged convoluted tubules (plate 5.4).

Histopathological examination of sections at higher magnification showed degenerative in each organ typified by breakdown of cell and nuclear membrane structure, loss of cell components, and margination of nuclear chromatin. Darkly staining PMNLs and leucocytes were also common (plate 5.5). These results are summarized in Table 5.2.

Immunofluorescence. The presence of L. pneumophila organisms in situ in tissue sections and homogenates was confirmed by specific immunofluorescence. Labelled organisms were visualized along with tissue damage in liver, heart, spleen and kidney sections of embryos previously inoculated with multiple doses of the $YSLD_{50}$.

Ultrastructural pathology. Electron microscopic examination of the liver, heart, spleen, and kidney of infected chick embryos revealed extensive pathological damage at the cellular and sub-cellular levels. These observations are summarized in Table 5.3. The ultrastructural tissue damage induced by the organism was similar in all organs examined and the earliest, most tenuous pathological changes were observed at three days post-inoculation. Mitochondrial damage was evident at this time (plate 5.6 and 5.7).

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Major ultrastructural changes occurred at four days post-inoculation and consisted of extensive degenerative changes which included fatty degeneration and cytoplasmic clearing. These changes were characterized by ballooning and breakdown of the cellular and nuclear membranes, nuclear clearing, margination of chromatin material, and cytoplasmic vesiculation (plate 5.8). A hemolytic process (plate 5.9) was evident in these tissues. During latter stages of the infection, many lysed, swollen or apparently empty mitochondria were distributed throughout the cytoplasm of affected cells. At this stage, the nuclei of many cells exhibited pyknosis or karyolysis (plate 5.10-5.12). In addition, lysosomal bodies were present within cells presumably involved with autolysis of these tissues. Occasionally, small numbers of legionellae were observed within cells of embryo organs (plate 5.13).

Antibiotic therapy studies. The MICs of ciprofloxacin, doxycycline, erythromycin and rifampicin for L. pneumophila are shown in Table 5.4. Of the four antibiotics, ciprofloxacin at 10 times the MIC proved most effective in delaying tissue damage and preventing an inflammatory cell response prior to eight days post-inoculation as assessed by histopathological examination. Erythromycin, doxycycline or rifampicin effected a delay in the development of these lesions, but their effect was much less pronounced. These results are shown in Table 5.4. Only embryos protected with ciprofloxacin survived to term. In addition, the efficacy of these antibiotics was evaluated in bacterial viable count assays performed on organs of embryos previously infected eight days previously with 100 times the $YSLD_{50}$ of L. pneumophila. Results are shown in

Figure 5.7. At 10 times their MIC, ciprofloxacin was more efficient at reducing organism numbers in these tissues than either erythromycin or rifampicin.

Figure 5.8 shows the results of embryo viability after inoculation with 100 times the $YSLD_{50}$ of L. pneumophila with administration 24 hours later of 10 times the MIC levels of ciprofloxacin, erythromycin or rifampicin. Ciprofloxacin gave the highest percentage of embryo survival throughout the trials. Eight days post-inoculation the survival rate for embryos protected with ciprofloxacin was 83% compared with rifampicin at 33% or erythromycin at 10%. By 14 days post-inoculation all rifampicin or erythromycin treated embryos had succumbed to infection.

Ultrastructural observations of infected embryo organs treated three days subsequently with 10 times the MIC of these antibiotics showed similar changes and are summarized in Table 5.5. Only viable embryos were processed for electron microscopic examination. Ultrastructural tissue damage in erythromycin (plate 5.14) and doxycycline (plate 5.15) treated embryos was less severe than unprotected tissues at four days post-inoculation. Indeed, the cellular changes at this stage in the presence of erythromycin were similar in appearance to those at day three of infection in the absence of antibiotic. Tissues from rifampicin treated embryos also showed less damage than controls and were comparable at four and eight days post-inoculation (plates 5.16 and 5.17). However, tissues from ciprofloxacin treated embryos were found to be consistently less damaged at eight days than at four days post-infection (plates 5.18 and 5.19) indicating that the degenerative process

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associated with infection at the ultrastructural level had been reversed.

5.5 Discussion

In keeping with current pressures and legislation to reduce vertebrate animal use in research and diagnosis, the self-contained embryonate hen's egg offers an alternative in vivo antibiotic assay system for L. pneumophila. Eggs are relatively easy to handle, inexpensive, and can be used in sufficiently large numbers to test each antibiotic so as to give significant assessment levels normally unattainable with other laboratory animals. In addition, no specialized animal facilities, equipment, personnel or licenses are required.

The LD_{50} studies revealed the high degree of pathogenicity of L. pneumophila for this animal. These findings reflected the relative few numbers of microorganisms required to achieve these mortality data and illustrated the extreme infectious nature of the bacterium for the chick embryo and its tissues. The rapid growth of L. pneumophila in the liver, heart, spleen and kidney following inoculation with multiple doses of the $YSLD_{50}$ indicated that growth of the organism in the chick embryo was characterized by a bacteremia with satellite infections in these organs. Microbial assays also revealed that irrespective of the inoculum dose, extensive organism growth occurred within organs all organs examined, most notably in the liver but to a lesser degree in the kidney. These data supported similar observations by other workers that organisms were found within the extrapulmonary organs of humans and animals previously infected with L. pneumophila.

Correlation exists between human legionellosis and experimental

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infections in various animal models. Pyrexia, antibody production, cell-mediated immunity and bacteremia have been demonstrated in various laboratory animals. In addition, the production of inflammatory cells in response to experimental and natural infections with the organism is often similar and consists of a mixed infiltrate of PMNLs and macrophages. Histopathological examination of the liver, heart, spleen and kidney of the chick embryo following inoculation with multiple doses of L. pneumophila revealed the simultaneous appearance of vascular cells in all organs and suggested a large scale inflammatory cell response consisting primarily of PMNLs and lymphocytes.

Experimental infection of fertile hens' eggs with L. pneumophila elicited severe cellular and sub-cellular pathological changes in all embryo organs examined by electron microscopy. That ultrastructural tissue damage was found to be similar and occurred synchronously three days post-inoculation in these organs supported histopathological observations of a systemic-type infection in this animal (128). The ultrastructural appearance of tissues of infected embryo organs was comparable to similar features exhibited in human legionellosis (20, 21, 30, 105, 111).

Although L. pneumophila is sensitive to a variety of antimicrobial agents in vitro, the ability of this pathogen to replicate intracellularly limits the use of many of these agents in vivo. Antibiotics which have been used successfully in treatment include erythromycin, rifampicin and the tetracyclines. Animal experiments with guinea pigs previously exposed to aerosol suspensions of L. pneumophila and treated with clinically proven antibiotics readily demonstrated

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the efficacy of rifampicin and to a lesser extent erythromycin. Effective antimicrobial agents were able to decrease mortality and clear viable L. pneumophila from the lungs but only rifampicin was able to reverse histopathological lesions (57). Similar results were obtained using L. pneumophila infected fertile hens' eggs in which mortality data was reduced by treatment with rifampicin and erythromycin administered either prophylactically or post-infection (83). To date, additional antimicrobial investigations have not been performed to corroborate and extend these results.

Ciprofloxacin has shown excellent in vitro activity against L. pneumophila in preliminary studies with agar and broth dilution tests as well as cell cultures (65). Effective ciprofloxacin treatment of experimental Legionella infection in guinea pigs has also been demonstrated. In these studies, ciprofloxacin was as effective as rifampicin and better than erythromycin in preventing death of infected guinea pigs (44).

In the present study, the fertile hen's egg was used as an in vivo assay system for the critical evaluation of clinically useful antibiotics. Antimicrobial agents were administered one day post-inoculation and tested for their ability to delay the development of histopathological lesions and the appearance of infiltrative cells, reduce organism numbers in tissues and decrease embryo mortality. Ciprofloxacin, erythromycin, doxycycline and rifampicin were all effective against L. pneumophila infection of the embryonated egg in all aspects of testing as compared with PBS controls. However, results from these investigations demonstrated the superior activity of ciprofloxacin in these in vivo

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trials.

The treatment of human legionellosis often commences well after the disease process is initiated. In this study, the progression of disease was monitored at the ultrastructural level and antimicrobial therapy administered to eggs to correspond with the appearance of the earliest cellular and sub-cellular pathological changes in tissues. It was evident that ciprofloxacin was the most effective antibiotic investigated based on its consistent ability to reverse ultrastructural lesions induced in response to infection with L. pneumophila.

Although no animal system completely simulates the conditions of human therapy, these results established the efficacy of the chick embryo to evaluate clinically putative antibiotics for the treatment of human legionellosis and to investigate the pathology associated with otherwise fatal experimental L. pneumophila infections. The usefulness of ciprofloxacin in the treatment of such infections in this animal was also demonstrated in all aspects of these trials. Rifampicin, erythromycin and doxycycline also showed antimicrobial activity in this assay system, but to a lesser degree. Evidence from this and other reports suggested that the use of ciprofloxacin for the treatment of L. pneumophila experimental infections in animals requires further investigation. In addition, studies are necessary to determine whether the use of this drug may be efficacious in the treatment of clinical legionellosis.

Table 5.1 LD_{50} values for <u>L. pneumophila</u> in the embryonated hen's egg.

Route of Inoculation	cfu/ml
allantoic sac	5.5×10^4
amniotic sac	1.4×10^2
chorioallantoic membrane (CAM)	3.3×10^4
yolk sac	6.6×10^2

Table 5.2 Histopathological observations on organs of embryos inoculated with 1, 10, 100 or 1000 times the $YSLD_{50}$ of L. pneumophila.

	Days post-inoculation	
	1, 2, 3, 4	5, 6, 7, 8[1]

Feature		
systemic degenerative changes in organs	-	+++
edema	-	+++
congestion of blood vessels	-	+++
cellular damage	-	+++
inflammatory cell response	-	+++

+++ = extensive damage, - = not found

[1]All features described reflect findings with inoculas of 1, 10 and 100 times the $YSLD_{50}$ with the exception of 1000 $YSLD_{50}$, which were found at four days post-inoculation.

Table 5.3 Ultrastructural observations on cells of embryo organs inoculated with 100 times the $YSLD_{50}$ of L. pneumophila.

Cell Damage	Days post-inoculation		
	1,2	3	4,5
Breakdown of cell and nuclear membrane	-	+	++++
Cytoplasmic clearing	-	+	++++
Fatty degeneration	-	+	++++
Ballooning of nuclear membrane	-	-	++++
Pyknosis	-	-	++++
Karyolysis	-	-	++++
Mitochondrial damage	-	+++	++++
Margination of chromatin and nuclear clearing	-	+	++++
Intercellular spaces and cellular vacuoles	-	+	++++
Hemolysis	-	-	++++

++++ = extensive in all cells examined; +++ = common in many cells;

+ = rarely found; - = never found

Table 5.4 Appearance of inflammatory cells in chick embryo organs infected with 100 times the $YSLD_{50}$ of **L. pneumophila** and protected with 10 times the MIC of each antibiotic.

Antibiotic	MIC (μg/ml)	Days post-inoculation
control (100 $YSLD_{50}$)	------	5
ciprofloxacin	0.08	8
doxycycline	0.80	6
erythromycin	0.50	6
rifampicin	0.03	6

Table 5.5 Ultrastructural cell damage in embryo organs previously inoculated with 100 times the YSLD$_{50}$ of _L. pneumophila_ and protected with antibiotic

Antibiotic	MIC (μg/ml)	Dose administered per egg[1] (μg/ml)	Days post-inoculation	
			4 (1 day post-therapy)	8 (5 days post-therapy)
Ciprofloxacin	0.08	28	+++[2]	+
Rifampicin	0.03	10.5	+++	+++
Erythromycin	0.50	175	+	Dead
Doxycycline	0.80	280	+	Dead

[1]Dose per egg was based upon 10 times the MIC for each drug and an average egg volume of 35 mls.

[2]Degree of ultrastructural damage observed, +++= marked, += slight
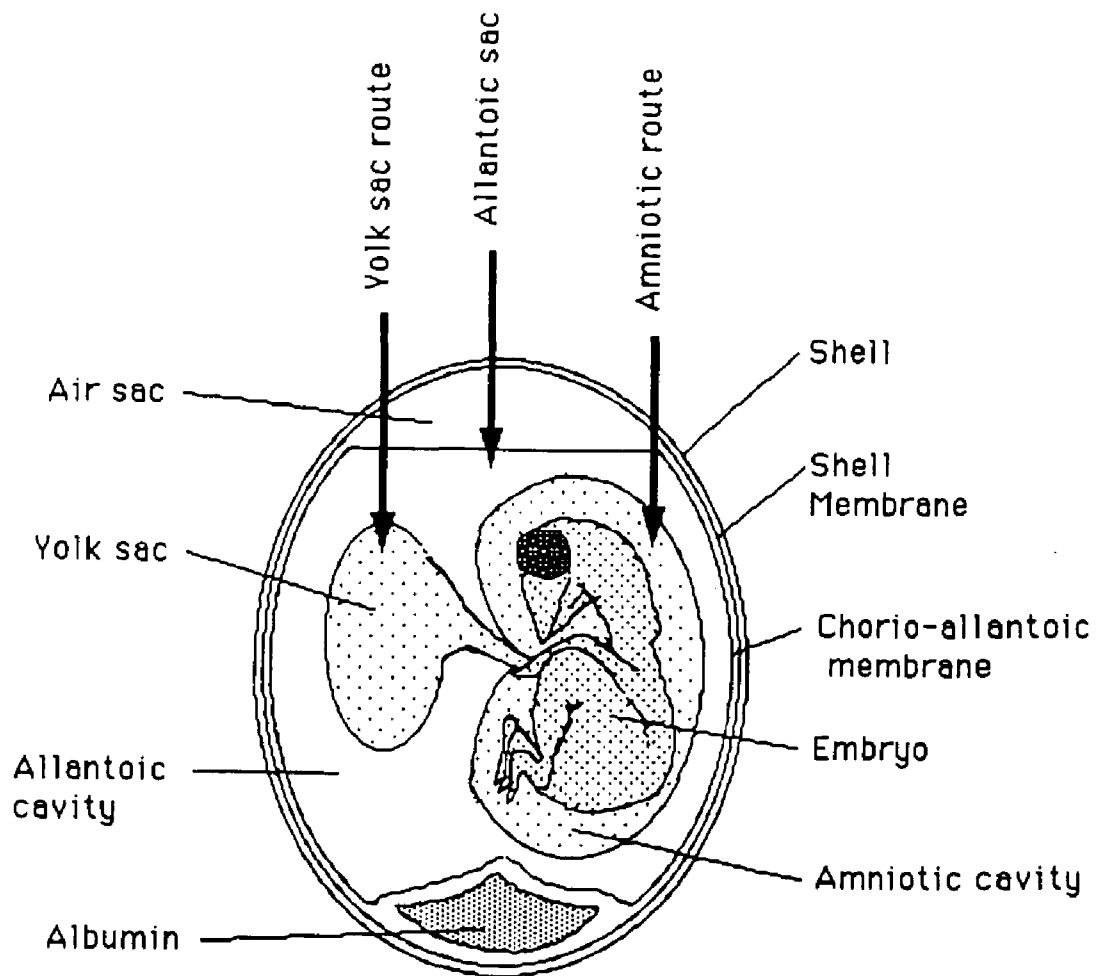
Figure 5.1 Schematic illustrating various routes of inoculation and compartments of the chick embryo.

140
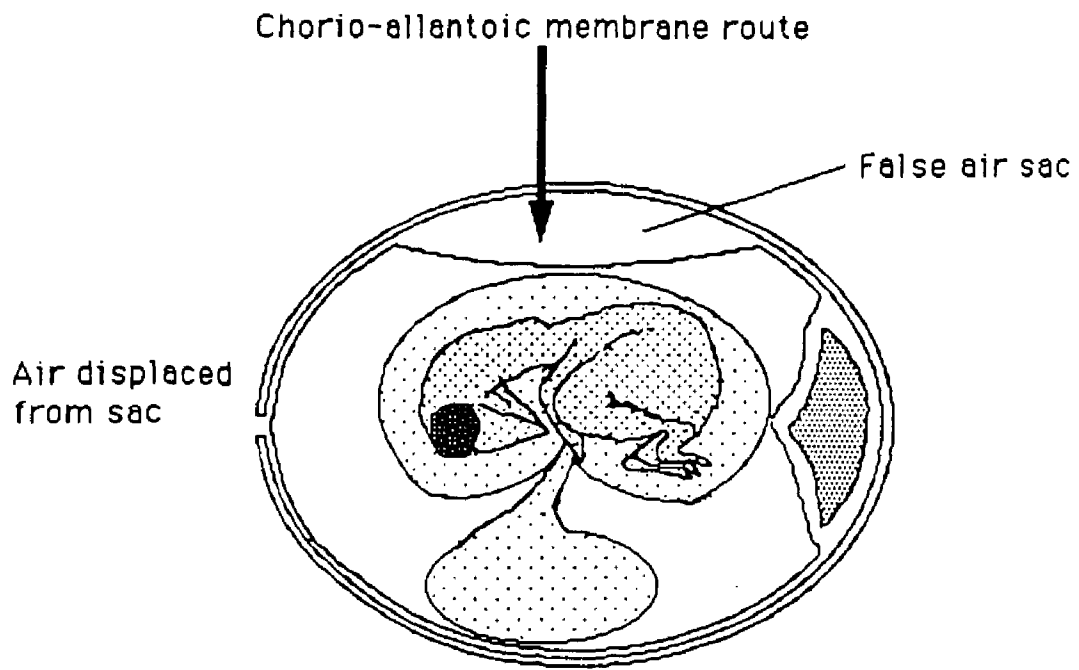
Figure 5.2 Schematic illustrating the chorioallantoic route of inoculation of the fertile hen's egg.

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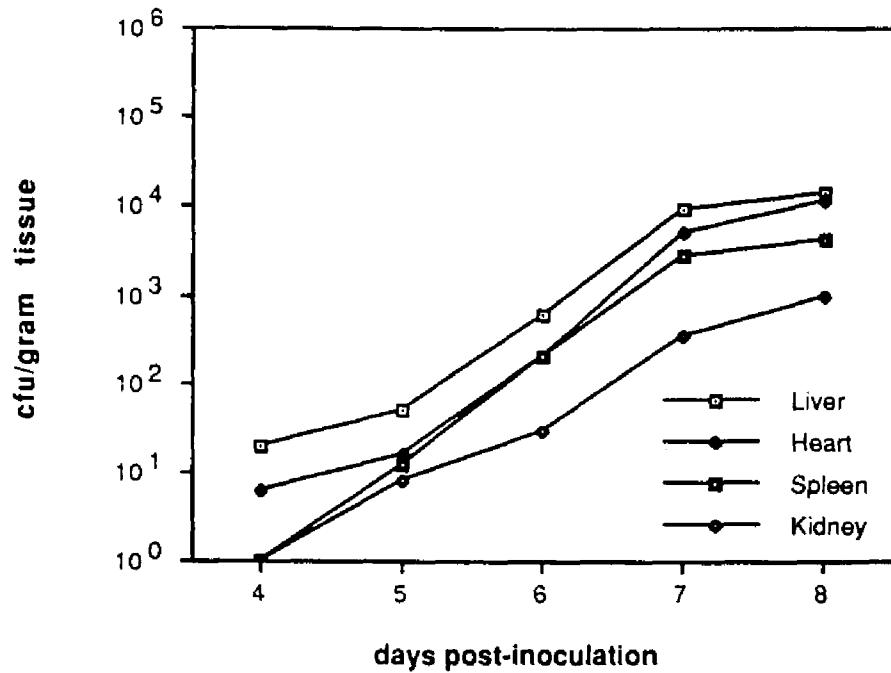
Figure 5.3 Bacterial viable counts assays on embryo organs from eggs inoculated with the $YSLD_{50}$ of L. pneumophila. Note rapid bacterial growth in all organs examined. Organisms were found most concentrated in the liver.
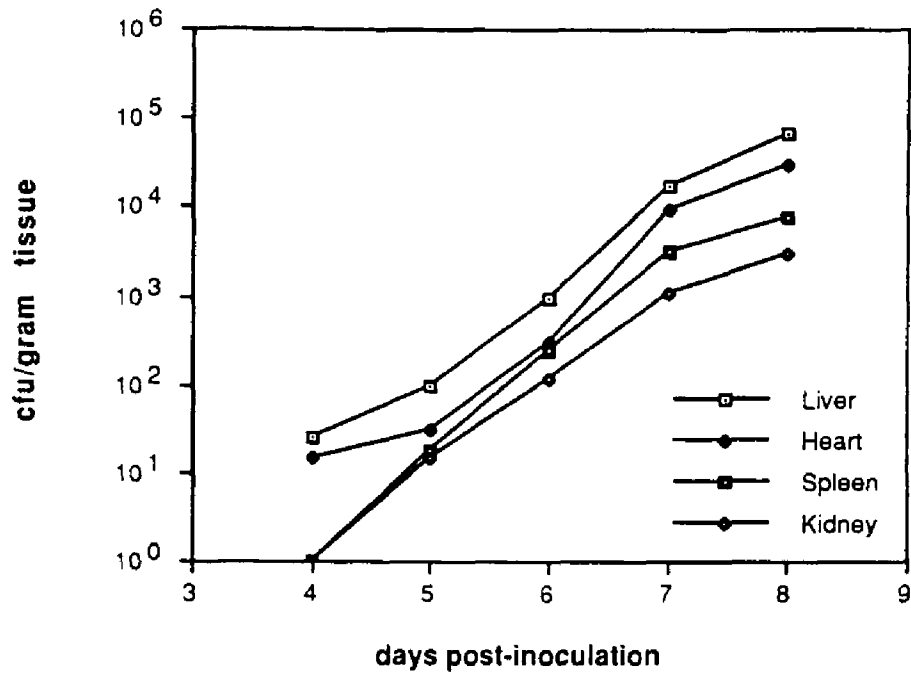
Figure 5.4 Bacterial viable count assays on embryo organs from eggs inoculated with 10 times the $YSLD_{50}$ of L. pneumophila. Note increased bacterial growth rate in all organs examined. The liver contained the densest microbial population.
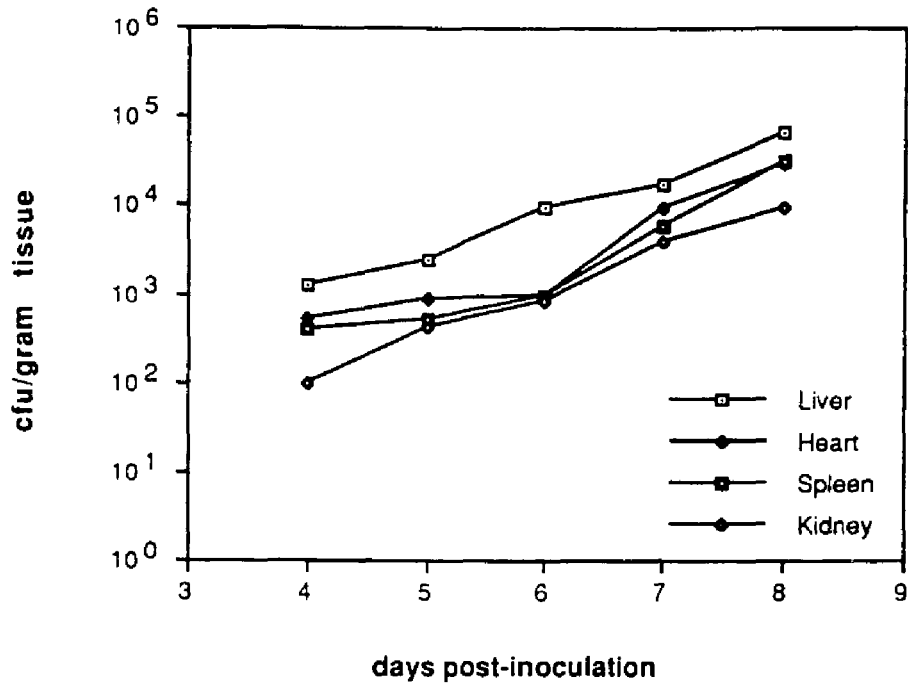
Figure 5.5 Bacterial viable count assays on embryo organs from eggs inoculated with 100 times the $YSLD_{50}$ of L. pneumophila. Bacterial growth in organs was similar in all organs examined but was less rapid than growth in organs of embryos inoculated with 10 times the $YSLD_{50}$.

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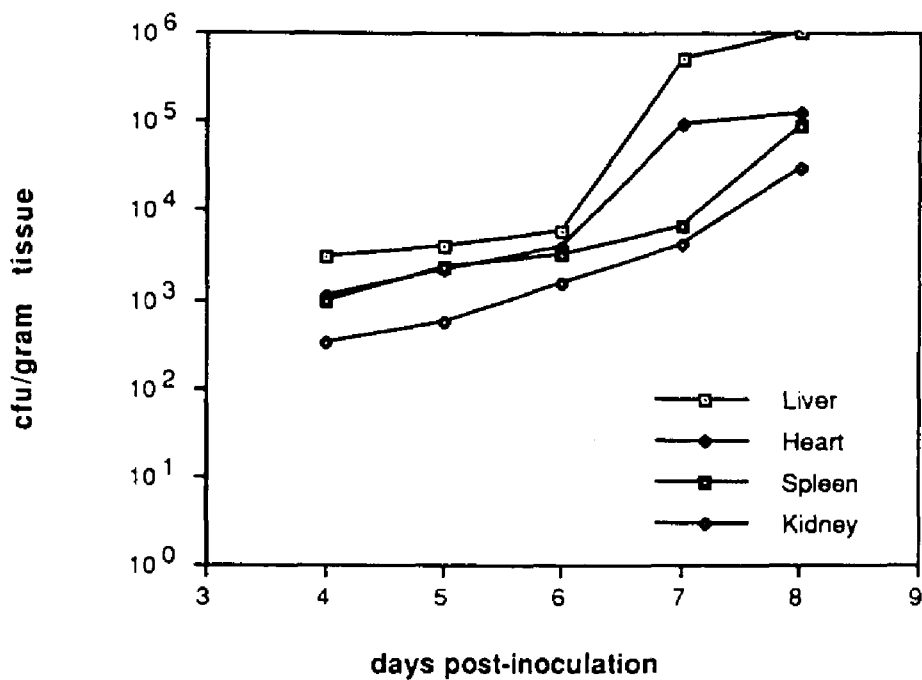
Figure 5.6 Bacterial viable count assays on embryo organs from eggs inoculated with 1000 times YSLD$_{50}$ of L. pneumophila. Note increased bacterial growth in liver and heart tissue after 6 days post-inoculation.

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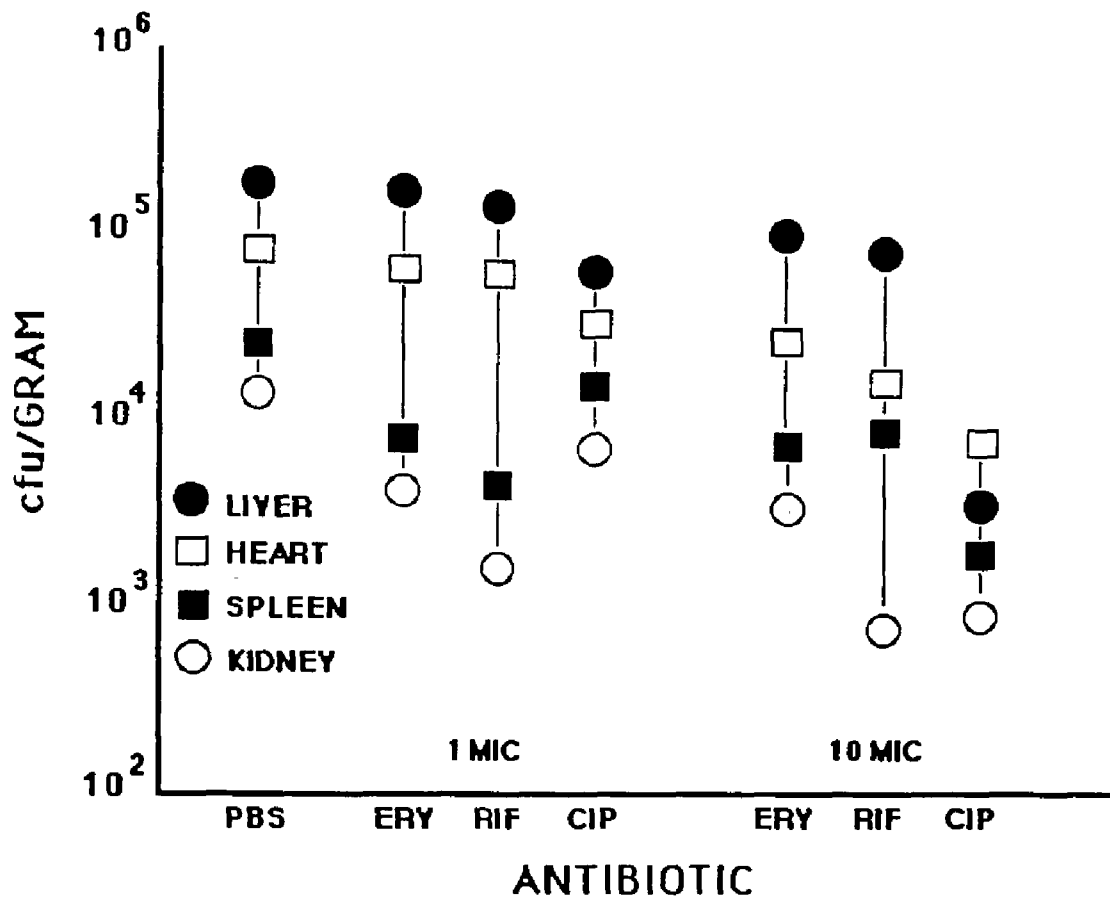
Figure 5.7 Bacterial growth of L. pneumophila in embryo organs previously inoculated with 100 times the YSLD$_{50}$ and treated one day post-inoculation with 1 or 10 times the MIC of antibiotic. Treatment with ten times the MIC of ciprofloxacin was most effective at reducing bacterial growth in the majority of organs examined.
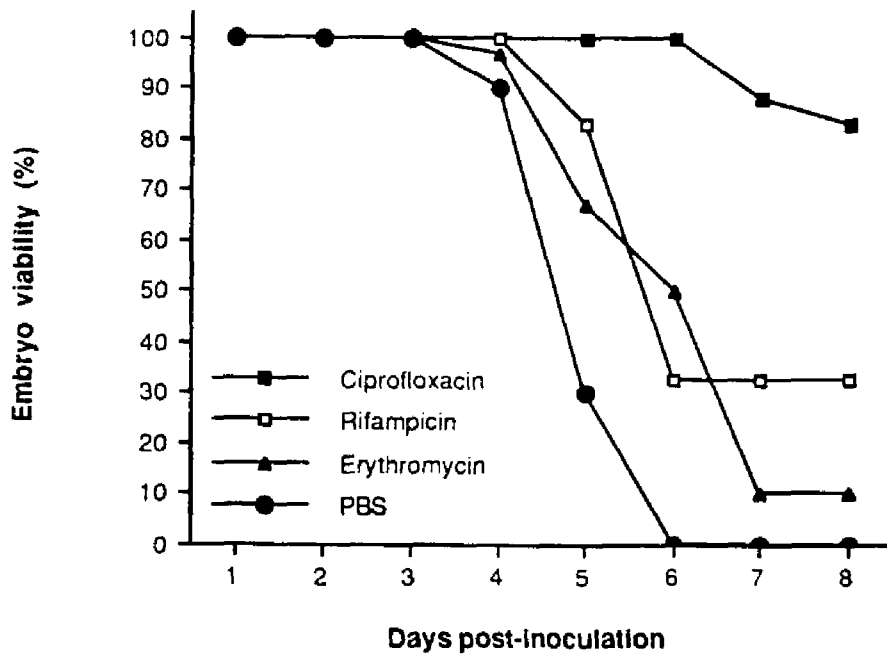
Figure 5.8 Viability rate of embryos previously infected with 100 times theYSLD$_{50}$ of L. pneumophila and treated with 10 times the MIC of each antibiotic. PBS control (●), ciprofloxacin (■), erythromycin (▲) and rifampicin (□). Ciprofloxacin was the most efficacious antibiotic used in these trials.
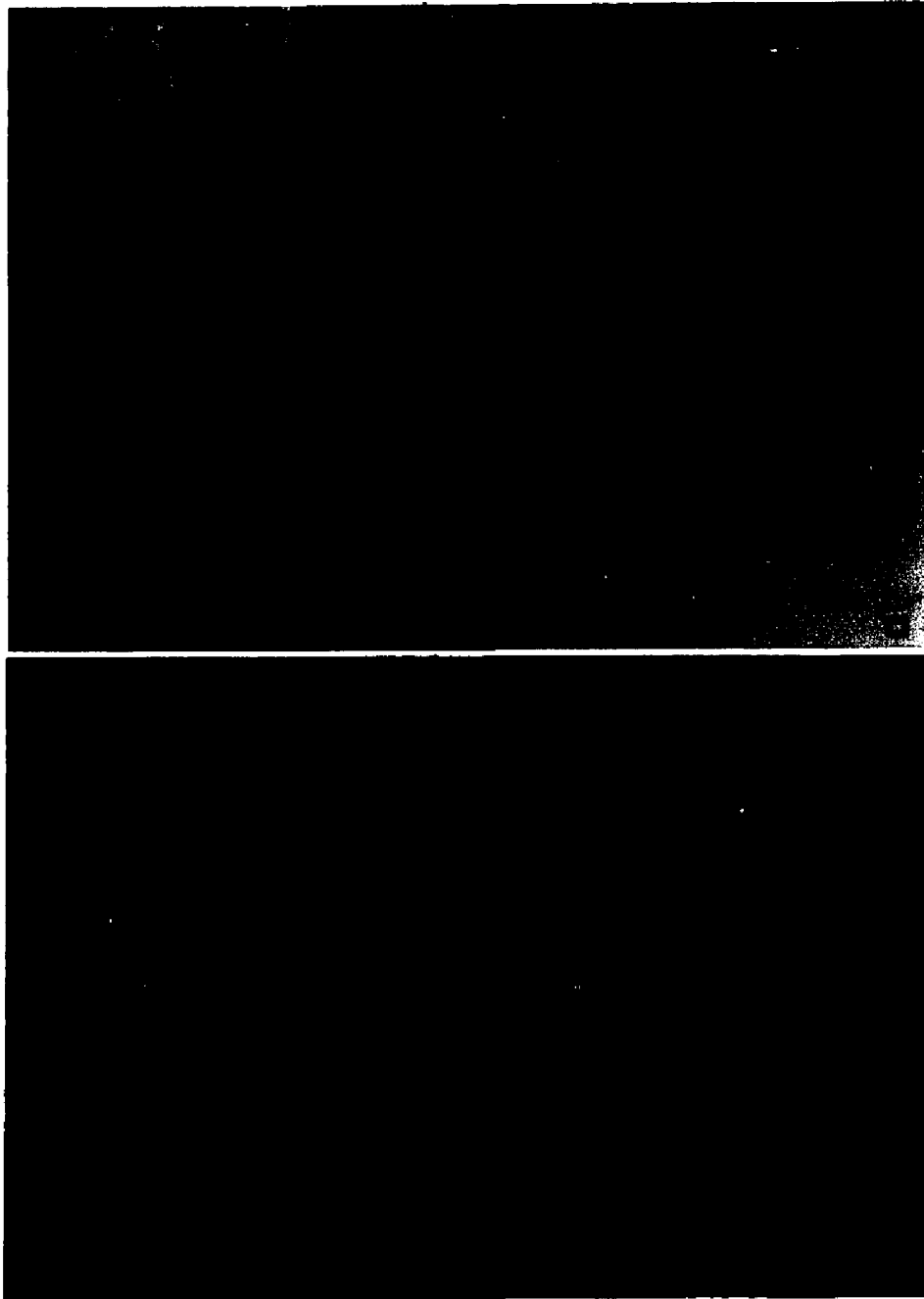
Plate 5.1 Histological sections of liver tissue from the chick embryo.
Hematoxylin and eosin stain.
a) normal tissue with dispersed red blood cells (RBCs) (X 165), b) liver tissue
five days after inoculation with the $YSLD_{50}$ of L. pneumophila. Note edematous
tissue and densely staining inflammatory response within congested central
vein (X660).

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Plate 5.2 Histological sections of heart tissue from the chick embryo. Hematoxylin and eosin stain.
a) normal tissue (X 165), b) heart tissue five days after inoculation with the YSLD$_{50}$ of L. pneumophila with marked vascular congestion (arrows) (X 165).
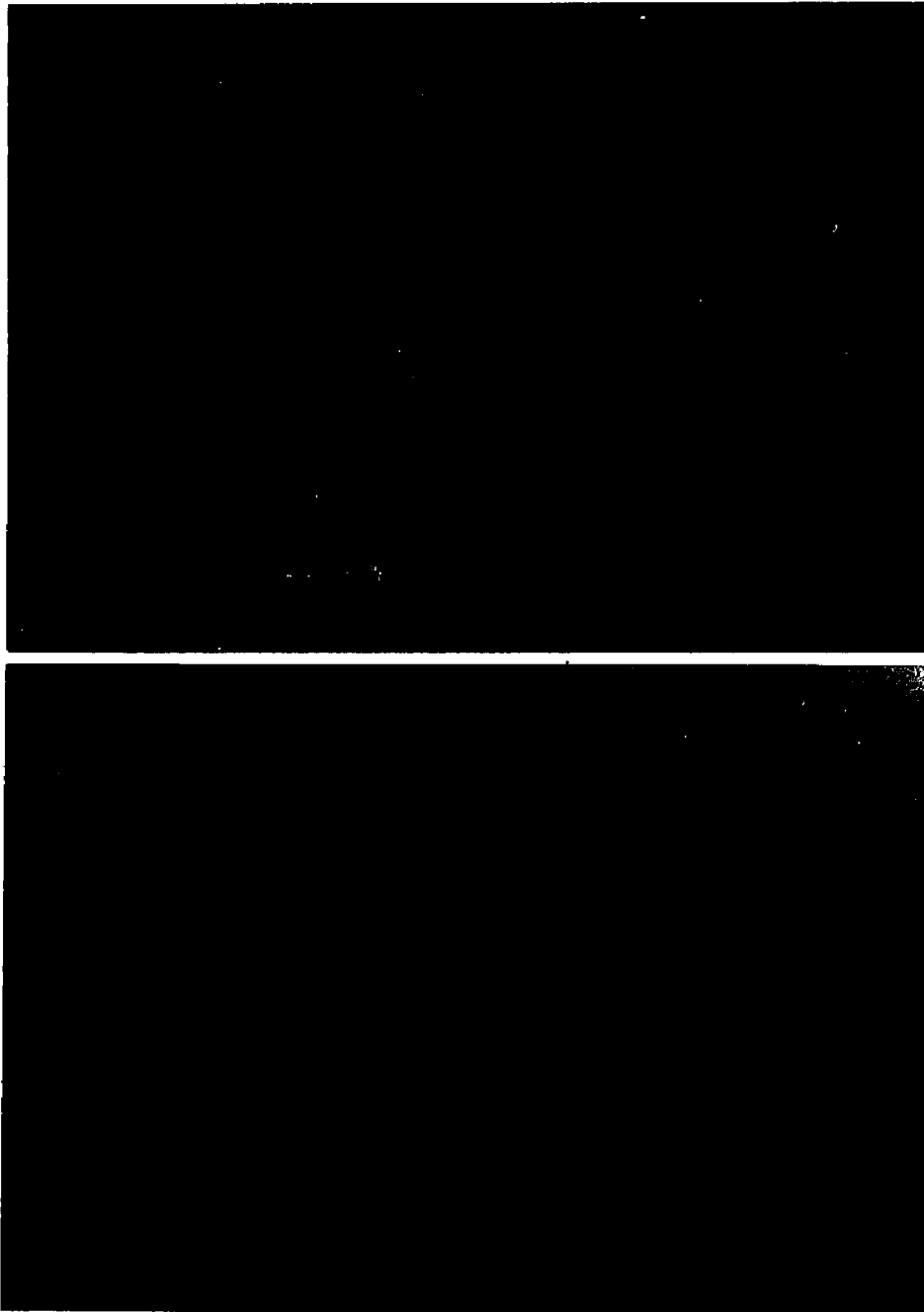
Plate 5.3 Histological sections of spleen from the chick embryo. Hematoxylin and eosin stain.

a) normal tissue (X 165), b) spleen 5 days after inoculation with the YSLD$_{50}$ of L. pneumophila. Inflammatory cells are evident within central artery and surrounding tissue (X165).

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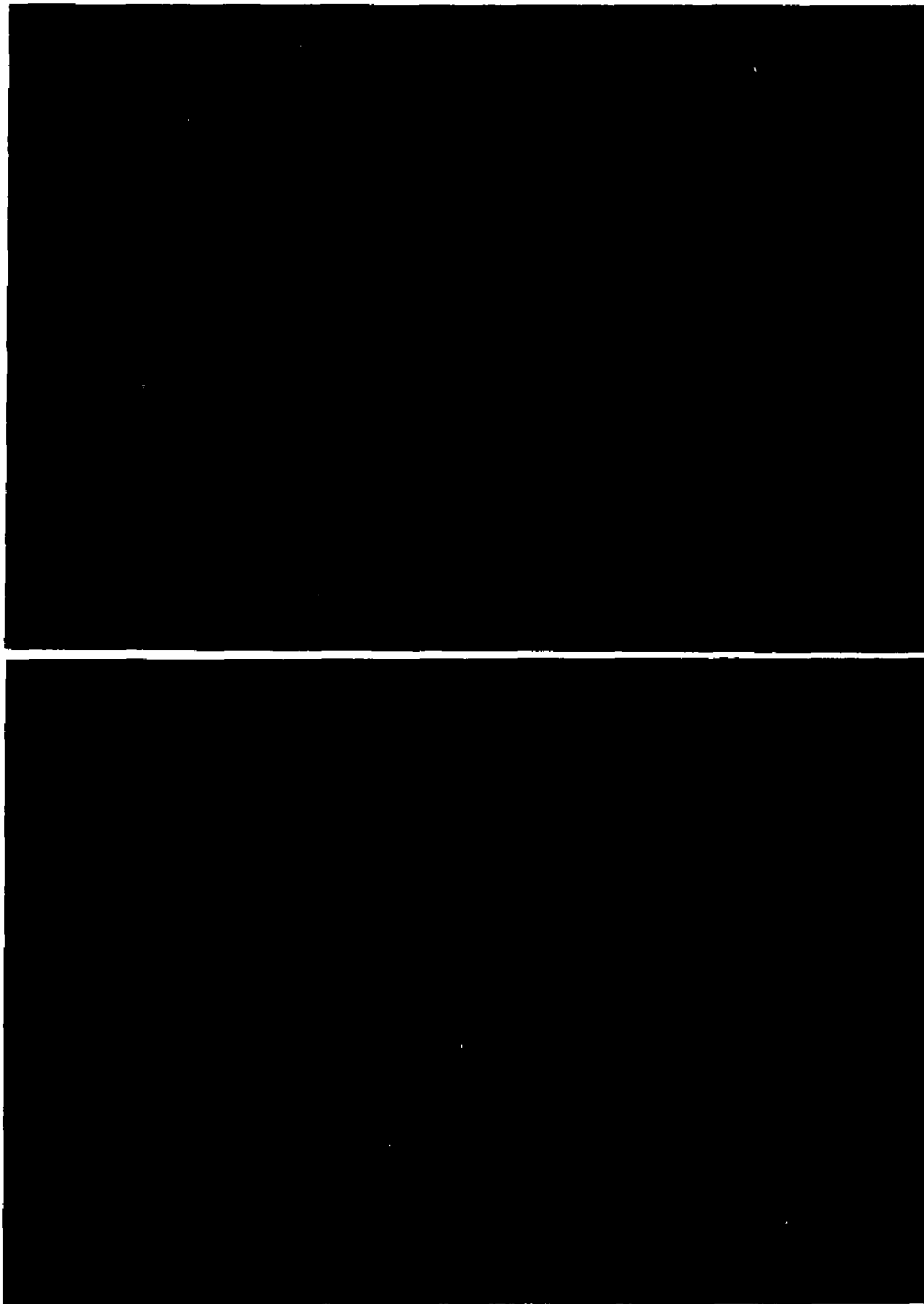
Plate 5.4 Histological sections of kidney from the chick embryo. Hematoxylin and eosin stain.

a) normal tissue showing glomeruli, convoluted tubules and circulating RBCs of the kidney (X 165), b) kidney five days after inoculation with the YSLD50 of L. pneumophila. Note damaged sustained by glomeruli (short arrow) as compared to the convoluted tubules (long arrow). Inflammatory cells are distributed thoughout tissue the tissue (X 660).
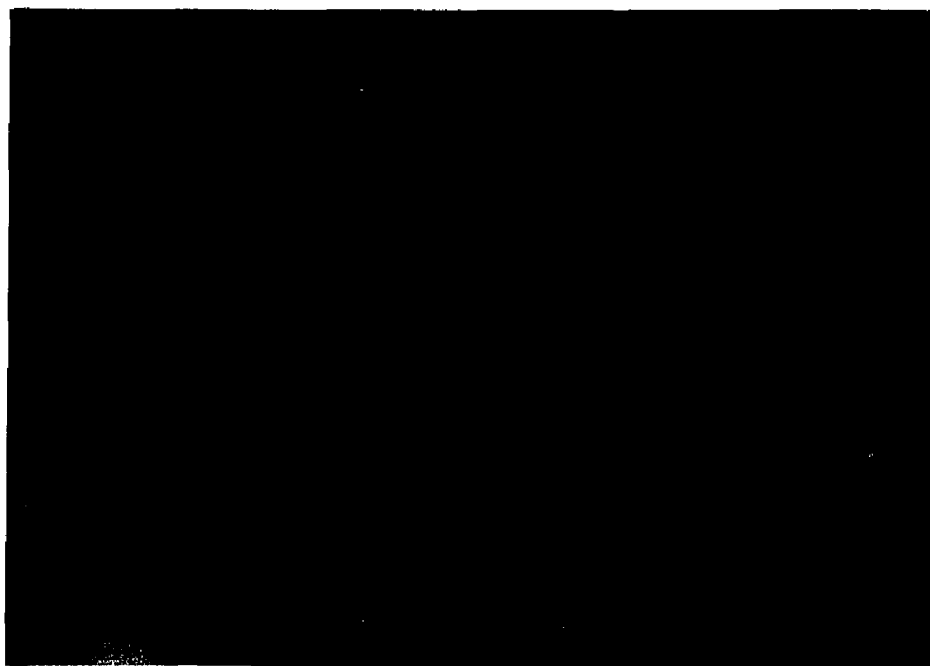
Plate 5.5 Histological section of liver at five days post-inoculation at high magnification showing influx of inflammatory cells. Note incidence of PMNLs (short arrow) and lymphocytes (long arrow) as well as damaged hepatocytes (X 1650). Hematoxylin and eosin stain.
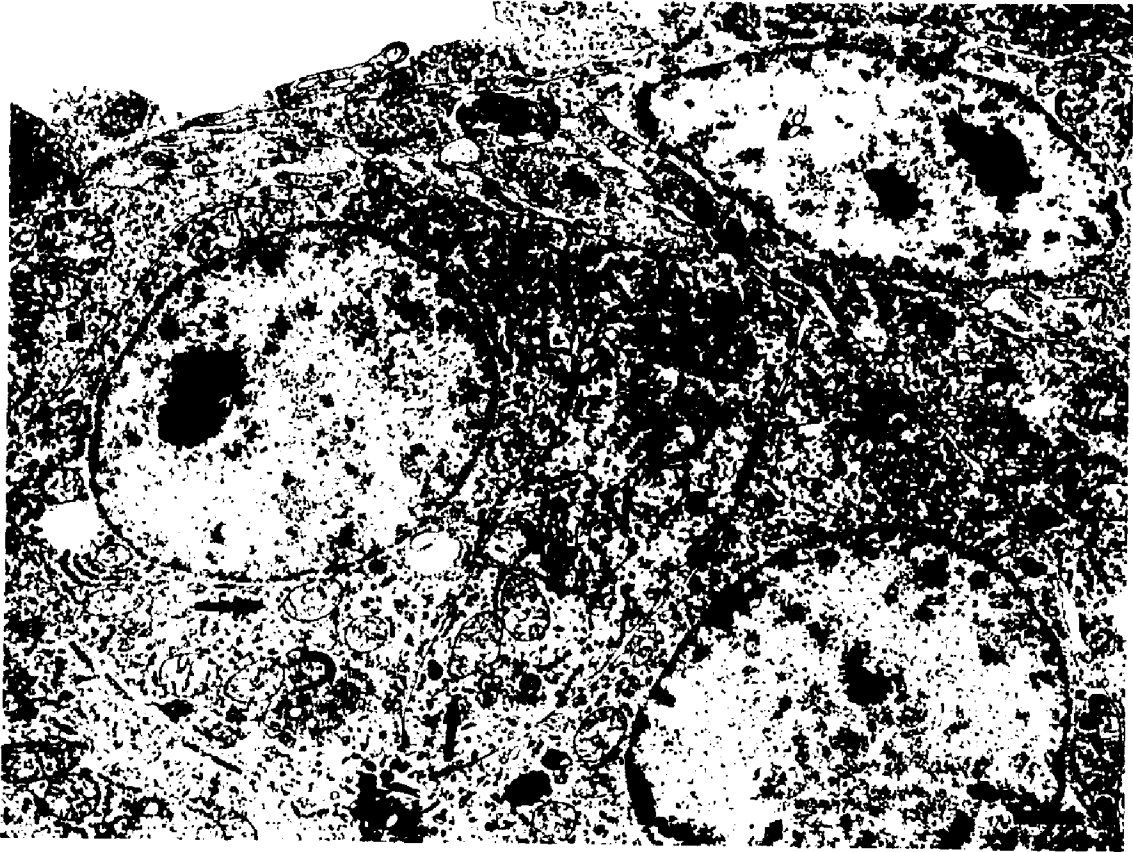
Plate 5.6 Electron micrograph of kidney three days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila. Subtle pathological changes within the mitochondria of cells were evident at this time (arrows) alongwith fatty degeneration and vacuole formation (X 8,000). Bar=1 μm.
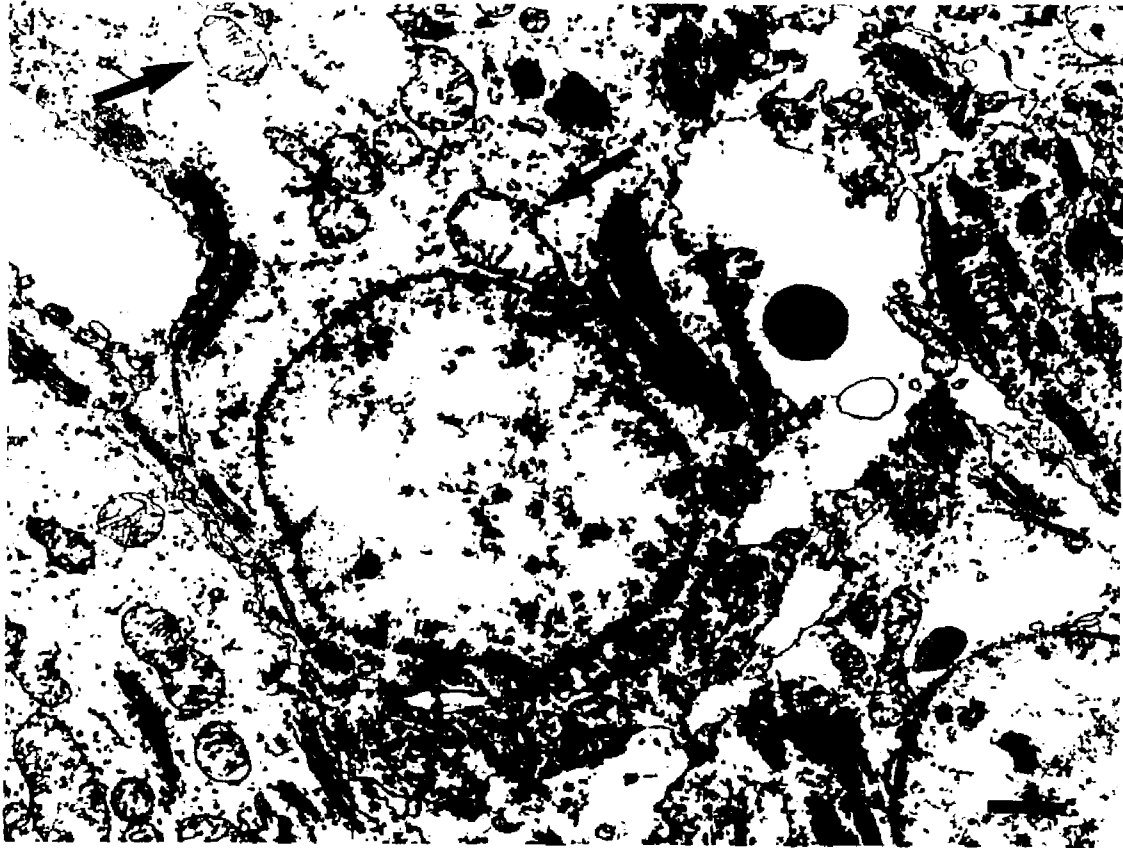
Plate 5.7 Electron micrograph of heart three days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila. Note extreme damage to mitochondria (arrows) and initial stages of cytoplasmic clearing (X 10,000).
Bar=1 μm.

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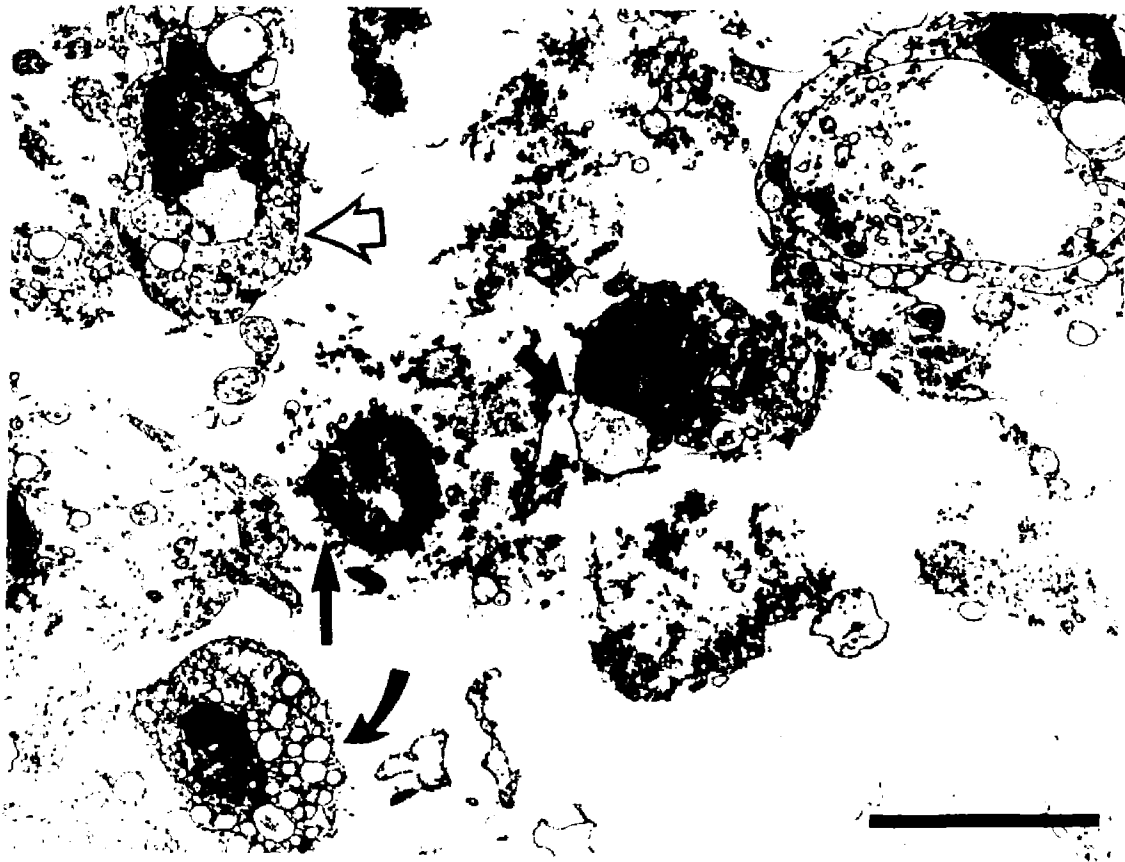
Plate 5.8 Electron micrograph of spleen four days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila illustrating ballooning of nuclear membranes (short arrow), margination of chromatin material (long arrow), vesicle formation within cytoplasmic space (open arrow) and the presence of "ghost mitochondria" (curved arrow) (X 6,000). Bar=5 μm.

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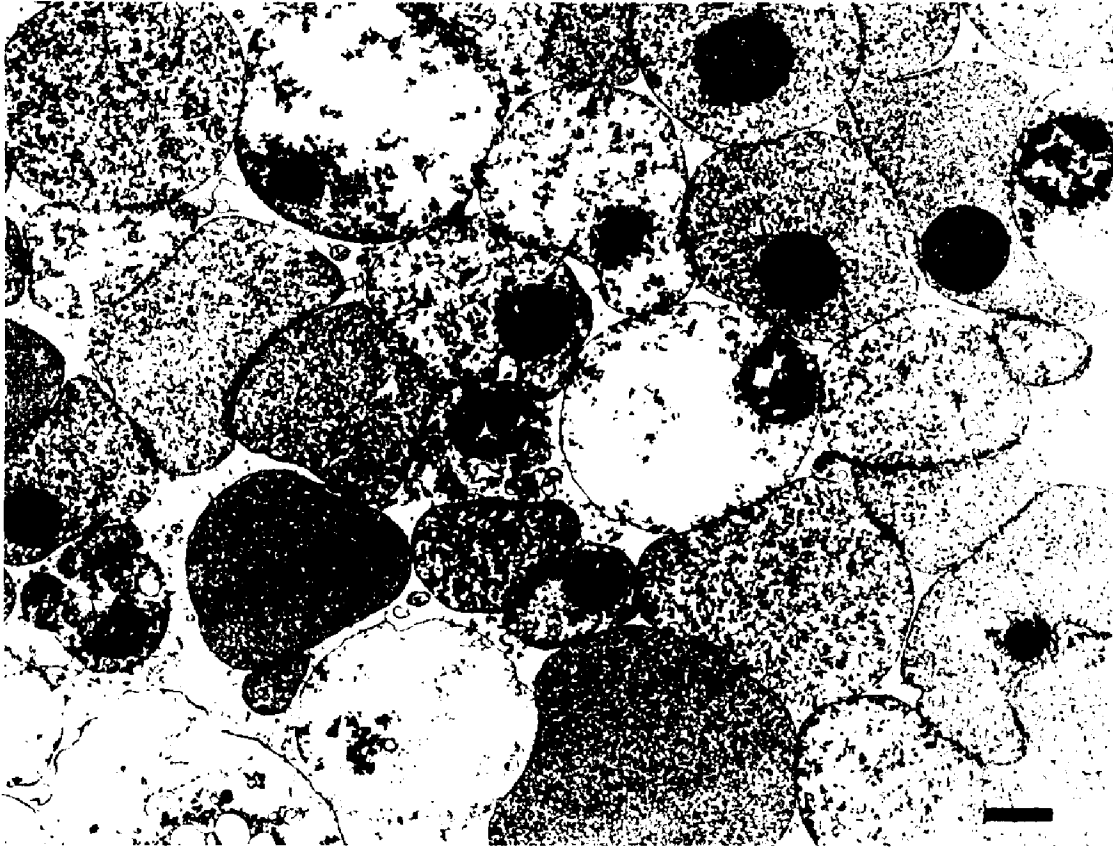
Plate 5.9 Electron micrograph of kidney four days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila. Note the various stages of the hemolytic process (X 8,000). Bar=1 µm.
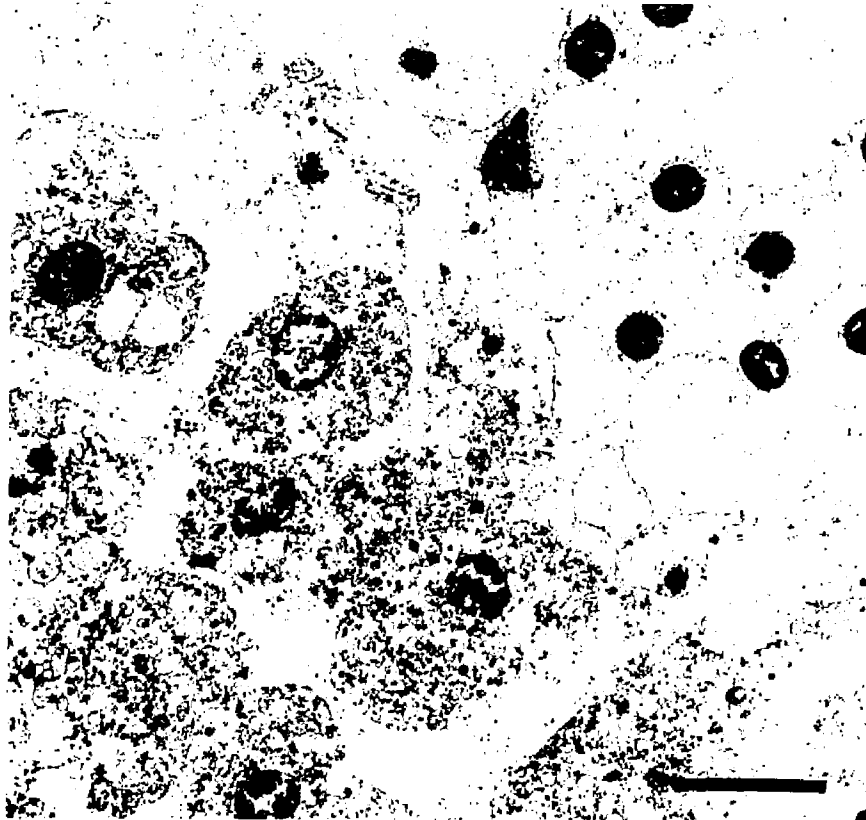
Plate 5.10 Electron micrograph of liver five days after inoculation with 100 times the YSLD50 of L. pneumophila illustrating advanced stages of the infection. Necrotic hepatocytes are bordered by hemolysed RBCS. Cell membranes of liver cells are intermittently lysed (X 4,000). Bar=5 μm.
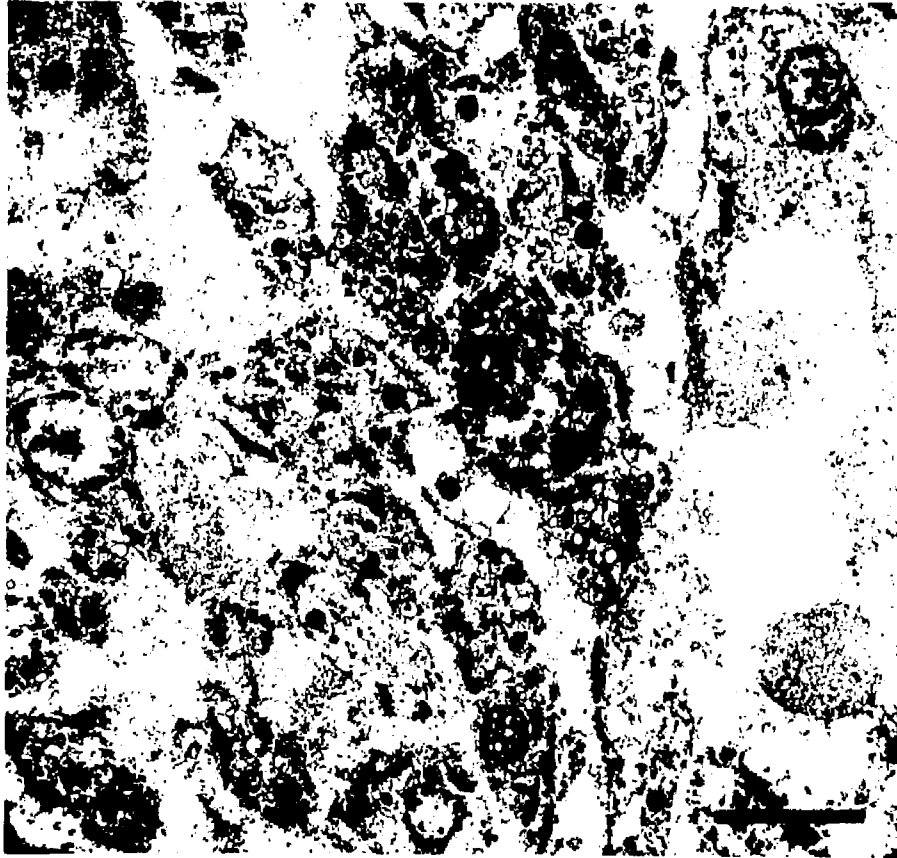
Plate 5.11 Electron micrograph of heart five days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila showing pathological changes at this stage of the infection. Fatty degeneration, intercellular and intracellular clearing, margination of chromatin material and membrane lysis are apparent (X 4,000). Bar=5 μm.
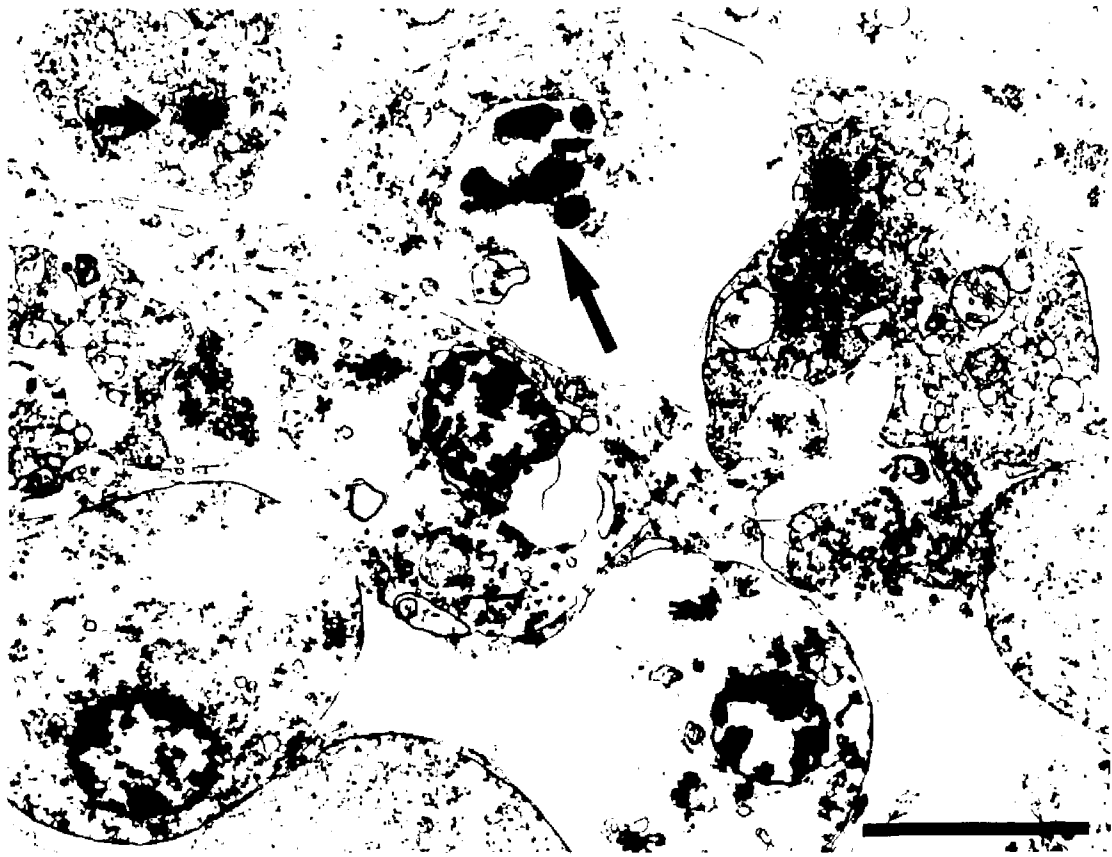
Plate 5.12 Electron micrograph of spleen five days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila. Pyknosis (short arrow) and karyolysis (long arrow) of nuclei are evident alongwith the formation of many cytoplasmic vesicles (X 6,000). Bar=5 μm.
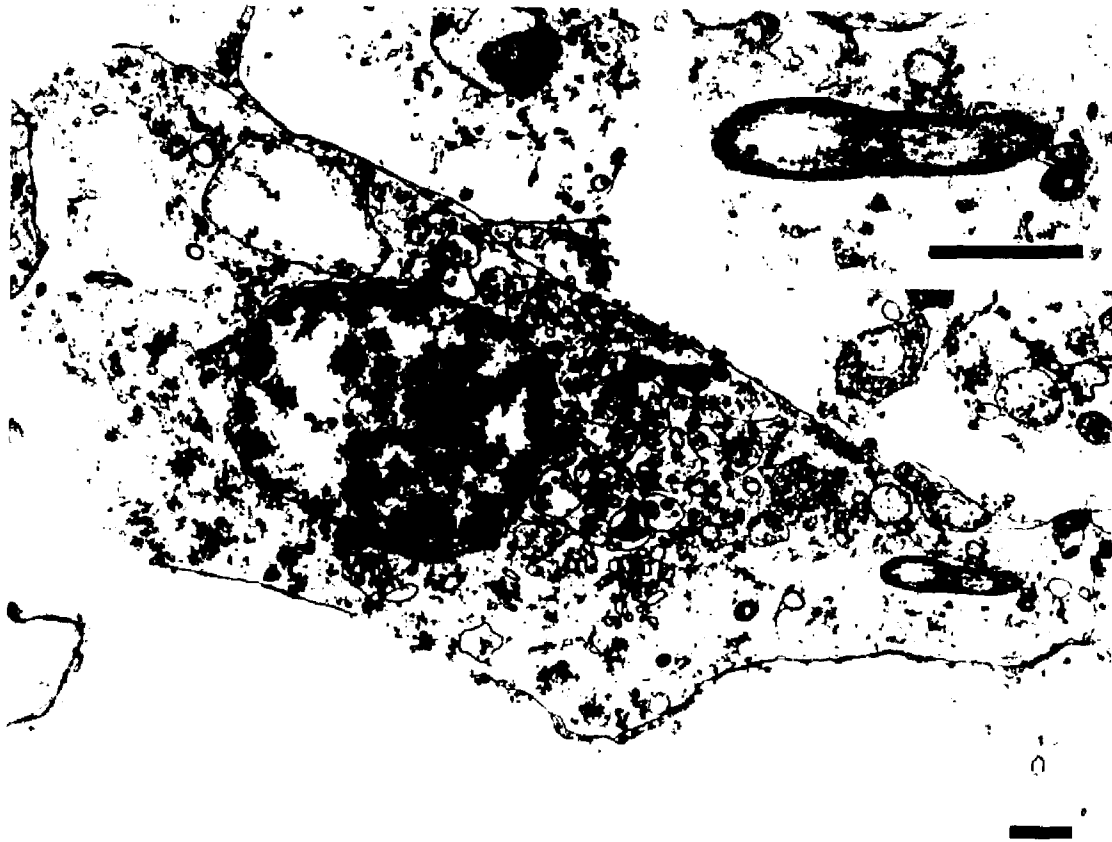
Plate 5.13 Electron micrograph of spleen tissue four days after inoculation with 100 times the YSLD$_{50}$ of L. pneumophila. Cell with intracellular organism associated with cellular and sub-cellular pathology (X 8,000). Inset: higher magnification of L. pneumophila organism (X 20,000). Bars=1 μm.
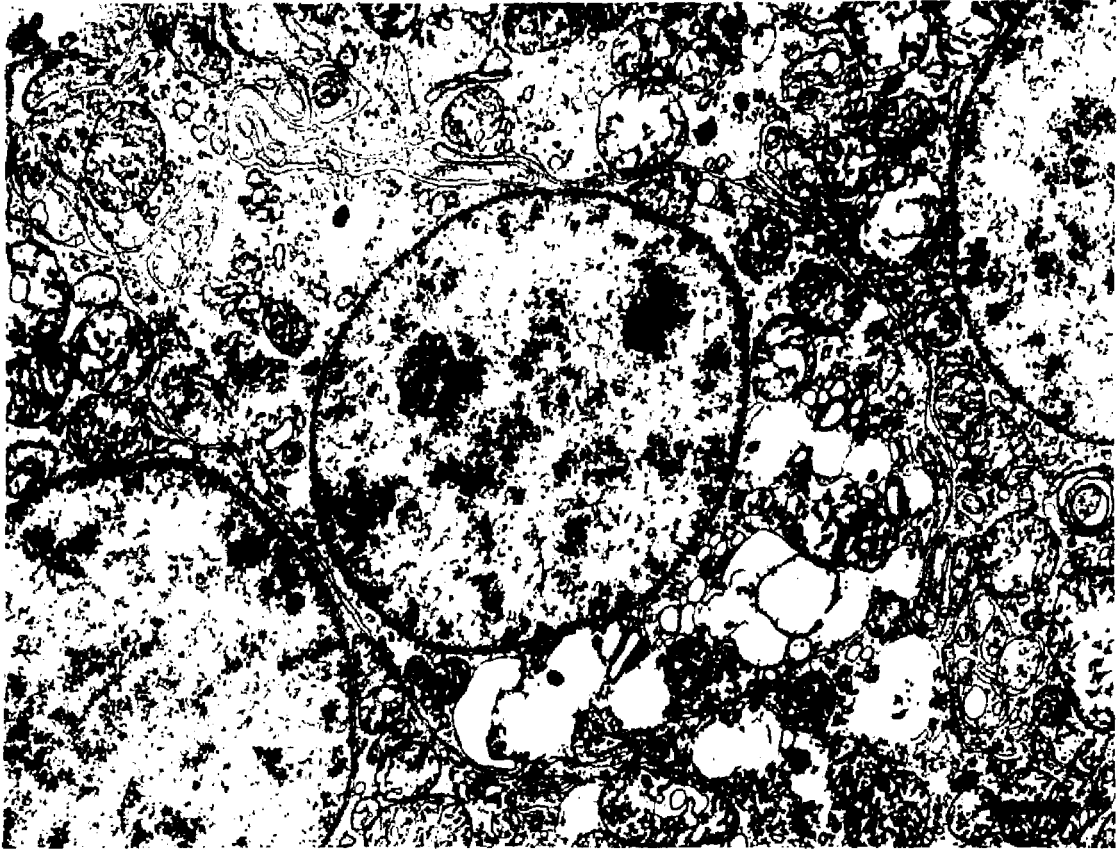
Plate 5.14 Electron micrograph of kidney from embryos treated with 10 times the MIC of erythromycin three days after bacterial infection. Thin-sectioned tissue at four days post-infection (one day post-therapy) showing improved tissue as compared to untreated controls at the same stage of infection. Erythromycin did not sustain this level of activity with time (X 10,000).

Bar=1 μm.

Plate 5.15 Electron micrograph of kidney from embryos treated with 10 times the MIC of doxycycline three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy) illustrating improved presentation of tissue as compared with untreated controls at the same stage of infection. Doxycycline was not able to maintain this level of activity with time . (X 8,000). Bar=1 μm.
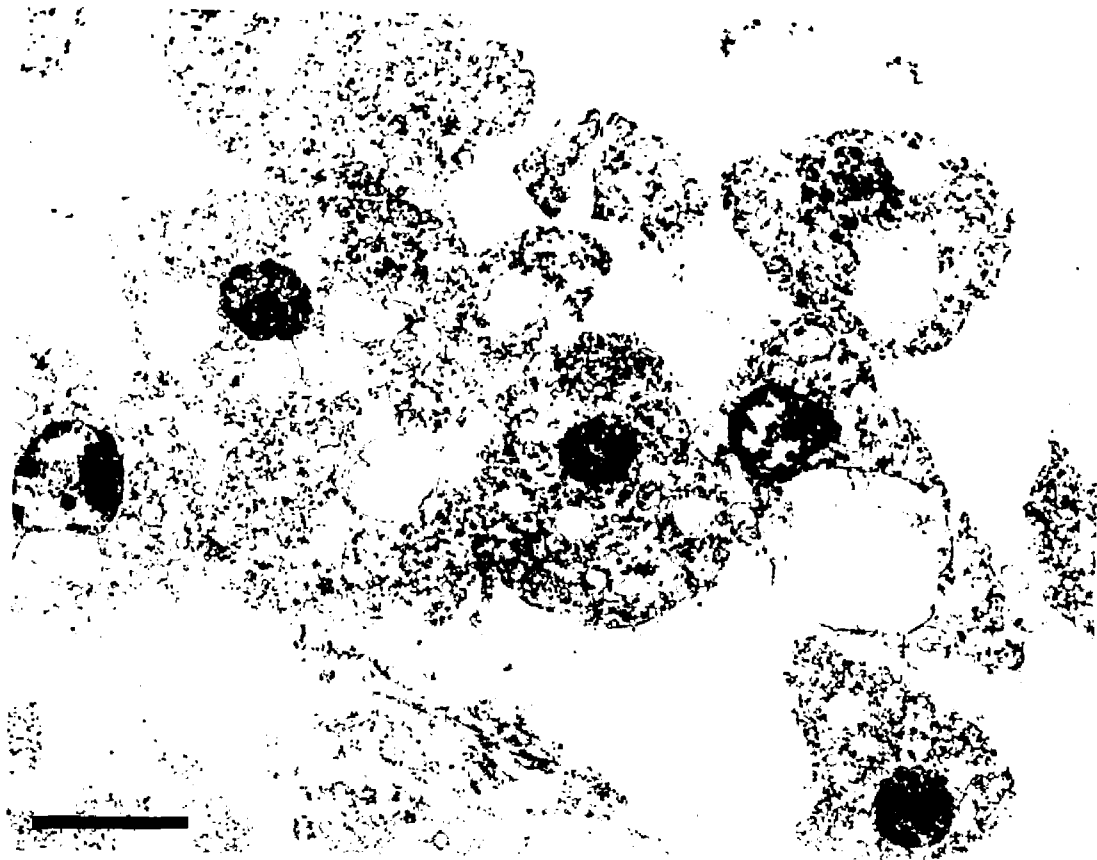
Plate 5.16 Electron micrograph of kidney from embryos treated with 10 times the MIC of rifampicin three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy). Rifampicin treated tissue showed some improvement as compared to untreated tissue at the same stage of infection (X 4,000). Bar=5 μm.
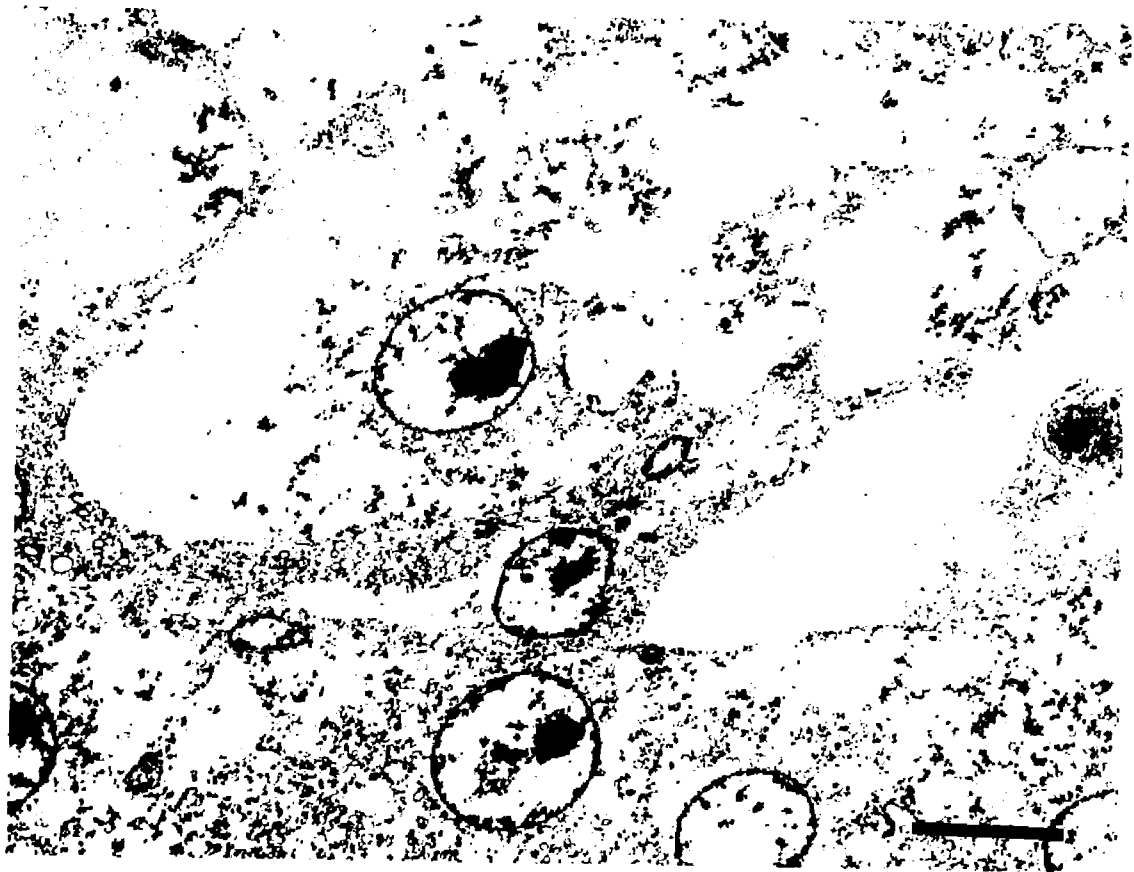
Plate 5.17 Electron micrograph of kidney from embryos treated with 10 times the MIC of rifampicin three days after bacterial infection. Thin-sectioned material at eight days post-infection (five days post-therapy). No improvement of the condition of the tissue was noted with time (X 4,000). Bar=5 μm.

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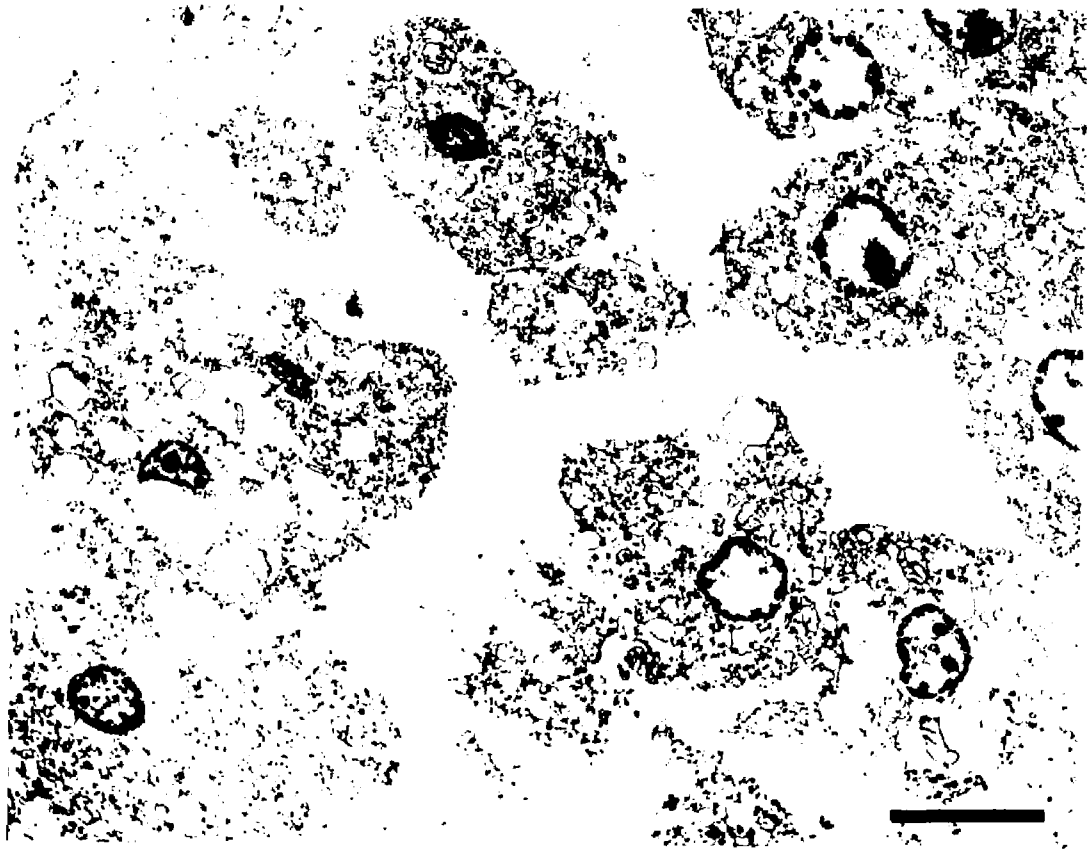
Plate 5.18 Electron micrograph of liver from embryos treated with 10 times the MIC of ciprofloxacin three days after bacterial infection. Thin-sectioned material at four days post-infection (one day post-therapy) exhibiting no significant improvement of tissue condition as compared to untreated controls at the same stage of the infection (X 4,000). Bar=5 μm.
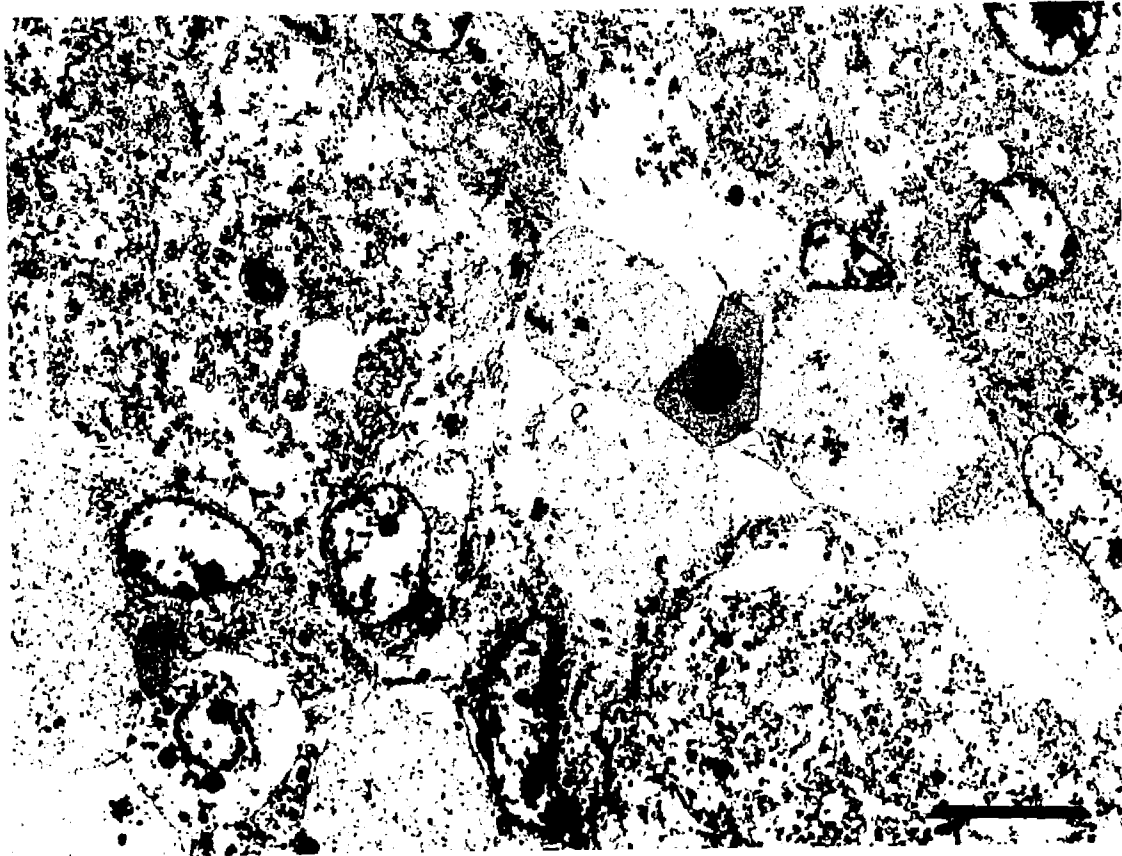
Plate 5.19 Electron micrograph of liver from embryos treated with 10 times the MIC of ciprofloxacin three days after bacterial infection. Thin-sectioned material at eight days post-infection (five days post-therapy). Note much improved condition of tissue as compared with treated embryos at 4 days post-infection (X 4,000). Bar=5 μm.

Section VI

6. General Discussion

Data from these studies indicated that a molecule or molecules with lectin-like activity on the surface of this organism may be responsible, at least in part, for L. pneumophila binding to host target cells. Further identification and isolation of L. pneumophila surface structures which facilitate attachment to target cells may lead to the development and production of potentially useful anti-adherence vaccines. The process by which L. pneumophila recognized and adhered to host cell surfaces represents the earliest, most critical stage of bacterial infection. The ability to block these initial events with such prophylactic treatments would illustrate a novel strategy for the prevention of clinical legionellosis.

The chick embryo was established as a powerful tool for the evaluation of L. pneumophila virulence and proved a viable alternative to other animal systems for the study of the pathology and chemotherapy of the extrapulmonary manifestations of experimental L. pneumophila infections. Because of the intracellular nature of this organism, in vitro MIC data do not reflect in vivo or clinical results. These studies demonstrated the fertile hen's egg as a readily accessible in vivo system for the evaluation of putative antimicrobial therapy for legionellosis and may prove of significant value for the evaluation of antibiotic regimens for other facultative intracellular pathogens such as Brucella, Yersinia, Listeria, Chlamydia and many others. Although, erythromycin (the drug of choice for Legionnaires' disease), rifampicin and doxycycline showed antimicrobial activity in this animal system,

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ciprofloxacin, a newer broad spectrum quinolone antibiotic was the most efficacious in these trials.

Studies which characterized the morphological response of L. pneumophila to clinically relevant antibiotics provided indicators of the bacteriostatic versus bactericidal nature of these agents and yielded some information concerning the structural nature of the organism. In addition, examination of the microbial damage induced by selected antimicrobials was necessary for the interpretation of results derived from further in vivo evaluations of these antibiotics.

L. pneumophila is a human pathogen that causes fatal disease. Immunocompromised individuals are particularly susceptible to infection and respond variably to the current antibiotic regimens. The present investigation partially characterized the adherence of L. pneumophila to host cells and explored the possibilities of novel preventative measures in the form of anti-adherence vaccines for human disease. Furthermore, these data delineated the pathogenesis and pathology of L. pneumophila in experimental animal infections and illustrated the role of clinically relevant antibiotics in the outcome of disease. These results may lead to the development of aggressive combination therapeutic regimens in which protective vaccines and new broad-spectrum antimicrobial agents can be used for the prophylaxis and control of clinical legionellosis. Finally, this work clearly demonstrated the superior antimicrobial activity of the quinolones and their potential for treatment of human Legionnaires' disease.

Section VII

7. Possible future studies:

This work has taken a number of interesting turns and has revealed a variety of problems which could usefully be addressed concerning the further characterization of the pathogenesis of L. pneumophila:

1) role of humoral and cell-mediated immunity of the chick embryo to L. pneumophila infection and the outcome of disease.

2) examination of the potential intracellular colonization of the inflammatory cells reported here by L. pneumophila.

3) characterization of the role of L. pneumophila toxins in the disease process in the chick embryo.

4) role of bacterial toxins and antibiotics in intracellular survival of L. pneumophila.

5) comprehensive characterization of specific L. pneumophila adhesins (outer membrane proteins, fimbriae, pili, lectins) involved in adherence to host cells.

6) identification of host cell receptors for L. pneumophila.

7) electron microscopic examination of L. pneumophila adherence to U937 and subsequent internalization processes.

8) effect of selected antibiotics (especially at sub-MIC levels) on L. pneumophila adherence to host cells.

9) evaluation of immunosuppressive regimens on the outcome of experimental legionellosis in the chick embryo system.

10) determination of the synergistic action of selected antimicrobial agents in vitro and in vivo.

11) characterization of the mechanisms of L. pneumophila uptake by host cells

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APPENDICES

Appendix 1

Media used in biological studies.

1. Cell cultures

1.1 De-ionized water

All water used to make up media was obtained from a Millipore Milli Q

filtration system. Ten Megohm water was collected, filter sterilized through a 0.2

μm filter, and one liter aliquots stored at 4º C.

1.2 Phosphate buffered saline (PBS)

Solutions of PBS were made in de-ionized water as follows and

sterilized by autoclaving and stored in 100 ml aliquots in glass bottles at 4º C.

component	grams/liter
NaCl	8.0
KCl	0.2
KH_2PO_4	0.2
Na_2HPO_4	0.15

1.3 Hanks' Balanced Salt Solution (HBSS)

A concentrated 10 X sterile solution of HBSS with 0.35 g/l sodium

bicarbonate and phenol red was obtained from Irvine Scientific. For use, the

stock solution was diluted ten-fold under aseptic conditions with sterile

de-ionized water. The stock and working strength solutions were stored in

sterile glass bottles at 4º C.

1.4 Sodium bicarbonate solution (NaHCO₃)

A stock 7.5 % solution of sodium bicarbonate was made by dissolving 75 g $NaHCO_3$ in 1 liter of de-ionized water and sterilized by filtration. Aliquots of 100 mls were placed in sterile bottles and stored at 4° C.

1.5 Bovine calf serum

Sterile bovine calf serum supplemented with iron was obtained from Hyclone Laboratories, distributed in 50 ml volumes in sterile plastic tubes and stored at -20° C.

1.6 Glutamine

This was obtained in powdered form from Sigma, dissolved in sterile de-ionized water to make a 100 X stock (300mM) solution and filter sterilized. Aliquots were stored at -20° C.

1.7 Minimal Essential Media with Earles salts and non-essential amino acids (MEM)

This medium was obtained from Irvine Scientific as a sterile 10 X concentrated solution with added phenol red. For use, stock solutions were diluted ten-fold with sterile de-ionized water in an aseptic manner. Stock and working strength solutions were stored in sterile glass bottles at 4° C.

1.8 Trypsin-EDTA

Trypsin and sodium versenate (EDTA) were obtained in powdered form from Sigma and dissolved in 100 ml of warm PBS to make a working solution of 0.05% trypsin and 0.02% EDTA. Aliquots of 10 ml were stored at -20°C.1.8

Growth medium

MEM with Earles salts was prepared with the following sterile constituents:

Component	Volume
MEM with Earles salts	450 ml
Bovine calf serum	50 ml
Sodium Bicarbonate (7.5%)	10 ml
Glutamine	5 ml

2. Organism growth

2.1 Solid medium

Buffered yeast extract agar supplemented with α-ketoglutarate (BCYEα) was obtained from Gibco Laboratories (Madison, Wisconsin) and prepared according to the manufacturer's instructions. Components of this medium were as follows:

Component	grams/liter
yeast extract	10.0
ACES buffer	10.0
ferric pyrophosphate	0.25
α-ketoglutarate	1.0
agar	15.0
charcoal, activated	2.0

Four ml of a filter sterilized solution of 10% L-cysteine HCl was then aseptically added to 1 liter of medium according to manufacturer's instructions.

2.2 Broth medium

Buffered yeast extract broth supplemented with α–ketoglutarate (BYEα) was prepared as follows:

Component	grams/liter
yeast extract	10.0
ACES buffer	10.0
ferric pyrophosphate	0.25
α–ketoglutarate	1.0

Constituents were added to one liter of distilled water, allowed to dissolve, filter sterilized through a 0.2μm filter and collected aseptically. Four ml of a filter sterilized 10% solution of L-cysteine HCL was aseptically added to the medium prior to use.

Appendix 2

1. Reagents and materials used in histological studies.

1.1 Harris' Alum Hematoxylin

This solution was comprised of the following:

Component	Amount
hematoxylin crystals	5.0 g
absolute alcohol	50 ml
aluminum ammonium sulfate	100 g
de-ionized water	1000 ml
mercuric oxide	2.5 g

Hematoxylin crystals were dissolved in alcohol and the aluminum ammonium sulfate dissolved in water. The two solutions were then mixed and rapidly brought to a boil. Mercuric oxide was added slowly and the mixture reheated for 15-20 min. This was kept covered in a glass coplin jar ready for use.

1.2 Stock 1% aqueous eosin solution

The following components comprised this solution:

Component	Amount
water soluble eosin	10 g
de-ionized water	1000 ml
glacial acetic acid	2.0 ml

This were mixed and the solute allowed to dissolve. The stock solution was kept in a glass stoppered bottle and stored at 20° C. A working solution was made by mixing 50 ml of the stock solution with 120 ml absolute alcohol, 30 ml de-ionized water and 1.0 ml glacial acetic acid.

1.3 Acid alcohol solution

This was made by adding 70% (710 ml of 95%alcohol with 290 ml

deionized water) to 10 ml concentrated hydrochloric acid. and stored in a stoppered bottle at 20° C.

1.4 Saturated lithium carbonate

This was made by dissolving 4.5 g of lithium carbonate in 450 ml de-ionized water and stored in a stoppered bottle at 20°C.

1.5 Buffered formalin solution

A 10% solution of formaldehyde (VWR) was prepared in PBS and stored in a stoppered glass bottle at room temperature.

1.6 Preparation of histological sections

Tissue samples were harvest from embryos and fixed in 10% buffered formalin. Samples were then embedded in paraffin with a Lab Tek Tissue Processor and trimmed with a razor blade. Four micron sections were cut with a American Optical rotary microtome, attached to glass slides and prepared for staining.

1.6.1 Hematoxylin and eosin staining procedure

The following staining procedure was used:

Treatment	Time
Histoclear (two changes)	2 min
100% alcohol (two changes)	1 min
95% alcohol (two changes)	1 min
de-ionized water	3 dips
Harris' Hematoxylin	6 min
Running tap water	2 min
Acid alcohol	6-8 dips
de-ionized water	3 dips
lithium carbonate	7 dips
running tap water	15 min
eosin	1-4 min
95% alcohol (two changes)	15 sec
100% alcohol (two changes)	15 sec

Appendix 3

Reagents and materials used in electron microscopy studies.

1. Negative staining

1.1 Grids

Copper 400 mesh grids were supplied by Electron Microscopy Sciences (EMS) and were used in all studies.

1.2 Formvar

This was obtained from EMS as a dry powder. A stock 2% solution was made by dissolving one gram of formvar in 50 ml electron microscopy grade ethylene dichloride (EMS). Remaining solid after vigorous shaking dissolved upon standing overnight. Stock solution was stored in glass stoppered bottles in a desiccator until use.

Working solution was made up freshly as a 0.2% solution by mixing 18 ml ethylene dichloride with 2 ml stock formvar solution.

1.3 Formvar coating of grids

A clean dry glass slide was dipped into 20 ml 0.2% working strength formvar solution in a Coplin jar, removed and drained. When dry, the film was cut in situ into suitable sized squares using a sharp scalpel, and these squares were floated off onto the surface of de-ionized water by carefully submerging at a shallow angle the formvar coated slide. In reflected light, the formvar films could be seen as pale opalescent squares floating on the surface of the de-ionized water. Copper grids were placed (matt side down) on top of the films and collected from the water by carefully placing pieces of Whatman No. 1 filter paper over the films. Filter papers with adherent formvar films and grids were

collected and dried.

1.4 Phosphotungstic acid (PTA) negative stain

A 1% (w/v) solution was made by dissolving potassium phosphotungstate in 100 ml de-ionized water and the pH was adjusted to 6.7 using fresh aqueous potassium hydroxide. Any turbidity remaining after standing at room temperature for 24 hours was removed by centrifugation at 10,000 RPM in a Beckman Microfuge 12. The stain was stored at room temperature and used as required.

1.5 Negative staining procedure

Equal volumes of a bacterial suspension of approximately 5×10^6 cfu/ml were mixed with 1% PTA on a glass slide and a drop of the resultant mixture was placed onto a formvar coated grid. The mixture was allowed to remain on the grid for about 10-15 seconds before being drained off by touching the edge of the grid with a piece of Whatman No. 1 filter paper. The rate at which the bacteria-stain mixture drained from the grid into the filter paper was indicative of the suitability of the preparation. Drops that drained quickly usually contained insufficient material.

2. Embedding and thin-sectioning

2.1 Grids

EMS 400 mesh copper grids were used in all studies.

2.2 Cacodylate buffer

This was made by adding the appropriate amount of sodium cacodylate (EMS) to one liter of de-ionized water to give the following concentrations:

Samples	Concentration
bacteria	0.05 M (supplemented with 10mM $MgSO_4$)
tissue	0.10 M

These solutions were adjusted to a pH of 7.2 using hydrochloric acid and stored at 4º C until required.

2.3 Glutaraldehyde

Glutaraldehyde was obtained from EMS as an electron microscopy grade 25% (v/v) aqueous solution. The stock solution was stored in at 4º C and working 3% (v/v) solutions were made freshly in cacodylate buffer as required.

2.4 Osmium tetroxide

A stock 2% (w/v) solution of OsO_4 (Stevens Metallurgic Corporation) was made by dissolving the contents of a 1g ampule in 50 ml cacodylate buffer. OsO_4 emits a dangerous vapor and was always handled in a fume hood. The solute was completely dissolved after standing overnight at 4º C. The stock solution was stored at 4º C in a tightly capped bottle covered with aluminum foil. The working 1% solution was made by mixing equal volumes of the stock 2% solution and cacodylate buffer.

2.5 Ethanol series

A graded ethanol series was made by mixing appropriate amounts of dry absolute ethanol and cacodylate buffer. The 50% and 70% solutions were stored at 4º C and the 90%, 95% and absolute ethanol solutions were stored at

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room temperature. (Percentages refer to the concentration of ethanol).

2.6 Propylene oxide

This was obtained from EMS and was supplied ready for use. The container was kept tightly stoppered at all times.

2.7 Resin mixture

The resin used in all studies is described in 2.7.1 below. All volumes were measured in disposable 50 ml tri pour beakers from VWR except for dibutyl phthalate and DMP-30 which were measured using one ml disposable syringes without needles.

2.7.1 Epon-araldite resin mixture

The resin mixture was comprised of the following:

Component	Proportion
1) Epon 812 (resin)	5 parts
2) Dodecenyl succinic anhydride (DDSA)	11 parts
3) Araldite (resin)	3 parts
4) Dibutyl phthalate (plasticizer)	0.8 parts
5) Methyl nadic anhydride (DMP 30) (accelerator)	0.4 parts

Components were added to a glass bottle in this order and vigorously shaken for 10 min with the exception of DMP 30 which was added just before resin was required for use. Complete resin (with DMP-30) was then vigorously shaken for an additional three to five min. This resin mixture was polymerized at 60° C for 24 h.

2.8 Embedding and thin-sectioning procedure

For bacterial samples, organisms were harvested, fixed in 3% buffered glutaraldehyde, washed three times in 0.05 M cacodylate buffer containing 10mM $MgSO_4$ and pre-embedded in 3% (w/v) Noble agar. Cubes of 1 mm^3 thickness were cut and post-fixed in 1% OsO_4 in cacodylate buffer for 1 h.

Tissue samples were harvested, rinsed in 0.1 M cacodylate buffer and fixed in 3% buffered glutaraldehyde. One mm^3 cubes were cut and post-fixed in 1% OsO_4 in cacodylate buffer for 1 h. All samples were rinsed in cacodylate buffer three times followed by a dehydration procedure of immersion for five min in each of 50%, 70%, 90% and 95% ethanol in cacodylate buffer. Dehydration of samples was completed by two rinses of ten min each in absolute alcohol. Ethanol was removed by pipette and the specimens rinsed twice in propylene oxide. Specimens were not allowed to dry during any step of this procedure.

Resin infiltration was done by placing the samples in a 2:1 mixture of propylene oxide and complete resin for one hour, and this mixture was then replaced by a 1:2 mixture of propylene oxide and complete resin for an additional hour. Specimens were placed in complete resin for one hour after which the resin was changed for fresh complete resin which had been degassed for 20 min in a vacuum desiccator. These remained in the degassed resin for one hour, removed and located in embedding molds. The molds were filled with degassed complete resin. Specimens were manipulated using wooden swab sticks sharpened at one end to form a flexible spatula which was pre-treated by dipping in degassed complete resin. These treated spatulas

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were placed at 60° C for one hour prior to use to allow the resin end to dry and so as to prevent the introduction of air bubbles around the specimen. Blocks were labelled with hardened paper and polymerized in a 60° C oven for 24 h. After removal from the oven, blocks were left at room temperature for two or three days to allow complete hardening of the resin prior to their removal from the molds. Embedding of samples was completed in one day and was followed by a 24 h polymerization, the whole procedure taking approximately 30 h to complete. The embedding schedule and times required are summarized in Table 1.

Polymerized blocks were placed in the round chuck of a LKB Ultratome III ultramicrotome and trimmed mechanically with a glass knife made with an LKB knife maker.

When blocks had been appropriately trimmed, ultra thin sections were cut using the thermal advance on the ultramicrotome and using a Diatom diamond knife filled with clean de-ionized water. The diamond knife edge was cleaned with a soft clean pith stick. Sections giving grey or silver interference colors (60-90 nm thickness) were chosen. Individual sections and small ribbons were picked up on copper grids. Grids were submerged in the boat and sections located above them with a mounted eyelash. Sections were removed by lifting the grid out of the water and the grids blotted dry by touching the edge to a piece of filter paper.

Ultrathin sections were stained with 5% aqueous uranyl acetate for 60 seconds by floating grids, sections downward, on a drop of stain placed on parafilm and covered by a glass petri dish lid. Grids were removed and

sections washed thoroughly in a running stream of de-ionized water using approximately 15 ml per grid, after which they were blotted and allowed to dry. The grids were then held in fine forceps and a drop of lead citrate placed on them for 20 seconds, after which they were washed as before using approximately 25 to 30 ml de-ionized water per grid. Grids were blotted and allowed to dry as before, Stain was removed from the stock bottles using a fine pasteur pipette with the tip located about 5mm below the meniscus. The first drop in the pipette was always discarded, thus ensuring minimal stain deposit.

Thin-sectioning was performed under strictly dust free conditions and all materials coming into contact with sections, knife or boat water were stringently cleaned and rinsed under running de-ionized water. Sections were examined and photographed in a Hitachi H600 electron microscope at 75 kV, using various instrumental manipulations.

2.9 Stains for ultra thin sections

2.9.1 Uranyl acetate

A 5% (w/v) solution of uranyl acetate was made by dissolving 1 g in 20 ml de-ionized water. The solid was found to dissolve completely upon standing overnight at room temperature. The solution was filtered through a Whatman No. 2 filter paper and stored in a dark glass-stoppered bottle at room temperature.

2.9.2 Lead citrate

One pellet sodium hydroxide was dissoved in 15 ml boiled cooled de-ionized water. This solution was added to 0.1 g lead citrate in a glass screw-capped bottle and dissolved by shaking. A further 10 ml boiled cooled de-

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ionized water was added, and the stain was stored tightly capped at room temperature.

3. Scanning electron microscopy (SEM)

3.1 Hexamethyldisilaxane (HMDS)

This was obtained from Polysciences, Inc. (Warrington, Pa.) and kept tightly capped in a fume hood prior to use.

3.2 SEM procedure

Bacterial specimens were fixed in 3% glutaraldehyde in cacodylate buffer, washed three times in buffer and dehydrated in 50%, 70%, 90%, and absolute ethanol. Samples were then treated with two changes of HMDS and applied to specimen stubs with double sided sticky tape. Stubs were coated with 20 nm gold/palladium at a target to specimen distance of 5 cm with a current of 15 mA for 4 min. Specimens were viewed in a Hitachi H600 electron microscope in the scanning mode at an accelerating voltage of 40 kV.

Table 1. Embedding of specimens for electron microscopy.

Procedure	Time
1) Wash in cacodylate buffer	15 min
2) Fix in 3% glutaraldehyde in cacodylate buffer	30 min
3) Wash in cacodylate buffer (ten changes)	30-40 min
4) Post-fix in 1% OsO_4	1 h
5) Wash in cacodylate buffer	5 min
6) Dehydrate in ethanol series in cacodylate buffer	20 min
7) Complete dehydration in absolute ethanol and propylene oxide	40 min
8) Resin / propylene oxide mixtures	120 min
9) Resin (two changes)	120 min
10) Polymerization	24 h

Total Time Required	Approximately 30 h

Appendix 4

1. **Production of polyclonal antisera against L. pneumophila.**

1.1 Rabbits

   White New Zealand rabbits were obtained locally and pre-bled to assess

pre-immunization level of antibodies against L. pneumophila.

1.2 Antigen preparation

   Whole cells of L. pneumophila (approximately 1×10^9 cfu/ml) were

formalin fixed in 1% (v/v) formaldehyde (VWR) in PBS and a small volume of

sterile egg yolk. Prior to immunization of rabbits, 1 ml of this solution was mixed

with an equal volume of Freund's Incomplete adjuvant (VWR) and

homogenized in a Sorval Omni Mixer.

1.3 Bleeding procedure

1.3.1 Ear bleed

   Samples of blood obtained for antibody titer analysis following

immunization were drawn aseptically from the central artery of one of the

rabbit's ears. Vasodilation of this artery was achieved by warming the rabbit's

ear with a 15 watt bulb of a portable lamp. When the artery was suitably

dilated, the ear was swabbed with alcohol and a 21 gauge needle was inserted

to release approximately 5 ml blood into a sterile test tube. The ear was then

swabbed again with alcohol and treated appropriately to stop the bleeding.

1.3.2 Cardiac puncture

   Once the antibody titer had reached a suitable level following

immunization, the final volume of blood was collected by cardiac puncture and

exsanguination of the rabbit. The rabbit was anesthetized with the addition of a

sodium solution of Nembutal into the peripheral vein of the ear and blood drawn through an 18 gauge needle and syringe inserted through the chest cavity into the heart. The rabbit was exsanguinated in a humane fashion with a final injection of Nembutal into the heart.

Blood obtained from ear bleeds and cardiac puncture was allowed to clot overnight at 4° C and the resultant serum decanted for titer analysis or stored frozen in 1 ml aliquots at -70° C.

1.4 Antibody titer assay

An indirect immunofluorescence assay (Materials and Methods, Chapter Four) was used to measure the titer of sera drawn from rabbits. Once obtained, doubling dilutions of the serum were made in PBS and used immediately in the analysis. Formalin fixed whole L. pneumophila cells were added to multiple spots on glass slides and allowed to dry. Dilutions of the serum were added to these areas as the primary antibody in an indirect immunofluorescence assay and allowed to incubate at 37° C for 1 hour. Three-fold washes were made with PBS whereupon goat anti-rabbit FITC conjugated serum was added to all spots on the glass slide and incubated for 1 hour at 37° C. These were washed three times and viewed with an BH-2 Olympus microscope in the immunofluorescence mode. Appropriate scores of immunofluorescence for each dilution were made and the most diluted spot which gave a positive score selected as the titer.

1.5 Immunization procedure

Rabbits were pre-bled and analyzed for pre-immunization antibody against L. pneumophila. A small area of the rabbits back was clean shaven,

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swabbed with alcohol and the antigen-adjuvant mixture administered into 10 sub-cutaneous sites with a 21 gauge needle and syringe. These sites were swabbed with alcohol again and examined for unusual reactions. The rabbits were boosted with a similar antigen mass 2 and 4 weeks later. Antibody titer was checked every week during this time.

REPRINTS OF PUBLICATIONS

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