Spring 1994

The role of opsonin-independent bacterial adherence in the pathogenesis of Legionnaires' disease

Frank Clinton Gibson
University of New Hampshire, Durham

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The role of opsonin-independent bacterial adherence in the pathogenesis of Legionnaires' disease

Gibson, Frank Clinton, III, Ph.D.

University of New Hampshire, 1994
THE ROLE OF OPSONIN-INDEPENDENT BACTERIAL ADHERENCE IN THE PATHOGENESIS OF LEGIONNAIRES' DISEASE.

BY

Frank C. Gibson III
B.S., University of New Hampshire, 1989

DISSERTATION

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

May 1994
This dissertation has been examined and approved.

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Dr. Robert M. Zsigay, Professor of Microbiology

4 May 1994

Date
TO MY MOTHER AND FATHER

WITHOUT YOUR LOVE AND EVERLASTING SUPPORT THIS WOULD NOT HAVE BEEN POSSIBLE.
ACKNOWLEDGMENTS

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Abstract

THE ROLE OF OPSONIN-INDEPENDENT BACTERIAL ADHERENCE IN THE PATHOGENESIS OF LEGIONNAIRES' DISEASE.

by

Frank C. Gibson III

Legionella pneumophila is a facultative intracellular bacterial pathogen which initiates infection of the human host by adhering to macrophages in the alveoli of the lung following which internalization and intracellular multiplication occurs. In this study a novel mechanism of opsonin-independent attachment for this organism to host cells was identified. L. pneumophila serogroup 1, strain Nottingham N7, and U-937 cells as well as primary guinea pig alveolar macrophages were used to study this adherence phenomenon.

A variety of assay methods were used to evaluate the nature of this binding event: transmission electron microscopy and scanning electron microscopy were used to visualize the uptake and replicative processes while organism enumeration as a measure of adherence was accomplished using viable bacterial cell colony counts and indirect immunofluorescence studies. It was shown that L. pneumophila adhered
to U-937 cells in the absence of complement and a complete infectious cycle for the organism was found. These data confirmed previously identified stages of phagocytic host cell interaction including uptake, intracellular replication and release of progeny bacteria.

The chemical nature of the *L. pneumophila* bacterial "adhesin" and the host cell "receptor" in binding studies were investigated. The results from both competitive binding and surface treatment studies suggested the nature of the putative adhesins and receptor moieties associated with successful attachment of *L. pneumophila* to these host cells. Glycolipids on host cell surfaces and proteins or glycoproteins on *Legionella* membranes function to facilitate binding of bacteria prior to engulfment.

Monoclonal antibodies specific for complement receptors CR1, CR3 and CR4 were used in blocking studies and these indicated that these molecules were not involved in the recognition of legionellae. These results indicate the importance of opsonin-independent binding mechanisms for *L. pneumophila* to host cells and shed new light on our understanding of cellular infection by *L. pneumophila*. 
SECTION I

HISTORICAL PERSPECTIVE OF THE GENUS LEGIONELLA AND
LEGIONNAIRES' DISEASE.

1.1 Background and Objectives of Study

*Legionella pneumophila* is the major organism responsible for the
potentially life-threatening human pneumonic illness called Legionnaires'
disease. Outbreaks of this disease either occur in explosive, spectacular
fashion or take the form of sporadic infections and in either case these
may be of community based or nosocomial origin (68, 77, 175). Although
the most notorious outbreak occurred 18 years ago among American
Legionnaires in Philadelphia, PA (68), to which this disease owes its name,
many outbreaks have occurred since then. Furthermore, numerous
outbreaks have been serologically diagnosed retrospectively prior to 1976
(97, 145, 173). It was from the Philadelphia epidemic that two very
important points were forthcoming: first, an entire family of previously
unidentified microorganisms were identified and second, physicians as well
as researchers were forcibly reminded that infectious diseases remain a
major problem afflicting humankind.

Since the late 1970’s, several new microbial diseases have been
identified and these include such newly recognized important infections as
Lyme’s disease, *Campylobacter* enteritis and acquired immune deficiency syndrome (AIDS) to name but a few. Understanding the physical nature of *Legionella*, identifying aspects of the environment that influence its proliferation together with resolving host involvements in the establishment of Legionnaires’ disease are essential if this pathogen is to be brought under control.

This bacterium is a facultative, intracellular, respiratory pathogen that induces lobar pneumonia in those infected. The inhalation of aerosolized water droplets contaminated with *L. pneumophila* is the route by which this organism gains access into the host’s respiratory network and begins the colonization of the resident macrophages in the lung (77). This process is a necessary prerequisite for the initiation of disease.

Aggressive antimicrobial chemotherapy has been shown to reduce the likelihood of patient mortality (68). Alternatives to traditional antibiotic treatment are currently being sought for the control of Legionnaires’ disease. This is particularly important as chemotherapy alone will not prevent the initiation of disease and may not on occasion prevent the outcome of severe infection. In addition, recurrent infections following antibiotic therapy have been reported. Treatment regimes often must be broad-ranging in their scope and, reflecting the importance of cell-mediated immunity, may require the use of immune system-enhancing molecules such as interferon-gamma (IFN-γ) and interleukins to augment chemotherapy (15, 69, 189, 190). Moreover, these treatments may be
most effective if used in conjunction with traditional antibiotic regimes. The development of combination therapy programs will undoubtedly assist in the effective control of this pathogen.

The purpose of this study was to investigate and evaluate an alternative mechanism of *Legionella* attachment to host cells involving an opsonin-independent process. U-937 cells, transformed human macrophage-like cells and guinea pig alveolar macrophage (GAM) cells were used to evaluate the adherence of *Legionella* by this mechanism. By identifying the bacterial attachment mechanisms the results of this work should augment current knowledge on understanding the fundamental processes of *Legionella* infection of host cells and may assist toward the development of a suitable vaccine for Legionnaires' disease.

1.2 The Problem

1.2.1 Background

Legionnaires' disease constitutes a medical problem of significant proportions and death due to infection is not uncommon. More data is required on the mechanisms of disease induction. Legionellosis is the common name for an illness which consists of two disparate disease conditions: Legionnaires’ disease and Pontiac fever. These conditions are caused by what appear to be phenotypically and genotypically identical organisms. Legionnaires’ disease is a lobar pneumonia, which received its name from the Philadelphia epidemic of 1976 (68, 114). It is the more serious condition caused by *L. pneumophila* and patient recovery
inevitably requires medical intervention. Pontiac fever, on the other hand, acquired its name from an outbreak in Pontiac, MI, in 1968 and is a self-limiting respiratory illness which presents with flu-like symptoms (77). Factors such as the health of the host, the infectious dose, the method of exposure and the expression of bacterial virulence determinants by the organism undoubtedly offer some explanation for these two variations in disease expression.

1.2.2 Philadelphia Outbreak

Immediately following the annual convention of the American Legion held in July and August of 1976 at the Bellevue-Stratford hotel in Philadelphia, PA, the world witnessed a spectacular epidemic of acute febrile respiratory disease (68). The etiology of this disease remained unresolved for several months. In the epidemic 216 people developed respiratory disease and 34 died (68). Analysis of the 216 identified cases indicated that 182 legionnaires contracted the disease and 29 died. The 34 remaining cases were people who were at or near the hotel during the outbreak and came down with so-called Broad Street pneumonia and of these 5 died (68).

During this outbreak, several hypotheses were proposed to explain the etiology of disease and establish the parameters for elucidating the causative agent of this outbreak. Initial identification protocols failed to establish the etiology of this disease in that commonly available culture media and state-of-the-art clinical identification procedures failed to
provide definitive information.

In early 1977 Dr. J. McDade from the Centers for Disease Control, using isolation procedures to identify rickettsia, demonstrate that the etiologic agent of Legionnaires' disease was a hitherto unidentified gram-negative, rod shaped bacterium (114). Isolation process involved inoculation of homogenized biopsied lung tissue from legionnaires' into the yolk sac of embryonated hens' eggs. The organism replicated in the yolk sacs which yielded pleiomorphic, carbol fuchsin staining bacilli (114) later identified as a new family of bacteria, the Legionellaceae (20). Subsequent studies have retrospectively indicated that Legionella infections of both epidemic and sporadic forms have occurred previously (77, 173).

1.2.3 Pontiac Fever Epidemic

Following the identification of the Legionnaires' disease bacterium, retrospective studies were conducted to determine whether previously unresolved outbreaks of pneumonic illness were due to infection by *L. pneumophila* (95, 144, 173). Although the Pontiac fever epidemic occurred in 1968, the etiologic agent was not identified at the time of the outbreak. This epidemic of acute, self-limiting, febrile respiratory illness involved nearly all employees of the Oakland County Health Department in Pontiac, MI, during July and August of 1968 and a total of 144 persons working at or visiting this facility became ill. In general Pontiac fever presents as a mild respiratory illness together with fever and/or chills.
generalized myalgia, malaise and headache (Table 1.1). The attack rate was 95% and the mode of transmission of the pathogen was determined to be the building's ventilation system (77). None of the infected persons died nor was there evidence of sequelae following this outbreak. Medical intervention is not normally required for this form of Legionella-induced disease.

It is not understood why Legionella-induced disease presents in these two quite different forms, nor are the critical factors governing disease expression known. Examination of variations in Legionella-host interactions will undoubtedly facilitate our understanding of the disparity in clinical presentations and will help develop strategies to prevent Legionella-induced deaths.

1.3 The Organism

1.3.1 Morphology

Legionellae are fastidious, gram-negative, pleiomorphic, bacilli approximately 1-2 µm in length and 0.3-0.9 µm in width (Table 1.2). In the lung of either infected humans or guinea pigs, short coccobacillary forms predominate (26, 149, 156). However, when these organisms are cultured on bacteriologic media longer forms abound and these can often be in excess of 20 µm in length. Legionellae stain weakly gram-negative with safranin but more strongly with carbol fuchsin. The weak safranin staining is most likely the result of hydrophobic
### Table 1.1: Symptomatic Presentation of Legionellosis*

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<th>Pontiac Fever</th>
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<td>Attack Rate</td>
<td>1-5 %</td>
<td>95%</td>
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<tr>
<td>Incubation Period</td>
<td>2-10 days</td>
<td>1-2 days</td>
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<td>Symptoms</td>
<td>Pneumonia</td>
<td>Flu-like illness</td>
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<tr>
<td>Other sites of Infection</td>
<td>CNS, G.I. Tract,</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Pericardium and Kidneys</td>
<td></td>
</tr>
<tr>
<td>Fatality Rate</td>
<td>0-40 %</td>
<td>0%</td>
</tr>
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</table>

* Legionellosis is the term applied to both illnesses. Legionnaires’ disease and Pontiac fever are the result of infection by genotypically and phenotypically identical organisms.
Table 1.2: Morphologic Identification of *Legionella pneumophila*

- Slender gram negative rods (*in vivo* forms are short 0.5 x 1-2μm)
  *in vitro* forms are elongated 0.4 x 2-3μm
- Pleiomorphic (long forms can be up to 20-50μm in length)
- Tapered ends
- Divide by non-septate, pinching, binary fission
- Possess lipopolysaccharide (major antigenic structure)
- No evidence of acid mucopolysaccharide capsule
- Motile by polar or sub-polar flagella
- Fimbriate
- Blebs on outer membrane surface
- Outer membrane and cytoplasmic membrane each 10nm thick
  separated by a thin peptidoglycan layer
- Ribosomes 25nm in diameter
- Internal PβH storage granules
interactions between the surface of *Legionella* and the safranin and this may be related to the copious amounts of branched chain fatty acids in the organism (124). *Legionella* do not form spores and with the exception of *L. micdadei* which is weakly acid-fast in lung tissue (127) they are not acid-fast.

*Legionellae* possess two lipid-rich membranes is separated by a peptidoglycan layer, typical of gram-negative bacteria. In addition, the organisms possess non-parallel sides with tapered ends (26, 150, 155). The outer membrane and cytoplasmic membrane are approximately 10 nm in width and are separated by a thin yet identifiable peptidoglycan layer. This structure has been demonstrated with biochemical procedures and has been visually identified by electron microscopy (64, 155). Division occurs by pinching binary fission (150). The lipids produced by *legionellae* are complex and are not of the types typically found on other gram-negative organisms. In addition, poly-β-hydroxybutyrate storage granules are commonly found in *Legionella* (26, 148, 155). The majority of organisms in the group are motile by polar or sub-polar flagella (150, 153) which are 14-20 nm in diameter and up to 8μm in length (135, 153, 154). The individual monomeric flagellin proteins have a molecular mass of 47 kD, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (136). Unlike organisms grown on laboratory media flagella are not usually found on organisms in lung specimens. The expression of flagella appears to be temperature-dependent (136).
Fimbriae (Rodgers) have been identified on the surface of *L. pneumophila* and lipopolysaccharide (LPS) is produced abundantly (131, 150). The chemical composition of *L. pneumophila* LPS is similar to that of many other gram-negative organisms and has been characterized by gas chromatography and mass spectroscopy (166). The composition of *Legionella* LPS is typical of that for other gram-negatives with a lipid A portion, a 2-keto-3-deoxyoctonate linker region forming 1-13% of the carbohydrate present and polysaccharide side chains. Species and serotypic differences in the composition of the *Legionella* LPS make this an excellent candidate for serogroup differentiation (25, 131). The LPS is smooth and is the primary structure that elicits an antigenic response in infected patients (71).

### 1.2.5 Taxonomy and Nomenclature

Brenner *et al.* (19) proposed a classification system for the Legionnaires' disease bacterium based upon DNA homology and % guanosine and cytosine content (mol %G+C). This study demonstrated that the organism responsible for the Philadelphia outbreak had not previously been described. The proposed family was similarly classified as unique and named the *Legionellaceae*. At the time, this group was determined to contain one genus *Legionella*, and one species *pneumophila*. Subsequently, numerous species have been identified and the family currently contains 1 genus, 37 species and 3 subspecies with numerous serogroups (Table 1.3). The range of DNA relatedness among the
legionellae is great and the serological variability is extensive. An alternative to the single genus nomenclature system has been suggested (74) with the family *Legionellaceae* consisting of 3 genera: (1) *Legionella* (*L. pneumophila*), (2) *Tatlockia* (*T. micdadei*) and (3) *Floribacter* (the autofluorescent species *F. baizemanae, F. dumoffii* and *F. gormanii*). However, the simpler and more convenient single genus system is by far the most universally accepted format. Isolation of *Legionella* from clinical or environmental samples requires specialized media while identification involves the use of molecular and serological procedures. Furthermore, it is known that legionellae grow exclusively on L-cysteine containing buffered charcoal yeast extract (BCYE-α) media (41, 184) and this feature greatly assists isolation procedures.

1.3.3 Biochemical Properties

Due to the cultural fastidiousness of this organisms and its strict requirement for both L-cysteine and ferric salts, commonly available laboratory media fail to grow legionellae. Indeed, initial studies dealing with the physiology of *L. pneumophila* were based on the development of media capable of cultivating the organism. The bacteria in the group are catalase positive while oxidase, hippurate, gelatinase, and β-lactamase expression are either variable or species dependent. In addition, some species show a brilliant blue/white or red autofluorescence under longwave UV and this may prove useful for identification (Table 1.3)
Using radiolabeled amino acids and carbon sources, the biochemical pathways of nutrient utilization for *L. pneumophila* have been elucidated. Pine et al. (139) demonstrated that carbohydrates were not used as energy sources for legionellae. Indeed, the production of metabolic energy is primarily derived from the breakdown of amino acids such as glutamate, serine and threonine. 14C labeled glutamate revealed biosynthetic utilization with approximately 29% of this substrate traced to lipid, 21% to protein, 16% to nucleic acid, 9% to miscellaneous polysaccharides and 24% to small molecular weight compounds (171). Legionellae are strict aerobes and the utilization of carbohydrates occurs via oxidative processes as opposed to fermentation (139, 180, 181). Glucose is metabolized and the resultant energy is used in a maintenance rather then in a biosynthetic fashion. When glucose metabolism occurs, it is preferentially broken down by Entner-Doudoroff and pentose-phosphate mechanisms rather then by glycolytic processes (172).

The cellular fatty acid content, in particular branched chain fatty acids, of *L. pneumophila* is not consistent with that of other gram-negative bacteria (124). Moreover, the fatty acid profile differs somewhat between species of the genus. The majority of lipids produced by *Legionella* are (iso-16:0) carbon compounds and in particular methyl-14-methylpentadecanoic acid. Such 16-carbon compounds make up greater than 30% of the total cellular fatty acid content of the organism. Other lipids are produced by *L. pneumophila* and include the polymeric poly-β-hydroxybutyric acid (PBH) which was demonstrated by sudan black
Table 1.3: Biochemical Identification for the Genus *Legionella*.

<table>
<thead>
<tr>
<th>Species</th>
<th># Serogroups</th>
<th>Source</th>
<th>Cat</th>
<th>Ox</th>
<th>Hip</th>
<th>Gl</th>
<th>β-lac</th>
<th>AF</th>
<th>M</th>
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<tr>
<td><em>L. pneumophila</em></td>
<td>14</td>
<td>H/E</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>L. micdaeii</em></td>
<td>1</td>
<td>H/E</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. bozemanii</em></td>
<td>2</td>
<td>H/E</td>
<td>+</td>
<td>V</td>
<td>-</td>
<td>V</td>
<td></td>
<td>BW</td>
<td>+</td>
</tr>
<tr>
<td><em>L. dumoffii</em></td>
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<td>H/E</td>
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<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>BW</td>
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<tr>
<td><em>L. gormanii</em></td>
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<td>H/E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>BW</td>
</tr>
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<td>2</td>
<td>H</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td>H/E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>L. oakridgensis</em></td>
<td>1</td>
<td>H/E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td><em>L. wadsworthii</em></td>
<td>1</td>
<td>H</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. feelii</em></td>
<td>2</td>
<td>H/E</td>
<td>+</td>
<td>-</td>
<td>V</td>
<td>-</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>L. saintheleni</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
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<td>+</td>
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<tr>
<td><em>L. anisa</em></td>
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<td>E</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
<td></td>
</tr>
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<td>H/E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>2</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>R</td>
<td>+</td>
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<td>H</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>R</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. speritensis</em></td>
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<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>BW</td>
<td>+</td>
</tr>
<tr>
<td><em>L. cherrrii</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>BW</td>
<td>+</td>
</tr>
<tr>
<td><em>L. stigerwaltii</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>BW</td>
<td>+</td>
</tr>
<tr>
<td><em>L. sainticrucis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. israeliensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. birminhamensis</em></td>
<td>2</td>
<td>H</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>YG</td>
<td>+</td>
</tr>
<tr>
<td><em>L. cincinnatiensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. moravia</em></td>
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<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>L. bruniae</em></td>
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<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. quinlivanii</em></td>
<td>2</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. tucsoniensis</em></td>
<td>1</td>
<td>H</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>BW</td>
<td>+</td>
</tr>
<tr>
<td><em>L. fairfeldensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>L. adelaidensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. shakespearei</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. geestiana</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. londoniensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. naurtarum</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. quateirensis</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>L. worsleienss</em></td>
<td>1</td>
<td>E</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+= positive reaction; - = negative reaction; V= variable results; w= weak
Cat= catalase; Ox= oxidase; β-Lac= β-lactamase; Hip= hipurate; M= Motility
Gl= gelatin liquefaction; AF= autofluorescence
H= human origin; E= environmental origin
YG= yellow-green; R= red; and BW= blue-white fluorescence.
staining and transmission electron microscopy (150, 181).

Numerous organic substrates and inorganic compounds are necessary for the laboratory cultivation of legionellae. Initial laboratory cultivation of legionellae on agar media demonstrated a requirement for hemoglobin and this specific requirement for high levels of iron is met by the addition of ferric pyrophosphate to BCYE-α media (143). Other ions which are essential to Legionella cultivation include trace amounts of Ca, Co, Cu, Mg, Mn, Na, Ni, V and Zn (143). In addition to these metal ions, charcoal and α-ketoglutarate are important facilitators for organism growth on laboratory media. The role played by these materials appears to be the detoxification of BCYE-α. It is suggested although not proven that they serve the purpose of either inactivating toxic activity of oleic acid or of scavenging toxic oxygen radicals generated from the process of autoclaving the yeast extract in the medium.

1.3.4 Physico-chemical Properties

In the environment legionellae experience a wide variety of conditions which often prove bactericidal in nature; these adverse conditions include pH variations, exposure to ultraviolet radiation (UV) and temperature fluctuations. *L. pneumophila* grow optimally on laboratory media between pH 6.85-6.95 at 37°C and in the presence of 2.5% CO₂. The bacterium is more sensitive to UV irradiation than many other gram-negative bacteria including *Escherichia coli*, *Salmonella typhi*, *Serratia
marcescens and Pseudomonas aeruginosa (5). Organisms from environmental or clinical isolates grow at temperatures ranging from 25°C-43°C; however, multiplication and growth do not readily occur at 50°C or above (65, 181). Legionella selection from clinical and environmental samples usually involves either acid or heat treatment. Edelstein et al. (47) showed a marked increase in Legionella recovery from clinical samples using heat treatment at 60°C for 1-2 min while Bopp et al. (18) and Buesching et al. (20) demonstrated the usefulness of acid treatment of pH 2.2 for 4 min to enhanced organism recovery from both environmental and clinical specimens. Legionella are sensitive to alkaline treatment (pH 8.0); however, their sensitivity to chlorine is inconclusive as organisms in association with protozoa appear to be more resistant to chlorine than free-living organisms (41, 97, 164).

1.3.5 L. pneumophila Growth on Bacteriologic Media

L. pneumophila are nutritionally fastidious organisms which require strict conditions for growth. Feeley and Gorman (57) first demonstrated that Legionella could be cultivated, but only poorly, on Muller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitaleX in the presence of 2.5% CO₂ (57). In subsequent media formulations Feeley et al. (57) optimized the growth of Legionella in the laboratory by demonstrating that this organism has an absolute requirement for L-cysteine and ferric pyrophosphate. This novel medium formulation was named Feeley-Gorman (F-G) agar. Further physiologic studies by Feeley et al. (56)
demonstrated that media with yeast extract and activated charcoal facilitated recovery and enhanced growth of legionellae. This new media was called charcoal yeast extract (CYE) agar (55). A further modification of CYE agar, introduced by Pasculle (136), involved buffering this media with N-2-acetamido-2-aminoethanesulfonic acid (ACES) buffer to give BCYE agar. The latest refinement introduced by Edelstein (42) added α-ketoglutarate to BCYE to give BCYE-α and this is the current standard for cultivation of legionellae.

Chemically defined culture media have been developed (101, 139) but these have been used primarily to determine the nutritional requirements of this genus. The use of defined media in conjunction with alternative growth systems may help to identify and define growth rate-dependent virulence characteristics and may aid in ascertaining the role these factors play in the infectious process.

1.3.6 Genetics and Molecular Biology of the Organism

Although the understanding of Legionella interactions with host cells at the cellular and molecular levels is paltry at best, even less is known about the genetic regulation and expression of those virulence factors responsible for the establishment of disease. The majority of early genetic investigations were directed toward the identification of Legionella in environmental or clinical samples. Genomic sizing studies demonstrated that the chromosome of the Legionella is approximately $2.5 \times 10^9$ daltons
Legionellae possess a guanosine + cytosine DNA content which ranges from 38-52 mol% and averages 39 mol% (48). Recently, 16S ribosomal RNA studies and DNA hybridization have indicated that the Legionellaceae are not closely related to other eubacteria (70, 160). In addition, several DNA probes are commercially available for the detection of legionellae from either clinical or environmental specimens (42, 174).

Extra chromosomal DNA in the form of plasmids is frequently isolated from legionellae (107, 116). The 80-megadalton plasmid is common among many strains of this organism; however, the role of these plasmids is unknown (19, 100, 107, 120, 135). Often the plasmids of other bacteria have been shown to harbor genes responsible for virulence traits such as antimicrobial resistance while others directly induce virulence factors such as toxin expression. This does not appear to be the case with Legionella in which the presence or absence of plasmids do not modify antimicrobial resistance patterns nor change expression of virulence traits. It would appear from this that plasmids apparently play no part in the expression or establishment of disease.

Cloning of Legionella DNA fragments into other organisms has been done successfully using E. coli as the recipient. Results however, are less then promising when plasmids of other organisms are inserted into Legionella (28, 37). In an early study in which Legionella fragments were cloned into E. coli an interesting virulence protein was revealed and this was involved with uptake of Legionella by macrophages (29, 51, 52). Other genes of L. pneumophila have been successfully cloned into E. coli.
including the gene responsible for the expression of the major outer membrane protein (80), a zinc metalloprotease (140) and a 19-kDa outer membrane peptidoglycan-associated protein (106). In addition, several highly conserved genes have been cloned into \textit{E. coli} including the recA gene (for homologous repair of DNA) and the groELS gene (for production of heat-shock proteins) (36, 81, 192). However, \textit{E. coli} although effective as a cloning system may not be best suited for expression of \textit{Legionella} inserts (80). Investigators may need to consider the use of alternatives to this vector system.

1.4 Pathogenesis

1.4.1 Human Implications

The precise processes by which legionellae infect host cells and thus induce human disease are not well understood. To characterize the mechanism of \textit{Legionella} infection and to define the role these processes play in \textit{Legionella} pathogenesis investigations on the infectivity of the bacterium have been performed using animal systems and a number of primary, secondary and transformed cell lines (Table 1.4). Various animal models have been evaluated for their ability to mimic the pathogenesis and pathology of human disease. Hamsters, several mouse and rat strains as well as guinea pigs have been proposed as models for human Legionnaires’ disease (10, 183, 191). The guinea pig has proven the most effective animal for the evaluation of systemic disease. Recently A/J mice have been investigated for their effectiveness at assessing several aspects
of mymicing the intracellular infection seen in human disease (191). Given that the mouse is well established for investigating other host-pathogen interactions and has a well characterized immune system the development of an effective mouse model for Legionnaires' disease would prove extremely useful for evaluating L. pneumophila interactions with the immune system in disease. Not only is the mouse immune system better understood than that of other animals but an extensive array of mouse immune modulators are available and functionally characterized for use in investigations. Such modulators include interferons, various interleukins, tumor necrosis and colony stimulating factors. Such a model could help elucidate the interaction of these modulating molecules with the pathogen and might eventually influence treatment regimes.

To better understand the events of Legionella infection at the cellular level researchers have used host cells from a variety of origins. These in vitro studies have used either transformed or non-transformed cells of epithelial, fibroblast, polymorphonuclear and mononuclear lineage (76, 87, 111, 134, 152). Most of these cells have been shown to support L. pneumophila infection; however, while human macrophages and monocytes support the growth of the organism polymorphonuclear leukocytes (PMNL) do not. Rather, these cells support an abortive type infection and this cell is relatively ineffective for studying cellular infectivity of legionellae (16, 88). These studies have proven important in defining which cells support Legionella replication and in outlining the ultrastructural events of intracellular infection. Furthermore, the establishment of an
Table 1.4: Stages of Cellular Infection for *Legionella pneumophila*.

- **Attachment**
  - **Weak Binding**
    - Hydrophobic interactions
    - Hydrogen bonds
    - van der Waals
    - Electrostatic interactions
  - **Strong Binding**
    - Structural Interactions
      - Fimbriae
      - Flagella
      - Surface proteins
      - Lipopolysaccharide

- **Uptake**
  - MIP Protein
  - Coiling phagocytosis
  - Banded enclosures

- **Intracellular Replication**
  - Inhibition of phagosome lysosome fusion
  - No acidification of phagosome
  - Ribosome-lined phagosome

- **Release**
  - Unknown
  - Protease?
appropriate cell model for the study of the cellular events in Legionnaires’ disease is necessary as human alveolar macrophages are not readily available.

Since the alveolar macrophage is the target cell in the lungs, macrophages or macrophage-like cells appear to be most useful for studying uptake, intracellular multiplication and release of bacterial progeny. In addition to studies with mononuclear phagocytes, investigations with epithelial and fibroblast cells which do not possess the same receptors as macrophages have shown that *L. pneumophila* binds to the cells and despite the lack of receptors these cells support a complete infectious cycle of the organism. These studies indicate that alternative mechanisms of cellular infection exist and that certainly those processes that occur independent of opsonins and/or complement receptors may constitute a primary mechanism by which cells are parasitized by *L. pneumophila* (75, 152).

A variety of studies have extensively investigated *Legionella* infection of monocytes and macrophage-like cells, as these cells are the most likely candidates for successful establishment of human infection (75, 82, 111, 138, 152). Those cells most frequently used include human peripheral blood monocytes and the cell lines U-937 and HL-60 (75, 87, 111, 138, 152). The U-937 cell has shown particular promise as a model for studying cellular infection as this cell has been used extensively in studies of both attachment and intracellular replication of *Legionella* (75, 76, 91, 138, 152). These cells possess Fc and complement receptors at levels consistent with
human alveolar macrophages (157). This, in conjunction with a similar intracellular replication kinetics profile for L. pneumophila, makes these cells an attractive model for studying L. pneumophila-host cell interactions. Furthermore, the U-937 cell has been useful for studying the interactions of other intracellular pathogens such as Salmonella with macrophage-like host cells (2).

Adherence of L. pneumophila to a host cell is a necessary prelude to intracellular infection (11). Recent studies suggested that the mechanism used by bacteria to adhere to cells was central to the establishment and outcome of disease (178, 182, 187, 188). Two mechanisms of L. pneumophila attachment to host cells have been reported (75, 88). One is mediated by opsonins while the other operates independent of opsonins. In the opsonin-dependent system Legionella uptake is mediated by the complement component C3bi and the host cell complement receptor type 3 (CR3). C3bi is deposited on the major outer membrane protein (MOMP) on the surface of Legionella and the MOMP/C3bi complex binds to CR3 on human monocytes (12). The opsonin-independent uptake mechanism occurs in an environment devoid of complement or specific antibody (75, 76, 152) and has been shown to produce a normal bacterial replicative cycle in U-937 cells (75, 152), as well as in MRC-5, HEp-2 and Vero cells (134).

Following complement-dependent adherence, Legionella organisms are internalized by monocytes via coiling phagocytosis (83) and internalized organisms are contained within banded enclosures (134). Shortly
thereafter, phagosomes containing organisms become ribosome-lined and are found in close association with mitochondria. The significance of these observations is, as yet, unknown; however, these processes may be important for the survival of this organism within host cells. Once the bacterium is internalized survival depends on the inhibition of phagosome-lysosome fusion within infected mononuclear cells (82). The mechanism by which *Legionella* inhibit phagosome-lysosome fusion is not understood. Research in this area would add to our understanding of the molecular events critical for *Legionella* survival in the inhospitable environment of the macrophage. Phagosome-lysosome fusion is one step in a series of normal degradative process by which foreign material is destroyed within phagocytes. The inhibition of these functions results in fulminant parasitism of infected macrophages and is closely followed by unabated intracellular replication of the organism.

Alternative mechanisms of intracellular survival are used by other facultative intracellular bacterial pathogens (60, 165). Some organisms such as *Listeria monocytogenes* breakdown the structural integrity of the phagosome and escape into the cytoplasm prior to phagosome-lysosome fusion (125). Others, like *Mycobacterium leprae*, persist within phagosomes that have fused with lysosomes and despite this bacteria actively multiply within this hostile environment (125).

Following the course of unchecked intracellular multiplication, legionellae escape from infected macrophages. The release of legionellae from the confinements of the host cell phagosome is then
followed closely by organism escape to the extracellular milieu. Release facilitates continuation of disease as progeny organisms initiate infection of other cells in the surrounding tissues. Legionellae are released from infected host cells by lysis (Table 1.4). The process by which Legionella organisms escape the phagosome and the host cell cytoplasmic membrane is as yet undefined; however, the major secretory protein of Legionella, a zinc metalloprotease with powerful cytotoxic potential, may play an important role in this process.

The terminal line of constitutive defense in the lung is the alveolar macrophage and this is important for the destruction of foreign material arriving in the lower respiratory tract. However, it is this cell which Legionella preferentially infects; the very cell designed to eradicate it. The cell-mediated branch of the immune response is critical to recovery from Legionnaires’ disease. However, it is unclear whether humoral defenses also play a part in recovery or immunity to infection (89, 91). Opsonization of Legionella with specific antibody enhances the adherence of this organism to mouse macrophages, as well as to U-937 cells and it has been suggested that without specific antibody complement may not enhance the uptake of L. pneumophila (91).

Iron is a requirement for the growth of most bacteria including pathogens whose virulence is frequently enhanced by iron (66). L. pneumophila has a strict requirement for high concentrations of iron to fuel metabolic activities. During infection of monocytes, L. pneumophila sequesters iron from the intermediary labile iron pools of the host cell.
However, the mechanism legionellae use to obtain the iron necessary for continuation of disease is not known. This iron pool is formed from iron-transferrin and iron-lactoferrin via transferrin and lactoferrin receptors respectively. Furthermore, iron is also available in the protein-bound form, ferritin. Intracellular replication of legionellae can be inhibited by treating monocytes with substances which decrease iron availability (22, 23, 24). These known inhibitory agents include iron chelators such as apolactoferrin and deferoxamine, agents which interrupt the evolution of iron from iron-transferrin like ammonium chloride and chloroquine as well as molecules that normally down-regulate transferrin receptors such as gamma interferon (IFN-γ) (22, 23).

*L. pneumophila* is incapable of intracellular replication within PMNLs whether or not these cells are in the presence of IFN-γ, specific antibody or complement. Moreover, PMNLs are not appreciably involved in the killing of legionellae, yet they may play a role in determining the eventual outcome of infection. Indeed, depletion of PMNL from the lungs of guinea pigs was shown to be detrimental to recovery (62). A mechanism that may explain the role of PMNLs during infection suggests a cooperative effect of PMNLs with monocytes or macrophages. At the site of infection PMNLs release large quantities of apolactoferrin that could be taken-up by infected cells. The concomitant reduction of iron availability following the uptake of this chelator would effect the reduction of intracellular replication of *L. pneumophila*. As cell parasitism occurs with or without
PMNLs, the inhibitory mechanism suggested would be only partially effective for control. Augmentation or enhancement of this bacteriostatic-type system may help to control infection and requires further investigations.

*L. pneumophila* has been shown to produce various toxins including legiolysin (a hemolysin), endotoxin (LPS) and a tissue destructive protease (13, 38). However, little is known about the role these molecules play in the development of disease. Cytopathology of destroyed lung tissues previously infected by *L. pneumophila* is suggestive of cytotoxic activities (10, 14, 183) (Table 1.5).

The protease of *L. pneumophila*, the major secretory protein, has been described as one of the more important molecules produced by the organism during infection. During the initial identification studies following the Philadelphia epidemic, a toxic etiology was suggested as an explanation of this outbreak (68). The organism produces a protease and this molecule was determined to possess both caseinase and gelatinase activities. Further investigations have identified the *Legionella* protease as a zinc metalloprotease with a molecular mass of 38,000 to 40,000 (38, 140). Hemolytic, cytotoxic and tissue destructive properties have been demonstrated for this molecule. Whether the protease is necessary as an important mediator of cell parasitism leading to establishment of infection or as a molecule essential for organism escape from infected cells is not known. Studies have indicated that the protease is produced primarily while the bacterium is within host cells and little is secreted when the
Table 1.5: Structural Virulence Factors of *Legionella pneumophila*:

<table>
<thead>
<tr>
<th>Structure</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fimbriae</td>
<td>Putative adhesin</td>
</tr>
<tr>
<td>Flagella</td>
<td>Common Antigen for Serogroups 1-3 Putative Adhesin</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>Activates Complement Cascade Potent Stimulator of Humoral Response Weak Endotoxin Putative Adhesin</td>
</tr>
<tr>
<td>Major Outer Membrane Protein</td>
<td>Genus Specific Epitope Species Specific Epitope C3 Acceptor Molecule Putative Adhesin</td>
</tr>
<tr>
<td>Legiolysin</td>
<td>Hemolytic properties</td>
</tr>
<tr>
<td>Major Cytoplasmic Membrane Protein</td>
<td>Heat Shock Protein (Hsp60/65) Potent Stimulator of Humoral Response Genus Specific Epitope</td>
</tr>
<tr>
<td>Macrophage infectivity Potentiator</td>
<td>Potentiates infection of mononuclear phagocytes Inhibits activity of host cell Protein Kinase C</td>
</tr>
<tr>
<td>Major Secretory Protein</td>
<td>Zinc Metalloprotease Cytotoxic for CHO cells Produced intracellularly within monocytes</td>
</tr>
</tbody>
</table>

*a Observations primarily associated with human disease*
bacterium is in the extracellular menstruum. However, strictly controlled physiologic *in vitro* studies have not been done to investigate these processes.

In addition to cytotoxic activity, hemolytic activity has been described for *L. pneumophila* (14, 69, 98, 188). Legiolyisin, the hemolysin of *L. pneumophila* is a 39-kDa protein which has been studied and has been cloned into *E. coli*. This hemolysin also confers the additional phenotypic characteristics of brown pigment formation on tyrosine containing media, as well as mediating colonial auto-fluorescence in longwave UV light (186). Despite cloning of the gene and partial characterization of this molecule, little is known about the role this structure plays in the pathogenesis of *Legionella* infections.

As is the case for the majority of gram-negative bacterial pathogens, *L. pneumophila* produces lipopolysaccharide (LPS). Initial investigations have provided contradictory evidence for the role of *L. pneumophila* LPS in virulence. LPS can activate the classical complement cascade (121); however, the precise nature of this process remains obscure. *Legionella* LPS is also the major immunogenic structure on the surface of this bacterium (71). Moreover, LPS can induce the production of immune modulators in mouse spleen cultures including interleukin-1 and interleukin-2, IFN-γ and tumor necrosis factor (16, 17).

Other *Legionella* proteins have been shown to play at least some role in the pathogenesis of Legionnaires’ disease. The macrophage
infectivity protein (MIP) was shown to be important for uptake of *L. pneumophila* by macrophages (30, 31). This molecule is a 24-kDa molecular mass protein the gene for which has been cloned into *E. coli* and sequenced (29, 51, 52). The MIP protein was shown to be conserved across the genus; however, there are notable variations in the migration pattern of the MIP protein between species (31).

### 1.4.2 Environmental Aspects

The ability of *L. pneumophila* to replicate within phagocytic freshwater amoebae was initially reported by Rowbotham (147). In subsequent years, several reports have confirmed that legionellae can multiply within a variety of protozoa including *Acanthamoeba castellanii, Naegleria fowleri, Hartmannella vermiformis* and the ciliated protozoa *Tetrahymena pyriformis* (59 122, 130). Several studies have identified environmental sources in which *L. pneumophila* organisms survived and have been shown to be responsible for several outbreaks of legionellosis (8, 58). In some cases, the protozoa isolated from these reservoirs have been shown to support the growth of *Legionella in vitro* (58). To date, there is no evidence to support the multiplication of *Legionella* organisms in an environment devoid of protozoa or host cells.

The mechanism by which *Legionella* parasite protozoa in the environment has yet to be defined. Furthermore, the characterization of a receptor on the surface of the amoebae that is responsible for the attachment of *L. pneumophila* has not been identified. Several research
groups have suggested that human infection by legionellae is a coincidental event in that *Legionella* may recognize a highly conserved structure on the surface of the alveolar macrophage which structurally mimics that on amoebae. A valid case can be made for coincidental human infection since humans form a dead-end host with no known mechanism for human to human transmission. This putative conserved surface structure located on amoebae and lung alveolar macrophages may allow for attachment and facilitate uptake, or allow for intracellular replication of *L. pneumophila* within these cells.

The mechanism of adherence for *L. pneumophila* to protozoa has yet to be identified; however, this organism has been shown to attach to human monocytes via two different molecular mechanisms, one of which requires complement, while the other process occurs in an opsonin-independent fashion (76, 88). In the natural aquatic environment, complement-mediated uptake is an unlikely process, but rather this mechanism would involve an "adhesin"-"receptor" complex arrangement between *L. pneumophila* and amoebae. Thus it is believed that *Legionella* may parasitize the alveolar macrophage at the earliest stage of organism recognition, in a manner similar to that by which these organisms parasitize fresh water amoebae.

1.5 Disease

1.5.1 Epidemiology

Legionellae are not primary pathogens for humans, but rather are
opportunist which cause severe disease. For infection to occur, \textit{L. pneumophila} must be presented to the host in aerosol form and the inhalation of small water droplets is necessary for colonization to occur (183). Confirmed cases of legionellosis must be reported to state health authorities and to the C.D.C. to facilitate national surveillance of this infectious disease. There are approximately 1300 cases of legionellosis annually and the incidence has shown a general rising trend since its first appearance (Fig. 1.1 and Fig 1.2). It has been suggested that the 1300 annual cases is a conservative or much underestimated figure and that there may be up to 100,000 cases of legionellosis per year (85). \textit{L. pneumophila} serogroup 1 is responsible for 85\% of all reported cases of community-acquired legionellosis while other serogroups of \textit{L. pneumophila} and \textit{L. macdadeii} make-up the remainder.

\textit{Legionella} spp. have been isolated from a wide variety of fresh water environments. The organisms is ubiquitous in lakes, ponds, streams, thermal lagoons, muds, sediments and potable water supplies (67, 126). In addition, legionellae have been isolated from shower heads, whirlpools, spas, respiratory therapy equipment (6, 126) and supermarket vegetable water misting systems (108). \textit{Legionella} have also been found to contaminate potting soil mixes (168).

In the environment, phagocytic amoebae are believed to play a major role in the survival and persistence of \textit{Legionella} and, prior to amplification of this organism followed by colonization of water supplies leading to outbreaks of disease is believed to be due to these protozoa
Figure 1.1: Total number of cases of legionellosis as reported by the Center for Disease Control and Prevention (CDC) Atlanta GA. Adapted from C.D.C. Morbidity and Mortality Weekly Reports, Summary of Notifiable Diseases, United States 1992.
Figure 1.2: Total number of cases of legionellosis per 100,000 people as reported by the Center for Disease Control and Prevention (CDC) Atlanta GA.. Adapted from C.D.C. Morbidity and Mortality Weekly Reports, Summary of Notifiable Diseases, United States 1992.
Figure 1.3: Total number of cases of legionellosis per 100,000 as reported by each state as documented by the Center for Disease Control and Prevention (CDC) Atlanta GA. Adapted from C.D.C. Morbidity and Mortality Weekly Reports, Summary of Notifiable Diseases, United States 1992.
The establishment of a cell model for investigating *Legionella* interactions with protozoa is necessary for understanding the normal events of *Legionella* persistence in the environment.

Inhalation of contaminated aerosolized water droplets is the normal route of infection; however, use of contaminated water on bandage dressings as a follow-up to invasive surgical procedures has lead to the development of systemic Legionnaires' disease (105). The normal operation of evaporative cooling systems generates and disseminates aerosols. Those small water droplets, approximately 5-15 μm in diameter, remain airborne and, when inhaled, are not entrapped by the constitutive defenses of the upper respiratory tract. On the other hand, they are large enough to contain *Legionella* organisms. On descending into the lower respiratory tract to water droplets impinge on the walls of the alveolar sacs where organisms are taken-up by alveolar macrophages. These cells are not readily able to destroy *Legionella* organisms without prior sensitization. The result of this phenomenon is development of severe lobar pneumonia especially in the immunocompromised.

Community-wide outbreaks are uncommon; however when they do occur these outbreaks are usually spectacular in nature and are most frequently attributed to evaporative condensers or cooling towers which harbor the organism (68, 77, 173). More frequently, nosocomial and sporadic cases contribute to the bulk of yearly reported incidence of legionellosis.

Men are more frequently infected by *L. pneumophila* than women.
and infection in children is rare. Immunosuppressed patients are most susceptible to infection while other at risk groups include patients with high alcohol intake, heavy smokers, people recovering from invasive surgical procedures and others suffering from underlying conditions or infections such as lymphomas, leukemia and AIDS (7, 105).

1.5.2 Clinical Presentation

The incubation period for Legionnaires’ disease is 2-10 days (68) and the prodromal symptoms include fever, chills, malaise, anorexia and weakness, and occasionally headache and myalgia (68). As infection proceeds, the acute onset of headache and fever occurs. Pulmonary disease predominates and within 3-5 days from the onset of symptoms, patients will likely require hospitalization and intensive care. The cough associated with early-stage Legionella infection is usually nonproductive; however, as the disease proceeds, the cough may become productive with a rust colored (blood tinged) or non-purulent sputum. Later in infection, lung function is compromised, purulent sputum is produced and patients undergo major systematic changes with abnormal liver function tests, blood gas and electrolyte imbalances and signs of confusion, respiratory collapse and cardiac failure. Patients require intubation and aggressive intervention-type therapy along with appropriate antibiotics. Despite these procedures, patients often fail to respond.

Radiological examination of the lungs of infected individuals shows a picture consistent with lobar pneumonia (184). Patchy unilateral infiltrates
are noted at the initial stages of disease, while bilateral infiltrates are noted in up to two-thirds of patients (54, 98). Extension of these infiltrates continues with subsequent consolidation evident in one or more lobes of the lung. The infiltrates can persist for up to several months after patient recovery. Nodular lesions may be present and can resemble the lesions of some fungal infections (33). Plural effusions are common but abscesses are rarely seen by radiologic examination. This is in contrast to the pathologic study of tissue specimens in that the lungs of fatal cases of Legionnaires' disease frequently show abscess formation (184).

Pathologic examination of lung specimens indicates that Legionnaires' disease is a multifocal pneumonia with coalescence in the late stages of disease (184). The distal airways including the alveoli and terminal bronchioles are primarily affected while the proximal bronchioles and bronchi are not usually involved. Lesions induced by this organism are distinct and are similar for all pathogenic species of Legionella (184); however, in some cases, these lesions are difficult to distinguish from classical pneumococcal pneumonia. Lung exudates are fibrinous and primarily contain neutrophils and macrophages; however, given this exudate development during infection the lack of sputum production is not understood.

Extrapulmonary involvement can occur during severe cases of disease, but these events are more common for L. pneumophila-induced disease than for disease caused by other members of the genus (3). Bacteremia has been documented (47) and extrapulmonary infections
include cerebral microglial granuloma, cutaneous and hepatic abscess formation, endocarditis, pericarditis and pyelonephritis (3, 31, 47, 53, 112, 113, 146). Intra-alveolar fibrosis is common and permanent sequelae including decreased pulmonary function are common (27, 102).

It is unclear whether resistance occurs in people who have previously been infected with the Legionnaires’ disease. Studies with guinea pigs have indicated that protective immunity is conferred on animals which were challenged by intraperitoneal injection of killed Legionella antigens, but similar studies using aerosol challenge to guinea pigs did not prevent infection (51).

1.5.3 Diagnosis

By comparison with those more frequent causes of pneumonia such as (Streptococcus pneumoniae and Hemophilus influenzae) the incidence of Legionnaires’ disease is relatively low. However, the reported incidence of Legionella-induced pneumonia from multi-center studies of pneumonic illness (100 or more cases of pneumonia) indicates a relatively high percentage of these cases are of Legionella origin (55, 184). Difficulties associated with the clinical diagnosis of L. pneumophila infections due to the similarity of symptoms and radiographic analysis of disease induced by Legionella and other microorganisms and viruses are common. To add to the difficulties in identifying Legionella-induced pneumonia, these organisms are intracellular pathogens and as such do not stain well using conventional grams stain procedures. Therefore, other identification
criteria including antigen or nucleic acid detection, and serological techniques are implemented to augment conventional laboratory culture when *Legionella*-induced pneumonia is suspected.

However, the gold standard for the diagnosis of *L. pneumophila* is cultivation (42). The legionellae are nutritionally fastidious and will not grow on conventional laboratory media. BCYE-α is the medium of choice for growth and identification of this pathogen. The sensitivity of cultivation ranges from 50-80%; it is, however, 100% specific (42, 185). *L. pneumophila* colonies possess a characteristic opalescent, cut glass appearance with smooth entire edges and these colonies, which present in a variety of sizes, appear 3-5 days after inoculation of clinical material onto this media. Several versions of BCYE-α have been developed to make this medium selective for legionellae from both clinical and environmental samples. Two semi-selective media have been created: the first, BMPA media, includes the antimicrobials cefamandole, polymyxin B and anisomycin (44) while the Wadowski-Yee formulation includes glycine a potent inhibitor of gram negative bacteria together with cefamandole, polymyxin B and anisomycin (179). These remove contaminants which would otherwise, quickly outgrow the legionellae. It has been suggested that all three media formulations should be used for the isolation of legionellae as some species, especially *L. micdadei*, are inhibited by the antibiotics present in these two selective media formulations. In addition to bacteriologic cultivation, laboratory identification frequently includes the use of direct
immunofluorescence, nucleic acid probes, radioimmunoassay (RIA) and polymerase chain reaction (PCR) technology (44, 79, 108, 168, 174).

Other additions to the basic BCYE-α media formulation include bromocresol purple and bromothymol blue, the presence of which enhance the recognition of some *Legionella* species, but affords no advantage or a disadvantage for organism growth. Although albumin enhances the recovery of *L. bazemanii* and *L. micdadei* from tissue samples it does not affect the recovery of *L. pneumophila* from specimens (123).

### 1.5.4 Antimicrobial Chemotherapy

Without an effective vaccine for the prevention of legionellosis a combination of aggressive antibiotic and clinical intervention is required for the successful treatment of infected patients. Following the Philadelphia outbreak, epidemiologic studies indicated that those who received erythromycin therapy fared better than those individuals who had received other antibiotics (68). Few antimicrobial agents have proven effective for the treatment of *Legionella pneumonia*. On the other hand, *in vitro* studies have demonstrated that legionellae are sensitive to an extensive array of antibiotics (151, 177, 178). The macrolides have proven most efficacious for the treatment of Legionnaires’ disease. Although erythromycin is the drug of choice for the therapy of legionellosis, this antibiotic is not well tolerated by some people and causes peripheral phlebitis and intestinal
upset. Other macrolides and alternative antibiotics may be used while others are continuously sought and tested for clinical use (45, 46, 78, 151).

A major criterion for the efficacy of any antimicrobial agent in the treatment of Legionnaires’ disease is membrane transportability. As legionellae are facultative intracellular pathogens antibiotics must enter the host cell for effective activity. Erythromycin penetrates cell membranes well and for this reason it is the drug of choice of Legionnaires’ disease. In severe, potentially life-threatening cases, rifampin and other antibiotics are often used in combination with erythromycin (9).

Several studies have suggested that other antibiotics may be effective for the control of this disease (45, 177). One such group of promising antimicrobials are the fluoro-substituted quinalones. Of this group, ciprofloxacin was shown to be particularly effective for treatment of Legionnaires’ disease both in vitro, as well as, in clinical trials (129, 177). Tzianabos and Rodgers (177) extensively studied the in vitro effects and demonstrated that ciprofloxacin, like erythromycin, was effective against intracellular organisms and hence Legionnaires’ disease. In subsequent studies, clinical trials have shown ciprofloxacin to be effective for the treatment of human legionellosis.

1.6 Comment

During the past 18 years, vast amounts of information have amassed concerning many of the major physiologic and pathogenic facets of L. pneumophila. In addition, procedures necessary for the diagnosis and
identification of the organism leading to the establishment of legionellosis have been forthcoming. However, *Legionella*-induced pneumonia is still a major cause of respiratory illness. This organism will continue to be of clinical importance until an effective vaccine has been developed. It is unlikely, however, that a vaccine will be available in the very near future since many of the immunological processes involved in the development or recovery from *Legionella*-induced pneumonia are still unclear. To help control legionellosis work must continue in the area of host-cell interaction, both in the environment in terms of *Legionella*-protozoa interactions, as well as, in the clinically significant areas of organism-macrophage interaction and intracellular survival. Indeed, given the intracellular nature of *Legionella*, a vaccine which prevents the intracellularization of the organism should interfere with the prosecution of the disease within the lungs. The development of such a vaccine should prove an effective and viable alternative to antimicrobial chemotherapy for the prevention and control of Legionnaires’ disease.
SECTION II

CHARACTERIZATION OF INTRACELLULAR REPLICATION FOLLOWING OPSONIN-INDEPENDENT ADHERENCE OF LEGIONELLA PNEUMOPHILA TO U-937 CELLS.

(The work outlined in this section has been published in the following articles:

2.1 Abstract

Legionella pneumophila is an opportunistic bacterial pathogen that infects alveolar macrophages in the human lung during the initial stages of disease. To define the interactions of this organism with human alveolar macrophages requires the use of cell models to mimic alveolar macrophages; cells which are difficult to obtain in a healthy state. As a consequence, many cell types have been used to simulate the interaction
of *L. pneumophila* with alveolar macrophages. The U-937 cell, a transformed human macrophage-like cell line, has been shown to support the growth of *L. pneumophila*. In some ways these cells are similar to alveolar macrophages in that they express complement and Fc receptors and in this respect they may be more representative of macrophages for the study of *Legionella* attachment and growth than many other cell models thus far described. Observations to elucidate the initial interactions of this pathogen with human cells identified a complement-dependent system for describing *Legionella* attachment to monocytes. It has been proposed that an alternative attachment mechanism occurs for *L. pneumophila* and that this binding process is effected independent of serum factor influences. The growth kinetics and stages of cell infection involving this opsonin-independent system as verified by electron microscopy have been characterized and are similar to those process described following complement-mediated attachment. Cellular and ultrastructural studies indicated that legionellae bound to U-937 cells within 1 h and replicated rapidly over a 30 h period within ribosome-lined phagosomes in association with mitochondria and endoplasmic reticulum. Release of organisms from infected cells occurred by lysis which commenced approximately 30 h after infection. This work establishes the U-937 cell as an effective model for understanding *Legionella* alveolar macrophage interactions.
2.2 Introduction

*Legionella pneumophila* is an intracellular bacterial pathogen that is the etiologic agent of a severe form of life-threatening lobar pneumonia. Approximately six months after the Philadelphia epidemic McDade *et al.* (155) demonstrated that the causative agent of this outbreak was a previously unidentified bacterium that was found in biopsied lung tissue by light microscopy. Subsequently, Rodgers *et al.* (146) demonstrated by electron microscopy that *L. pneumophila* functioned as a facultative intracellular pathogen replicating inside host lung cells. The intracellular nature of the Legionnaires’ disease bacterium is critical for the establishment of disease in humans. Furthermore, it aids organism survival by facilitating the evasion of the immune defence mechanisms of the host. Since the early 1980’s studies have endeavored to elucidate the infectious process of *L. pneumophila* within host cells (75, 87, 111, 132, 136, 152). As a number of studies have shown, defining infection of host at the cellular level as well as understanding the interaction between the organism and the immune system of the host have proved problematic. Although, the general stages of *Legionella* infection, at the cellular and systemic levels, have been identified and a clearer picture of the pathogenesis of Legionnaires’ disease has evolved, much remains to be elucidated on the nature and role of bacterial virulence factors as well as host-mediated responses.

*Legionellae* are environmental organisms and are ubiquitous in freshwater habitats. While in these aquatic environments, *L. pneumophila*
has a close relationship with phagocytic amoebae. Legionellae parasitize these cells and the resultant intracellular replication and release of bacterial progeny perpetuates these organisms in the aquatic environment. Preliminary investigations outlining Legionella-amoeba cell interactions were described in 1980 (146). Since then several studies have identified many host cells that support the intracellular replication of \textit{L. pneumophila} (57, 120, 128). It is commonly believed that human disease is coincidental and offers no ecological advantage to the organism. These bacteria when presented in aerosolized form are taken up by the host and preferentially infect alveolar macrophages which, it is postulated, are infected in a fashion similar to amoebae in the environment. Several useful models for describing \textit{L. pneumophila} interaction with protozoa have been reported (59, 131). The stages of protozoal infection are very similar to those observed with human cells and include such important processes as ribosome-lining of phagosomes containing organisms, as well as similar intracellular replication kinetics (120).

In human disease, the alveolar macrophage is the target cell for \textit{Legionella} infection. Due to the inaccessibility and unavailability of human alveolar macrophages, a suitable cell model simulating alveolar macrophages was required. A wide variety of cells have been used to study \textit{Legionella}/host cell interactions and the ultrastructural development of intracellular \textit{Legionella} and these include MRC-5, Vero, HEp-2, HL-60 and U-937 as well as primary explants of human peripheral blood monocytes and human, guinea pig and simian alveolar macrophages (50, 75, 99, 111,
136, 152). None of these cells is ideal in that each only approximates some of the normal activities of human alveolar macrophages in vivo.

Initial work centered on the role of the immune response of the host to this pathogen and as a consequence, a complement-mediated receptor binding mechanism was outlined (12, 128, 188). Recently, a novel attachment process for this organism has been suggested that occurs independent of serum influences (75, 76, 152). Host cell recognition is followed by attachment of the bacteria to surfaces that possess appropriate receptors. Direct binding of bacteria to host cells have been described for group B streptococci and Yersinia pseudotuberculosis (4, 92, 93). Furthermore, recent work studying uptake of bacteria by phagocytes demonstrated that the mode of attachment (complement/antibody or direct binding) affected the subsequent intracellular fate of the organisms (187). It is possible that opsonin-independent binding mechanisms are necessary for intracellular infection to develop and that this may play an important role in the maintenance of disease.

2.3 Materials and Methods

2.3.1 Organism Cultivation: L. pneumophila serogroup 1, strain Nottingham 7 is a highly virulent clinical isolate from a fatal case of Legionnaires’ disease. Initially the organism was isolated from sputum on low sodium, enriched, blood agar. Colonies were sub-cultured once onto buffered charcoal yeast extract agar supplemented with α-ketoglutarate (BCYE-α)
(Difco, Detroit, MI) and stored as stock cultures frozen at -70°C in 1% serum sorbitol (Appendix 1). Thawed aliquots were passaged once for 72 h on BCYE-α at 37°C in a humid atmosphere with added CO₂ prior to experiments. Colonies were harvested and resuspended in buffered yeast extract broth containing α-ketoglutarate (BYE-α) to give 58 Klett units (equivalent to 1-2x10⁸ colony forming units/ml). Tubes containing 5 ml sterile BYE-α were inoculated with 100 μl of this 58 Klett unit suspension and were grow for 24 h at 37°C with vigorous shaking. Following growth, 1 ml samples were placed into eppendorf tubes and centrifuged at 8,000 x g for 15 min in a Beckman microfuge 12. Supernates were drawn off and pellets were resuspended in 1 ml sterile Hank’s balanced salt solution (HBSS). This process was repeated 3 times and the organism containing samples were resuspended with fresh HBSS prior to infection assays.

Periodic assessment of *Legionella* virulence using the fertile hens’ egg assay (Fig. 2.1) was conducted during the course of these studies to ensure that organism virulence was maintained (176).

### 2.3.2 U-937 cell Cultivation:

U-937 cells were grown in RPMI-1640 cell culture media (Sigma, St. Louis, MO.) supplemented with 10% fetal bovine serum (FBS) in T-75 cm² cell culture flasks (Costar, Cambridge, MA.) at 37°C with 5% CO₂. Cells were grown to a density of 1-2 x 10⁶ cells/ml, harvested by centrifugation at 250 x g for 10 min in 50 ml conical tubes (VWR Scientific). Supernatant fluids were removed and cells were resuspended
Figure 2.1: Diagrammatic representation illustrating the compartmentalization of the fertile hens' egg. The yolk sac route of inoculation was used to monitor virulence of *L. pneumophila* in this model (176).
to give $5 \times 10^5$ viable cells/ml in 20 ml of fresh RPMI-1640 with 10% FBS.

Differentiation of the U-937 cell to the macrophage-like status was done by adding 25 $\mu$l of a $10^{-5}$ M solution of phorbol myristate acetate (PMA) to these cultures for 24 h. Adherent cells were washed 3 times with HBSS, physically removed from the culture flasks by gentle scraping with a silicon rubber-coated glass rod and harvested by centrifugation at 250 x g for 15 min. Collected cells were resuspended in fresh RPMI-1640 with serum at $5 \times 10^5$ cells/ml and aliquoted into either T-75 cm$^2$ flasks (20 ml) for thin section transmission electron microscopy (TEM), 6 well plates (3 ml) each containing a 22 mm$^2$ glass coverslip for scanning electron microscopy (SEM) or 24 well plates (1ml) for viable bacterial cell colony (VBCC) counts studies cells were allowed to readhere in culture vessels for 24 h.

2.3.3 Infection Assay

U-937 cells were inoculated with *L. pneumophila* at a multiplicity of infection (MOI) of 100 bacteria per host cell at 37°C for 1 h in fresh HBSS within an atmosphere containing 5% CO$_2$. After the infection period monolayers were washed 3 times with HBSS. At this stage, fresh RPMI-1640 medium with serum was added to each sample. Samples were collected at 1, 6, 12, 24, 48 and 72 h post infection. For TEM and SEM, infected monolayers were washed 3 times with cacodylate buffer containing 10mM MgSO$_4$ pH 7.4 (CB), fixed for 18 h in 2% (v/v) glutaraldehyde (Electron Microscopy Science, Ft. Washington, PA) in CB and washed 10 times with CB in situ.
2.3.4 **Viable Bacterial Cell Colony Count:** In conjunction with electron microscopic investigations of the attachment, intracellular replication and release events of *L. pneumophila* infection of U-937 cells, VBCC counts studies were performed to assess the rate of cell infection and intracellular replication of this organism. Following the 1 h infection of U-937 cells, supernates were removed and each well was washed three times with HBSS to remove unbound bacteria. The three washings along with the supernates were collected and pooled for each sample time. Serial 10-fold dilutions of these pooled samples were made in 1% (w/v) peptone and duplicate samples of 25 μl were plated on BCYE-α agar for colony counting evaluations.

At each of the prescribed time intervals, cultures were assessed for both intracellular as well as extracellular bacteria. Following collection of supernatant fluids together with each of the 3 x 1 ml HBSS washings, the cells in each well were lysed by adding 1 ml sterile Milli-Q water for 30 min. The cells were then physically disrupted with vigorous aspiration and expulsion using a sterile pasteur pipette. For each time interval, a 100 μl aliquot was removed from each of the supernatant fluids plus wash samples as well as each lysate. These samples were each 10-fold serially diluted in 1% (w/v) peptone and duplicate 25 μl samples were plated on BCYE-α agar for the enumeration of intracellular and released legionellae.

2.3.5 **Transmission Electron Microscopy:** For TEM, glutaraldehyde-fixed U-
937 cells in CB were physically removed from the T-75 cm² culture flasks with the aid of a silicone rubber-coated glass rod and harvested by centrifugation at 200 x g in a Beckman model TJ-6 centrifuge. Samples were pre-embedded in molten 1% (w/v) Noble agar maintained at 55°C. The agar embedded specimens were allowed to set at room temperature, were cut into 1 mm³ blocks and post-fixed in 1% (w/v) osmium tetroxide (OsO₄) in CB for 24 h. Samples were then dehydrated in an ethanol series in CB consisting of 50%, 70%, 75%, 90%, and 95% for 5 min each and then into two changes of absolute ethanol for 10 min each. Samples were equilibrated in two changes, 10 min in propylene oxide (epoxy propane) and embedded in an epon-aryldite resin mixture (Appendix 2). At no time were samples allowed to dry. After polymerization at 60°C for 24 h, blocks were sectioned on a LKB ultramicrotome III using a diamond knife. Sections of 60-90 nm thickness were collected onto 400 mesh copper grids (Electron Microscopy Science), were stained with 1% (w/v) uranyl acetate for 1 min. and 0.4% (w/v) lead citrate for 30 sec. and examined in a Hitachi H-600 scanning-transmission electron microscope at 75 kV in the transmission mode.

2.3.6 Scanning Electron Microscopy: For SEM, glutaraldehyde-fixed samples were washed thoroughly with PBS at pH 7.2 and dehydrated in a graded ethanol series to absolute ethanol. Specimens were fully dried from hexamethyldisilazane (EMS, Fort Washington, PA) in air. Monolayers on coverslips thus dried were sputter coated with 20 nm gold-palladium in a
Hummer V sputter coater with a specimen to target distance of 5 cm for 4 min at 15 mA and observed in an AMR 1000 scanning electron microscope at 60 kV.

2.4 Results

The virulence of the stock cultures of this strain remained unchanged from previous studies (176). Adherence of *L. pneumophila* to U-937 cells in the absence of opsonins was observed within the 1 h incubation period (Fig. 2.2). By comparison with normal control (uninoculated) U-937 cells (Fig. 2.3), analysis of the 1 h post-inoculation specimens by TEM showed that wash-resistant attachment involved apposition of *L. pneumophila* membrane blebs and host cell surface membranes (Fig 2.4). SEM observations of similar monolayers showed numerous legionellae adherent to U-937 cells. In addition, SEM data suggested that little host cell change occurred following these initial adherence steps of virulent bacteria to host cells (Fig. 2.5). VBCC counts data indicated that approximately $1 \times 10^6$ bacteria had attached to $5 \times 10^5$ U-937 cells. From this it was clear that approximately 2 bacteria had bound to each U-937 cell in the monolayers and this constituted 2% of the initial inoculum (Fig. 2.2). Neither coiling phagocytosis (83) nor the presence of banded enclosures (Oldham and Rodgers) previously reported for human monocytes and other cells were observed; however, these differences may have reflected the absence of opsonic components in the adherence medium in the present study.

Rapid intracellular bacterial multiplication was observed (Fig 2.2).
Intracellular Development of \textit{L. pneumophila} in U-937 cells

![Graph showing the development of \textit{L. pneumophila} in U-937 cells over time.]

Figure 2.2: Attachment and intracellular replication of \textit{L. pneumophila} N7 in U-937 cells measured by VBCC counts. U-937 cells at 5x10^5 cells/ml were infected with a MOI of 100 \textit{L. pneumophila} and washed 1 h after infection. Intracellular replication (○—○) and release (■—■) of legionellae. Assays were the average of three trials and bars represent standard error.
Plate 2.3: Untreated U-937 cells observed by transmission electron microscopy (A) and scanning electron microscopy (B). Magnification A = x 17,000; B = x 3000. Bars: A = 2 μm and B = 10 μm.
Plate 2.4: *L. pneumophila* bound to a U-937 cell 1 hr after infection. Inset: higher magnification of host cell-bacterium interaction. Note electron lucent zone between organism and cell. Magnifications A = x 17,000; B = x 60,000. Bars: A = 2 μm and B = 300 nm.
Plate 2.5: *L. pneumophila* bound to U-937 cell at 1 hr after infection by SEM. Note several bacteria bound to the surface of the host cell. Magnification = x 5000. Bar represents 5 μm.
Plate 2.6: TEM (A) and SEM (B) of *L. pneumophila* 12 hr after infection of U-937 cells. Organisms are contained within loose phagosomes which are ribosome-lined. Effects of infection on host cells include blunting and a decrease in the number of microvilli on the surface of U-937 cells.

Magnification: A = x 20,000; B = x 3000. Bars: A = 3 μm and B = 10 μm.
Plate 2.7: TEM (A) and SEM (B) of *L. pneumophila* 24 hr after infection of U-937 cells. TEM of organism infected U-937 cell. Note fulminant infection of cell as well as the release of organisms from the phagosome. B. SEM of infected cells. Cells are rounded and show a decrease in the number of microvilli which show marked blunting. Magnification: A = x 13,600; B = x 3000. Bars: A = 2 μm and B = 10 μm.
Plate 2.8: TEM (A) and SEM (B) of *L. pneumophila* 48 hr after infection of U-937 cells. TEM of organism infected U-937 cell. Note bacteria no longer confined to phagosomes and some bacteria can be seen in the extracellular milieu. B. SEM shows almost complete loss of microvilli.

Magnification: A = x 25,500; B = x 3000. Bars: A = 2 μm and B = 10 μm.
Plate 2.9: TEM (A) and SEM (B) of *L. pneumophila* 72 hr after infection of U-937 cells. Host cells are seen at the terminal stages of infection. Cell lysis and lack of bacteria within cells are noted in both TEM and SEM presentations. Magnification: A = x 13,600; B = x 2000. Bars: A = 2 μm and B = 10 μm.
Intracellular bacterial replication, which occurred by pinching binary fission, was evident by 12 h post-infection of cell monolayers (Fig. 2.6). *Legionella* replication occurred initially in tight, ribosomes lined vacuoles and later these phagosomes were much distended. Host cells at this time possessed fewer microvilli at the surfaces. By 24 h post infection, intracellular *L. pneumophila* were confined to loose vacuoles within the U-937 cell (Fig. 2.7). At this stage of the infectious process, cytoplasmic clearing was evident and extensive intracellular damage was also noted. Continued loss and blunting of microvilli were apparent as the infectious process continued. Gross morphologic surface changes were evident by 48 h post-infection and these included cell lysis with a concomitant increase in cellular debris as was evident by TEM and SEM (Fig. 2.8). Although bacteria were observed within cells at 48 h post-infection they were present within disrupted vacuoles. At this stage of the infectious process the integrity of the plasma membrane was breached in numerous cells and released bacteria predominated. By 72 h cellular infection and disruption was complete with >95% of the U-937 cells detached and lysed (Fig. 2.9). VBCC counts of washed samples (extracellular organisms) and U-937 cell lysates (intracellular organisms) showed maximal intracellular *Legionella* numbers by 24 h of infection (Fig. 2.2). As intracellular replication continued toward the later stages of infection (72 h) an increase in the number of bacteria was found in the extracellular milieu with a simultaneous decrease in the number of intracellular organisms. In addition, *L. pneumophila* did not replicate in either HBSS or RPMI-1640 with or without added FBS, whether
previously conditioned with U-937 cells or not.

2.5 Discussion and Conclusions

*Legionella pneumophila* infections constitute a significant source of medical problems each year and an understanding of the infectious process at the cellular level is essential prior to the potential development of vaccines for the control of this pathogen. Investigations of biopsied lung tissue strongly suggested the alveolar macrophage as the most likely candidate for the establishment of human disease (183). Although cell models have been proposed to mimic human alveolar macrophage infection at the cellular level these cells are generally difficult to obtain. Horwitz and Silverstein (88) initially described the interaction of *L. pneumophila* with human peripheral blood monocytes and since then many cells have been shown to support intracellular replication of the *Legionnaires' disease* bacterium (75, 111, 128, 134, 138, 152). Although the organism has been shown to replicate within epithelial, fibroblast and mononuclear cells; they appear to be incapable of replication within PMNLs (62, 88, 134).

Several transformed cells have been proposed as potential cell models the HL-60 cell which expresses polymorphonuclear leukocyte-like or macrophage-like characteristics and the U-937 cell, a histiocytic lymphoma cell line which can express either monocyte or macrophage-like cell surface markers (170). The U-937 cell has been used frequently as a model cell to represent *Legionella* host cell interactions (111, 138). Pearlman *et al.*
(138) demonstrated that L. pneumophila could infect the transformed human histiocytic lymphoma cell line U-937. These studies suggested that U-937 cells may be an appropriate model for reliable investigations of the Legionella/host cell interactions. The inducible dual monocytic and macrophage-like nature of these cells, which is dependent on cell differentiation, makes them amendable to studies of L. pneumophila interactions with monocyte-like and macrophage-like cells. In vitro, U-937 cells are monocyte-like; however, they can be differentiated into macrophage-like cells when stimulated with agents such as phorbol myristate acetate (PMA), gamma interferon (IFN-γ), 1α,-dihydroxyvitamin D3 (a vitamin D metabolite), human recombinant interleukin-6, or retinoic acid (143, 161). In the undifferentiated state, U-937 cells are non-adherent and have the ability to divide in culture. However, when differentiated, these cells possess receptors analogous to macrophages, adhere to culture vessels, and are incapable of cell replication. In addition, the U-937 cell has been studied extensively in order to determine the role of the macrophage in infection and to elucidate features of cell mediated immunity (34, 61, 142). These cells express both high and low Ig G binding receptors as well as the complement receptors type 3 (CR3) and type 4 (CR4) at levels similar to alveolar macrophages (63, 110, 159, 161). Furthermore, the U-937 cell is rapidly becoming the cell of choice to study host-microbe interactions and in investigations to better understand the effects of modulation of the human immune system. U-937 cells have also
been used extensively to study the production and expression of proteins by intracellular pathogens such as *Salmonella typhi* and *L. pneumophila* when these organisms are within host cells (2, 101). This cell is considered a versatile model in the areas of immunology, pathogenesis and microbiology (110, 115, 138, 141).

Attachment is the first interaction to occur between a facultative intracellular bacterial pathogen and its appropriate host cell (11). Many adherence mechanisms and the structures involved have been elucidated for a variety of prokaryotes and their eukaryotic host cells (49, 92). Bacterial adherence of an organism to a host cell is believed to be a two stage phenomenon in which an initial weak association occurs, mediated by forces such as electrostatic, van der Waals and hydrophobic interactions, and these are followed by a second, much stronger binding event. Attachment of legionellae to human monocytes was initially shown to be dependent on the deposition of complement on the *Legionella* MOMP (12). This organism adheres to and infect cells which are not professionally phagocytic such as fibroblastic MRC-5 cells, and the epithelial HEp-2 and Vero cells (134). Since these cells do not possess complement and Fc receptors normally found on the surface of mononuclear cells, it is not clear how they become colonized and support infection of the organism. Furthermore, colonization and intracellular infection of fresh water amoebae clearly occurs in nature in the absence of opsonic components (8). In addition to the broad range of cells that are parasitized by *L. pneumophila*, work by Reynolds and Newbold (145)
demonstrated that the distal bronchioles and the alveolar spaces of the human lung typically contain very low levels of complement. Therefore, the opsonin-independent system of binding may prove an important mechanism for the initial recognition events of \textit{L. pneumophila} and alveolar macrophages. Studies to better define these alternative processes are necessary.

Uptake studies conducted in media containing complement components showed that \textit{Legionella} entered host cells by a process defined as coiling phagocytosis (83). Oldham and Rodgers (134) noted organism uptake by host cells occurred within banded enclosures. These were not observed in the present experiments conducted in the absence of serum. Recently, Horwitz (85) has indicated that when uptake studies are conducted in the presence of complement, coiling phagocytosis occurs for many \textit{Legionella} species; however, it was noted that not all bacteria in a population are taken up in this fashion. This variation may indicate organism-based differences or may represent two mechanisms of organism uptake are occurring simultaneously. The present studies suggest that uptake under opsonin-independent conditions does not occur via coiling phagocytosis. Other studies addressing \textit{Legionella} uptake have described a 24 kD macrophage infectivity potentiator or MIP protein as a necessary factor for \textit{L. pneumophila} uptake by phagocytic cells (31, 51). Yet it is clear that this protein plays no role in the adherence process of \textit{Legionella} to macrophages. Isogenic mutants of \textit{L. pneumophila} devoid of the MIP protein, binds to host cells, but are not taken-up (29).
Following uptake of *L. pneumophila* by monocytes or other cell types, the organism is contained within tight-fitting phagosomes. The recruitment of both ribosomes and mitochondria occurs and this recruitment event appears to be necessary for intracellular replication (87, 134). It is not understood why this organelle recruitment should occur; however, it may be an important factor for organism survival within host cells, or may be involved in the supply of nutrients to the organism. Intracellular survival with subsequent multiplication of *Legionella* was apparently dependent on the ability of the organism to uncouple both the acidification of the phagosomes as well as prevent lysosome fusion with bacteria-containing phagosomes in the macrophage (86). Inhibition of phagosome-lysosome fusion was a crucial factor in the persistence and subsequent intracellular replication of legionellae in host cells. The later stages of U-937 cell infection occurred from 48-72 h after infection as documented within these cells and demonstrated phagosome distention with a concomitant increase in the number of bacteria within these structures. Fulminant cellular infection was followed by bacterial escape into the cytoplasm of the host cell. Subsequent to this event, release of *L. pneumophila* occurred into the extracellular milieu following lysis of the cells. The factors governing the process of cell escape is not known, however the *L. pneumophila* protease, the major secretory protein of the organism may be central to bacterial release from the phagosome and final escape from terminally infected host cells.

This study adds further to the suggestion that opsonin-independent
processes may be necessary for the initial establishment of macrophage infection and that complement mediated processes could follow as cellular destruction and local inflammation proceed (91). This idea may be important as it has been shown that *L. pneumophila* can bind to a cells in the absence of serum (75, 152). In the absence of specific antibody, complement did not promote the adherence of *L. pneumophila* to U-937 cells, guinea pig alveolar macrophages or J774 mouse macrophages (91). The present work taken together with that of Husman and Johnson (91) indicate that an opsonin-independent attachment mechanism may exist for *Legionella* and the establishment of infection may be directly related to this process.

These experiments identified the attachment of *L. pneumophila* in an opsonin-independent fashion and documented the morphological events of intracellular replication following *Legionella* infection of U-937 cells. In addition, this study supports the usefulness of U-937 cells as a model for macrophage infection in that the intracellular development of *Legionella* and the cell-induced changes were similar to those described for guinea pig alveolar macrophages and human peripheral blood monocytes. The use of these cells may further assist in clarifying the cellular and molecular events involved in the development of Legionnaires' disease (75, 138, 152).
SECTION III

PARTIAL CHARACTERIZATION OF THE ADHERENCE OF LEGIONELLA PNEUMOPHILA TO U-937 AND GUINEA PIG ALVEOLAR MACROPHAGES.

(The work outlined in this section has been published in the following article: Gibson, III, F. C., F. G. Rodgers, and A. O. Tzianabos. 1993. Opsonin-independent Adherence of Legionella pneumophila to MRC-5 and U937 cells. In Barbaree, Breiman and Dufour: Legionella: Current Status and Emerging Perspectives. ASM. Washington D.C. pp. 78-82.)

3.1 Abstract

Adherence of Legionella pneumophila, strain Nottingham N7, to host cells occurred in salt solutions devoid of opsonins. Two cell types, U-937 cells and primary explants of guinea pig alveolar macrophages were used to study and partially define the opsonin-independent adherence mechanisms for L. pneumophila. "Adhesin" and "receptor" characterization studies were performed by assaying the numbers of Legionella organisms which had adhered to cells using viable bacterial cell colony (VBCC) counts and indirect immunofluorescent assays (IFA).
Receptor saturation studies indicated that at a multiplicity of infection of 100 2%-8% of the non-opsonized bacterial inoculum was taken-up following the addition of \textit{L. pneumophila} to host cells. The single saturation plateau noted with VBCC counts and IFA suggested a first order kinetic relationship of one bacterial "adhesin" per host cell "receptor" for \textit{L. pneumophila} to each of the 2 cell types studied. The binding structures were modified to determine the broad chemical make-up of the bacterial adhesins and host cell receptors in competitive binding assays. In addition, surface membrane treatments were performed to determine the interruption of binding due to the enzymatic loss or destruction of particular groups of molecules; a process of determining the nature of function, through loss of function. With the exception of the aldehydes, these treatments had no effect either on the viability of either the microorganism or the host cell. Adhesin evaluation was made using chemical treatments of the bacterial membrane surfaces and these studies indicated that the adhesin was a complex structure consisting of protein material with lectin-like or carbohydrate characteristics suggestive of a glycoprotein. However, the role of lipopolysaccharide could not be excluded. Modification of host cell membranes indicated that carbohydrate moieties suggestive of glycolipids on the host cell surface acted as receptors responsible for binding \textit{L. pneumophila}. However, the nature of these carbohydrate receptors differed in structure from those saccharides examined in competitive binding assays.
3.2 Introduction

Bacterial recognition of host cells by pathogens is a necessary event prior to the establishment of infection (11). The concept of bacterial adherence to substrates is well described and many studies have demonstrated that bacteria bind efficiently either to particular substrates in the environment or to cells in host tissues (11, 49, 74). Work on the microflora of the mouth established microbial attachment-dependent colonization in humans by microbes and demonstrated the adherence of microorganisms to both tooth and mucosal surfaces (74). Other studies suggested that bacterial colonization was frequently dependent on a prior attachment or binding event (11). Some microorganisms bind to a variety of "inert" or non-living substances and some have been shown to bind to eukaryotic cells while others possess the ability to do both (11, 74). To define the interaction of a bacterium with a host cell, current terminology identifies the bacterial attachment structure as the "adhesin" and the structure to which the organism binds as the "receptor" and attachment can occur to epithelial cells, endothelial cells or macrophages. (40, 87, 92, 134). Fimbriae and the glycocalyx of some bacteria are well-established as adhesins for several species of bacteria; however, structures such as outer membrane proteins and lipopolysaccharide are also frequently associated with adherence (11, 49, 91, 133, 187, 188).

Attachment is a multifactorial event and is dependent on many factors including availability of appropriate receptors, spatial distribution of adhesin and receptor moieties, attractive and repulsive forces, physical
factors such as pH and flow rate and the degree of hydrophobicity of the interacting membranes (11, 48). Moreover, the stages of binding usually involve a sequential two-step process in which a preliminary or weak binding event is followed by a firm highly specific attachment process. However, the presence or absence of these diverse bacterial structures are dependent on the phase of growth of the organism and for pathogens, the stage of the infectious process. One such process is phase variation of fimbrial expression.

A rapidly emerging hypothesis is that pathogens may possess several mechanisms by which they interact with a host cell either at different stages of the infectious process or when the participants interact in differing environments such redundancy has been demonstrated for *Escherichia coli*, *Salmonella typhi*, *Mycobacterium tuberculosis* and *Legionella pneumophila* (12, 60, 75, 165). This concept may be an important feature either for determining the conditions necessary for the establishment of disease or as a microbial strategy for evading the immune response of the host (132, 165). Recently, it has been demonstrated that several pathogens take advantage of a series of immune system-specific receptors known as integrins and gain entry into various cells through these relatively conserved group of molecules (4, 12, 93, 162). The integrins most frequently utilized by microorganisms include the β₁ and β₂ integrin subsets, while the majority of organisms which abuse these receptors are facultative intracellular pathogens (4, 93, 162). The β₁ integrins are used by
organisms that possess invasin on their surface, while the $\beta_2$ group of receptors including complement receptor type 3 (CR3) are exploited by organisms that bind directly to these cells either in the presence or in the absence of complement or immune modulators (4, 39, 75, 93, 152, 162).

Opsonins, such as complement and antibody, are phagocytosis-enhancing molecules and are well-recognized as facilitators for the destruction of foreign material by phagocytes. Components of the immune system of the host, specifically antibody and complement, are deposited on bacteria, thus facilitating the processing mechanism leading to engulfment by macrophages. In contrast, the mechanisms of direct recognition of bacteria by macrophages, independent of these modulators, is poorly understood at best (4, 11, 75, 152). In addition, a role for opsonin-dependent bacterial attachment is well-established in the field of adherence. Direct bacteria-host cell binding in the absence of these immune mediators has established that direct attachment may be an important mechanism of recognition, especially for some facultative intracellular pathogens (75, 94, 152, 162). *L. pneumophila* attachment to host cells, mediated by complement, has been shown while bacterial attachment between adhesin and host cell receptors may also occur. Candidate binding structures for *L. pneumophila* include fimbriae, flagella, outer membrane proteins and lipopolysaccharides (148). The precise nature of microbial adherence is critical to understanding the mechanisms by which pathogens establish human disease. Indeed, it is probable that
Figure 3.2: Schematic defining the putative *L. pneumophila* adhesins and host cell receptors. (Reproduced from reference, Rodgers, Zbl Bakt. I Abt. Orig. A. 1983).

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the manner in which bacteria bind to cells directly affects the fate of the 
organism in the host cell (178, 182, 188).

*L. pneumophila* is the causal agent of Legionnaires’ disease; a 
severe form of lobar pneumonia. This pathogen parasitizes a wide variety 
of mammalian cell types, as well as freshwater protozoa (59, 75, 87, 111, 
122, 134). In the environment the numbers of legionellae increase when 
protozoa are present (59). This amplification prior to colonization of water 
supplies and their aerosolization forms a necessary prelude to the 
establishment of human infection. The inhalation of *Legionella*- 
contaminated aerosols into the lower respiratory tract (LRT) constitutes the 
initial stage of disease and once there, organisms infect alveolar 
macrophages (AMØ). *Legionella* adheres to human derived cells including 
monocytes and macrophages in the presence of the serum opsonins 
complement or specific antibody (12, 89, 91). Although the guinea pig has 
been adopted as the model of human disease, an acceptable cell model 
for the study of legionellosishas not been forthcomming. Guinea pig 
alveolar macrophages (GAM), human peripheral monocytes, U-937 cells 
and HL-60 cells have been used to investigate bacteria/alveolar 
macrophage interactions; however, none are apparently ideal. U-937 cells 
were derived from a human histiocytic lymphoma (170) and these have 
been shown to exhibit macrophage-like characteristics such as Fc and 
CR3 expression following treatment with phorbol myristate acetate (PMA) 
(103, 16, 163, 167). Pearlman et al. (138) documented uptake and survival 
of virulent legionellae within U-937 cells in the presence of serum.
Previous work centered on the role of complement and specific antibody-mediated uptake of this facultative intracellular pathogen (12, 89, 91). *Legionella* attachment to peripheral blood monocytes and macrophages involves the deposition of complement 3b (C3b) on the bacterial major outer membrane protein (MOMP) of *Legionella* (12). Opsonized bacteria are then recognized by host cell complement receptors. Following opsonin-mediated attachment, legionellae are apparently taken-up by a novel coiling phagocytotic process (83).

It is possible that uptake of *L. pneumophila* by host cells may not use an opsonin-dependent mechanism in the initial stage of infection (75, 152). The rationale for this is based on the observation that respiratory secretions and the environment of the normal human lung are low in opsonins (145). In the light of these observations opsonin-independent adherence mechanisms for microbial attachment were investigated and attempts were made to identify and characterize the adhesins and receptors in the binding process.

3.3 Materials and Methods

3.3.1 Bacterial Growth and Maintenance

*L. pneumophila* strain Nottingham N7 was grown and maintained as previously described (see Section 2.3.1).

3.3.2 Cultivation of U-937 cells

U-937 cells were grown and phorbol ester treated as previously
described (see Section 2.3.2).

3.3.3 Collection and Cultivation of Guinea pig Alveolar Macrophages

Hartley outbred guinea pigs of 600-650 g (Charles River Labs, Wilmington Mass.) were euthanized with an intraperitoneal injection of sodium pentobarbital. The animals were then bled by lacerating the dorsal aorta prior to the collection of macrophages. Alveolar lavage was performed with intratracheal injection of 10ml sterile HBSS (pH 7.2) from a syringe through an 18-gauge needle followed by aspiration of the HBSS-macrophage suspension (Appendix 3). Lavage was repeated 4 times per guinea pig and this yielded approximately 5 x 10^6 macrophages per animal. GAM cells were held on ice prior to enumeration. Cells were placed in 50 ml conical centrifuge tubes, centrifuged at 250 x g, counted in an American Optical hemocytometer and resuspended in RPMI-1640 medium containing 10% FBS (Appendix 2) to yield 2.5 x 10^5 cells/ml. Macrophages were then distributed to 6 or 24 well cell culture plates and allowed to adhere for 2h in a humid environment at 37°C in 5% CO_2 prior to use.

3.3.4 Treatments

All treatments were prepared fresh in HBSS, adjusted to pH 7.2 and filter sterilized through a 0.22 μm pore-size filter prior to use. Concentrations, treatment times and the rationale for cell-surface modifying agents as well as monosaccharides are shown in Tables 3.1, 3.2 and 3.3.
3.3.5 Infection Assay

Monolayers of U-937 cells or guinea pig alveolar macrophages in each 6 or 24 well plates were washed 4 times with HBSS to remove serum opsonins from the culture media and fresh HBSS without serum was added. The cells were then inoculated with L. pneumophila N7 at a MOI of 100. For competitive binding studies sugars were added to cells in each well 10 min prior to the addition of organisms. Surface modification of either the prokaryotic or eukaryotic cells was done prior to adding organisms to host cells. Following each treatment regime, legionellae were allowed to adhere for 1 h. Monolayers were then washed 3 times with HBSS to remove non-adherent bacteria. Adherence was assayed by VBCC counts and IFA and the data was compared with untreated controls.

3.3.6 Detection: Viable Bacterial Cell Colony Counts

Inoculated monolayers in 24-well culture plates were lysed at room temperature by adding to each 1 ml sterile distilled water for 30 min followed by vigorous aspiration with a sterile pasteur pipette. The resultant lysates were serially 10-fold diluted in 1% peptone and 25 μl of each dilution was plated in duplicate on BCYE-α agar. Colonies were counted at 72 h and the number of bacteria in each original sample was evaluated.

3.3.7 Detection: Immunofluorescent Assay

Prior to adding U-937 or GAM cells to 6-well cell culture plates, 22
### Competitive binding Treatments

#### Table 3.1: Competitive Binding Experiments

<table>
<thead>
<tr>
<th>AGENT*</th>
<th>CONCENTRATION</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-D-glucosamine (NAc Glu)</td>
<td>100mM</td>
<td></td>
</tr>
<tr>
<td>N-acetylgalactosamine (NAc Gal)</td>
<td>100mM</td>
<td></td>
</tr>
<tr>
<td>N-acetyleneuraminic acid (NAc Neu)</td>
<td>100mM</td>
<td>Competitive binding</td>
</tr>
<tr>
<td>α-D(-) fucose</td>
<td>100mM</td>
<td></td>
</tr>
<tr>
<td>β-D(+)-glucose</td>
<td>100mM</td>
<td></td>
</tr>
<tr>
<td>D(+)-mannose</td>
<td>100mM</td>
<td></td>
</tr>
<tr>
<td>D(+)-galactose</td>
<td>100mM</td>
<td></td>
</tr>
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</table>

*Each of the select treatments were added to host cells 10 min prior to infection.
## Eukaryotic Cell Treatments

**Table 3.2: Eukaryotic Treatments**

<table>
<thead>
<tr>
<th>AGENT</th>
<th>CONCENTRATION Activity (units/ml)</th>
<th>TREATMENT TIME (min)</th>
<th>FUNCTION</th>
</tr>
</thead>
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<tr>
<td><strong>Enzymes</strong></td>
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</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.1</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
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<td>30</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>1.0</td>
<td>30</td>
<td>Degrades sensitive receptor</td>
</tr>
<tr>
<td>Pepsin</td>
<td>100.0</td>
<td>30</td>
<td>on host cells</td>
</tr>
<tr>
<td>Protease</td>
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<td>30</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
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<td>30</td>
<td></td>
</tr>
<tr>
<td><strong>Agent</strong></td>
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<tr>
<td>Glutaraldehyde</td>
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<td>10</td>
<td>Immobilization of protein moieties</td>
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<tr>
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<tr>
<td>Nonidet P40</td>
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<td>60</td>
<td>Oxidation of lipids</td>
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<td>Oxidation of carbohydrates</td>
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<tr>
<td>Cytochalasin B</td>
<td>3µg/ml</td>
<td>10</td>
<td>Inhibition of microtubule formation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Concanavalin A (Con A)</td>
<td>100µg/ml</td>
<td>60</td>
<td>Binds to mannose</td>
</tr>
<tr>
<td>Wheat Germ Agglutinin (WGA)</td>
<td>100µg/ml</td>
<td>60</td>
<td>Binds to N-acetylglucosamine</td>
</tr>
</tbody>
</table>
### Prokaryotic Cell Treatments

#### Table 3.3: Bacterial Treatments

<table>
<thead>
<tr>
<th>AGENT</th>
<th>CONCENTRATION</th>
<th>TREATMENT TIME (min)</th>
<th>FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enzymes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B-galactosidase</td>
<td>100</td>
<td>60</td>
<td>Degradation of protein/sugar</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>250</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>100</td>
<td>60</td>
<td>containing</td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>20</td>
<td>60</td>
<td>moieties on the surface of L. pneumophila</td>
</tr>
<tr>
<td>Protease</td>
<td>5</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>250</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td><strong>Fixative</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.1%</td>
<td>10</td>
<td>Immobilization moieties</td>
</tr>
<tr>
<td>of protein</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Oxidizing Agent</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium metaperiodate</td>
<td>10mM</td>
<td>60</td>
<td>Oxidation of carbohydrate moieties</td>
</tr>
</tbody>
</table>
mm² sterile glass coverslips were added and the cells were allowed to adhere to the coverslips. After competitive binding and prokaryotic or eukaryotic cell surface treatments had been performed, monolayers were inoculated with L. pneumophila as described in the infection assay (3.3.4).

After 1 h incubation, host cells were washed 3 times to remove unbound bacteria after which cultures were fixed with 10% formalin in PBS (pH 7.2) for 1 h at 25°C. After three further washings with PBS, monolayers were treated with rabbit polyclonal L. pneumophila strain N7 antiserum for 1 h at 37°C. Unbound globulin was removed by washing 3 times with PBS and goat anti-rabbit FITC conjugated antibody (Boehringer Mannheim, Indianapolis, IN) was added for 1 h. After multiple washings with PBS to remove unbound globulin, cells were counter-stained with 0.01% propidium iodide in PBS for 30 min and were further washed three times with PBS. Cells on coverslips were air-dried and mounted in glycerol containing 1% 1,4-diazobicyclo (2.2.2) octane (DABCO) (Sigma) as a anti-quenching agent.

3.3.8 Detection: Criteria for Assessing Data

VBCC counts were an average of 10 trials, each performed in duplicate and the number of bacteria bound either to control host cells or to cells following the various treatment regimes were calculated as colony forming units (cfu). Adherence was expressed as a percentage of control cells. VBCC count data were statistically analyzed by determining standard error using Statview software package (Abacus Concepts) on a Macintosh Cetris 610 microcomputer. IFA results were the average of 3 trials.
enumerating adherent bacteria on the first 200 cells observed per trial. Data were calculated as the number of bacteria bound to each host cell and expressed as a percentage of control cells. A 50% change in adherence data was the criterion used to assess significance of a particular treatment for both VBCC counts and IFA studies.

3.3.9 Binding Kinetics

Wells containing U-937 cells (5 x 10^5 cells/ml) or GAM cells (2.5 x 10^5 cells/ml) in either 6 or 24 well cell culture plates were washed 5 times with HBSS to remove serum contained opsonins. Fresh HBSS was added to each well containing cell monolayers and these monolayers were infected with increasing 10-fold multiplicities of infection (MOI) of \textit{L. pneumophila} organisms. Following a 1 h incubation period at 37°C, unbound bacteria were removed by multiple HBSS washings. Infected cell monolayers were then processed for VBCC counts or IFA detection respectively.

3.4 Results

Saturation kinetics for U-937 and GAM cells indicated that host cell receptor saturation was effected at the equivalent of 1000 bacteria per host cell or 2.5 x 10^8 bacteria added to 2.5 x 10^5 host cells. These findings were similar for each cell type and assay method (Fig. 3.2.3.3.3.4, 3.5). Due to unacceptable levels of non-specific binding to the plastic culture plates at receptor saturation a MOI of 100 was selected for all subsequent studies. This gave an inoculum size close to receptor saturation levels and
minimized non-specificity while retaining the effectiveness of 50% or greater changes in binding data.

Following, a 1 h incubation with a 100 MOI of virulent *L. pneumophila*, host cells bound approximately 2% to 8% of the bacterial inoculum as indicated both by VBCC counts and IFA (see Fig. 3.2, 3.3, 3.4, 3.5).

3.4.1 Competitive Binding Treatments

Co-incubation of *L. pneumophila* with host cells in the presence of monosaccharides in a competitive fashion indicated that none of the tested simple sugars outlined in Table 3.1 inhibited opsonin-independent attachment. Similar data was generated by VBCC and IFA counts (Fig. 3.6, 3.7, 3.8, 3.9). However, carbohydrate oxidation by sodium metaperiodate treatment of either the organism or host cells indicated the importance for carbohydrates, other than those tested, in the attachment of *Legionella* to GAM cells.

3.4.2 Host Cell Surface Modification

In an effort to determine the general chemical moiety of the host cell receptor used by *L. pneumophila* to attach to host cells, cell surface membranes were chemically treated to digest or remove potential receptors. These studies indicated that U-937 and GAM cells possessed somewhat similar profiles for their adherence to receptors (Fig. 3.10, 3.11, 3.12, 3.13) treatments outlined in Table 3.2. The receptor structure was apparently carbohydrate in nature with likely lipid components (Fig. 3.18).
Metaperiodate oxidation of saccharides on the surfaces of host cells strongly inhibited bacterial attachment and was suggestive of carbohydrates in the receptor complex. Likewise, lipids may be involved with adhesin recognition as Nonidet P40 solubilization of lipids slightly interfered with the binding of legionellae to U-937 and GAM cells while lipid cleavage by lipase treatment gave variable results. Host cell surface proteins modified by trypsin, chymotrypsin protease or pepsin did not significantly influence the adherence of the organism to these cells. In addition, neuraminidase did not abolish adherence suggesting no role for neuraminic acid residues in binding. The addition of the lectins wheat germ agglutinin (WGA) or concanavalin A (Con A) strongly interrupted attachment, supporting a role for lectins or lectin-like molecules on the bacterial surface as required for the attachment event. WGA has binding specificity for N-acetyl-glucosamine and neuraminic acid while Con A attaches to mannose residues. These data suggested an inhibitory effect would be expected for these saccharides tested in competitive binding experiments; however, this was not the case.

Inhibition of microtubule formation by pretreating host cells with cytochalasin B, thus uncoupling the processes of organism attachment from organism uptake, showed no effect on bacterial binding. Treatments of host cell surfaces indicated essentially similar receptor profiles; however, some differences were noted between these cell types. With the exception of the aldehyde fixatives, the host cell treatments did not significantly affect host cell viability.
3.4.3 Bacterial Surface Treatment

Adhesin modification studies performed using the treatments outlined in Table 3.3 indicated that a complex bacterial structure was involved in this opsonin-independent attachment process. All treatments with the exception of neuraminidase produced significant decreases in *L. pneumophila* attachment to the host cells. From these data, the adhesin of *L. pneumophila* would seem most likely to be a protein containing carbohydrate and possibly lipid components (Fig. 3.14, 3.15, 3.16, 3.17). The most likely structure is a glycoprotein located on the bacterial surface which interacts and binds to a glycolipid on the host cell membrane (Fig. 3.18). All of the protein modifying agents tested, trypsin, protease and chymotrypsin significantly reduced bacterial attachment. In addition, the data from the metaperiodate oxidation of organism surface indicated that carbohydrates were necessary for binding of *Legionella* to both U-937 and GAM cells while lipase treatment moderately affected attachment. Noted that none of these adhesin modifying agents affected bacterial viability as determined by VBCC counts.
Receptor Saturation U-937 VBCC counts

Figure 3.2: Binding of *L. pneumophila* to U-937 cells as measured by VBCC counts. U-937 cells were seeded at $5 \times 10^5$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. Ratios of cfu per U-937 cell were determined by dividing the resultant lysate value by the number of host cells per well. Host cells approached saturation with *L. pneumophila* with an inoculum of $5 \times 10^7$ cfu/ml and this approximated 1.7 bacteria per U-937 cell. Each point is the average of 3 trials.
Figure 3.3: Binding of *L. pneumophila* to U-937 cells as measured by IFA. U-937 cells were seeded at 1 x 10^6 cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. Each point is the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells. Host cells approached saturation with *L. pneumophila* with an inoculum of 1.5 x 10^8 cfu/ml and this approximated 3 bacteria per U-937 cell.
Figure 3.4: Binding of *L. pneumophila* to guinea pig alveolar macrophages cells as measured by VBCC counts. Guinea pig alveolar macrophages were seeded at $2.5 \times 10^5$ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. Ratios of cfu per guinea pig alveolar macrophage were determined by dividing the resultant lysate value by the number of host cells per well. Host cells approached saturation with *L. pneumophila* with an inoculum of $2.5 \times 10^7$ cfu/ml and this approximated bacteria per guinea pig alveolar macrophage. Each point is the average of 3 trials.
Figure 3.5: Binding of *L. pneumophila* to guinea pig alveolar macrophages as measured by IFA. Guinea pig alveolar macrophages were seeded at 7.5 x 10⁵ cells/well and increasing 10-fold multiplicities of infection of bacteria were added to the cells. Each point is the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells per trial. Host cells approached saturation with *L. pneumophila* with an inoculum of 7.5 x 10⁷ cfu/ml and this approximated bacteria per U-937 cell.
Competitive inhibition U-937 VBCC counts

Figure 3.6: Competitive inhibition of *L. pneumophila* binding to U-937 cells as measured by VBCC counts. Each value represents the average of 10 trials enumerating percent bacterial adherence of controls with coincubation of legionellae with the saccharides listed in Table 3.1.
Figure 3.7: Competitive inhibition of *L. pneumophila* binding to U-937 cells as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells per trial as percent adherence of controls with co-incubation of legionellae with the saccharides listed in Table 3.1.
Figure 3.8: Competitive inhibition of *L. pneumophila* binding to guinea pig alveolar macrophages as measured by VBCC counts. Each value represents the average of 10 trials enumerating percent bacterial adherence of controls with co-incubation of legionellae with the saccharides listed in Table 3.1.
Figure 3.9: Competitive inhibition of *L. pneumophila* binding to guinea pig alveolar macrophages as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells per trial as percent adherence of controls with co-incubation of legionellae with the saccharides listed in Table 3.1.
Surface Membrane Treatment U-937 cell: VBCC counts

Figure 3.10: Surface membrane treatment of U-937 cells with agents in Table 3.2. Percent adherence of legionellae to U-937 cells as measured by VBCC counts. Each value represents the average of 10 trials.
Figure 3.11: Surface membrane treatment of U-937 cells with agents in Table 3.2. Percent adherence of legionellae to U-937 cells as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 cells per trial.
Figure 3.12: Surface membrane treatment of guinea pig alveolar macrophages with agents in Table 3.2. Percent adherence of legionellae to guinea pig alveolar macrophages as measured by VBCC counts. Each value represents the average of 10 trials.
Figure 3.13: Surface membrane treatment of guinea pig alveolar macrophages with agents in Table 3.2. Percent adherence of legionellae to guinea pig alveolar macrophages as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 cells per trial.
Surface Treatment *L. pneumophila* VBCC counts: U-937

![Graph showing surface membrane treatment of *L. pneumophila* with agents in Table 3.3. Percent adherence of legionellae to U-937 cells as measured by VBCC counts. Each value represents the average of 10 trials.](image)

Figure 3.14: Surface membrane treatment of *L. pneumophila* with agents in Table 3.3. Percent adherence of legionellae to U-937 cells as measured by VBCC counts. Each value represents the average of 10 trials.
Surface Treatment *L. pneumophila* IFA: U-937

Figure 3.15: Surface membrane treatment of *L. pneumophila* with agents in Table 3.3. Percent adherence of legionella to U-937 cells as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells per trial.
Surface Treatment *L. pneumophila* VBCC counts: GAM

Figure 3.16: Surface membrane treatment of *L. pneumophila* with agents in Table 3.3. Percent adherence of legionellae to guinea pig alveolar macrophages as measured by VBCC counts. Each value represents the average of 10 trials.
Figure 3.17: Surface membrane treatment of *L. pneumophila* with agents in Table 3.3. Percent adherence of legionella to guinea pig alveolar macrophages as measured by IFA. Each value represents the average of 3 trials enumerating the total number of fluorescent bacteria bound to 200 host cells per trial.
3.5 Discussion and Conclusions

Legionellae are facultative intracellular pathogens. They have an intimate relationship with the cell-mediated immune (CMI) system of the host in that they primarily infect alveolar macrophages and mediating CMI is critical to recovery. *Legionella* organisms can be controlled with antimicrobial therapy using antibiotics such as erythromycin, tetracycline, ciprofloxacin or rifampin, normally used in combination therapy (151, 177). However, people often succumb to Legionnaires' disease despite the implementation of appropriate and aggressive therapy soon after pneumonia is diagnosed (68). A fuller understanding of the interactions between this organism and the cells it infects is necessary for the development of alternative treatment regimes. In the absence of a toxic etiology for disease, binding is frequently the first event in bacterial infection which eventually leads to cell death or tissue invasion. This recognition process is especially necessary for diseases associated with facultative intracellular pathogens such as *L. pneumophila* (75, 87, 92, 162). The binding process is complex and probably multi-factorial in form and organisms may possess several sets of adhesins which are used under different circumstances for binding to alternative cell receptors (11). Moreover, to add to the already existing complexity of measuring adherence, the methods used to assess attachment inevitably produce contradictory results making interpretation all the more difficult. In this study, the criterion used to deem a treatment significant for modification of *L. pneumophila* adherence was a 50% reduction of the experimental or treatment data.
compared with untreated controls.

Lectins are well known attachment factors for bacteria (133). For Legionnaires’ disease to develop, the organism and cells of the undergo a recognition event which is followed by wash-resistant attachment (50, 152). Several bacterial structures have been suggested as adhesins such as mucopolysaccharide capsules, lipoteichoic acid, various lectins, fimbriae, flagella, lipopolysaccharide and outer membrane proteins (11, 40, 49, 84, 132, 133, 188). Although the adhesins for many bacteria and their counterpart host cell receptors have been identified, few binding mechanisms have been described in detail (92, 93, 132). For \textit{L. pneumophila} flagella, fimbriae, lipopolysaccharide and outer membrane proteins have been proposed as potential adhesins (149), but none has been directly implicated. However, it is clear that the characterization of the binding phenomenon of \textit{L. pneumophila} to host cells is central to elucidating the pathogenesis of this disease. Work by Gibson and Rodgers (75) demonstrated that \textit{L. pneumophila} binds to various host cells in the absence of opsonins in a wash resistant manner (see Section 2 and this report). These studies demonstrated that the Legionnaires’ disease bacterium bound to U-937 cells in the absence of either complement or specific antibody. In this study it was demonstrated that a MOI of 100 was appropriate for studies involving adherence of \textit{Legionella} to macrophages. In addition, the preliminary characterization of the bacterial and host cell molecules involved in binding are described. This mechanism of bacterial attachment to host cells would appear to be a relatively
Figure 3.18: Schematic Representation of *L. pneumophila* adhesin and host cell receptor for opsonin-independent attachment of this organism to U-937 or guinea pig alveolar macrophages.
conserved process as many similarities exist between adhesin and receptor profiles for U-937, GAM and MRC-5 cells (75, unpublished observations).

To identify the process by which *Legionella* were taken-up, cells were pretreated with cytochalasin B, a potent inhibitor of microtubule formation (50). This was used to separate active invasion or phagocytic uptake from adherence. The cytochalasin treatment had no effect on attachment of *L. pneumophila* to U-937 or GAM cells. Complement-dependent uptake of *L. pneumophila* by phagocytes requires the preliminary deposition of the complement components C3b or C3bi on the surface of the MOMP of the organism. Following this coating process, opsonized organisms were taken-up via the complement receptors (CR) type 1 or CR type 3 on human monocytes (12). Preliminary data on the identification of the adhesin(s) and receptor(s) necessary for binding *L. pneumophila* to host cells in the absence of complement or serum suggested that this process occurred in a different manner. The use of U-937 and GAM cells for these experiments was justified by the observation that this pathogen infects the resident macrophages of the lung. The monosaccharides selected for competitive inhibition studies did not interfere with binding. Clearly, mono-, di-, or oligosaccharides other than those examined may have blocked binding; however, the requirement to screen many thousands of compounds rendered this unfeasable. Subsequent studies examined the role of carbohydrates in attachment by oxidizing these compounds on bacterial or host cell surfaces. Indeed, treatment of *L. pneumophila* with surface
modifying agents indicated that the adhesin was a complex structure containing protein, carbohydrate and possibly lipid. It is possible that the adhesin in question was the MOMP of *Legionella*; as is the case for opsonin-dependent binding. The lipopolysaccharide of *L. pneumophila* is very closely associated with MOMP to the point where purified LPS is inerodibly contaminated with MOMP and vice versa. LPS is an important structure for both the establishment of disease and as an activator of the immune system (119). Isolated LPS from various organisms has been shown to bind to human monocytes via several key structures including the CD 11/CD18 Leu-CAM complex (a 80-kDa protein), the glycolipid CD 14 (a lectin-like molecule), as well as LPS binding proteins found in human serum (72, 104, 188, 189). LPS requires further examination in binding studies.

Receptor modification studies suggested that the proposed host cell receptor responsible for binding *L. pneumophila* was comprised of carbohydrate and lipid material but lacked protein and was possibly a glycolipid moiety. Whether this is identical to the previously described glycolipid CD14 is not clear. However, it does seem clear that in the absence of complement the glycoprotein receptors CR1 and CR3 were not involved in binding *L. pneumophila*. The non-opsonin adhesin/receptor process outlined in Figure 3.17 may be an alternate even critical mechanism for *Legionella*-host cell attachment especially early in disease when inflammation and hence increased opsonin levels have not occurred (75, 152). This becomes of greater significance given the observations of Reynolds and Newbold (145) that the lung and secretions are almost
devoid of the opsonins complement and IgM. Following Legionella-induced tissue destruction, the increase in opsonin concentration may well augment opsonin-mediated uptake and thus facilitate disease.

It was clear that metaperiodate oxidation and treatment with various lectins strongly inhibited L. pneumophila attachment. Lectins are proteinaceous structures which possess the ability to recognize specified carbohydrate moieties. The type 1, Pap and S fimbriae often found in members of the Enterobacteriaceae frequently show lectin activity. Indeed, for these organisms fimbriae are believed to be central to attaching bacteria to surfaces (90, 118, 123, 132). Type 1 fimbriae bind to D-mannose residues (1, 133). Although, the major subunits vary widely from species to species the tip is a conserved structure across most type 1 fimbriae of the Enterobacteriaceae (49). This structural organization is common among other fimbriae such as the Pap pili and S fimbriae that recognize α-Gal (1→4)β-Gal and sialic acid containing glycoconjugates (90, 121). In these studies, D-mannose did not compete with L. pneumophila for binding sites for U-937 or GAM cells. Although L. pneumophila possess fimbriae, it seems that Type 1 fimbriae are not involved in opsonin-independent adherence described. In addition to these findings the use of galactose, N-acetyl neuraminic acid and neuraminidase failed to inhibit L. pneumophila adhesion to these cells. The contradictory data from the competitive assays with mannose and N-acetyl-galactosamine, and the lectins Con A and WGA (these binding
ligands for mannose and N-acetyl galactosamine respectively) may reflect steric phenomena involving lectin recognition of the appropriate saccharides in the proximity of the host cell receptor. Data from the metaperiodate oxidation of carbohydrates supported these suggestions.

It is also possible that Legionella possess a myriad of attachment molecules and that the organism can modulate their expression as required. A comprehensive characterization of both adhesin and receptor will require their isolation and purification; however, these procedures are complex (11, 60, 74, 165). Mucopolysaccharide capsules are important for the adherence of bacteria to oral tissue cavity (74), while Bacteroides fragilis possess polysaccharides which are potent mediators of abscess formation (175). Capsules have also been shown to decrease both antigenicity and decrease the phagocytotic expression of macrophages for encapsulated bacteria (125). Although data is conflicting, it would appear that Legionella do not produce a capsule (150). Studies are further complicated in that traditional laboratory batch growth procedures tend to select for organism populations not expressing adhesins. This is brought about because nutrients are present to excess and so there is no advantage for their expression. Variations in culture conditions for bacterial populations used in adherence study may produce widely divergent results.

Facultative intracellular bacterial pathogens such as L. pneumophila, L. monocytogenes, M. tuberculosis, S. typhi and Y.pestis belong to this group of facultative intracellular pathogens and all have demonstrated
fascinating strategies for infecting and surviving within host cells (60, 83, 75, 125, 166). Legionellosis has serious implications for the health of the nation especially given the spectacular outbreaks that occur from time to time. Antimicrobial intervention which is not preventative, may not successfully eradicate the pathogen. In addition, resistant strains have been reported and organisms cannot be removed from the environment entirely. As a consequence alternatives to conventional therapeutic regimes are required for to prevent severe cases of legionellosis among susceptible individuals. A vaccine to augment antibiotic therapy is required. Attachment to the alveolar macrophage is the first stage of Legionella-induced disease; therefore, it may serve as a potential attack site for vaccine research. A vaccine that is capable of interrupting or preventing this binding event may prove an efficacious addition to the clinical armamentarium for the prevention of Legionnaires' disease.
SECTION IV

ASSESSING THE ROLE OF COMPLEMENT RECEPTORS FOR ATTACHMENT OF

LEGIONELLA PNEUMOPHILA TO U-937 CELLS.

(The work outlined in this Section has been published in abstract form:


4.1 Abstract

In order to define the role of CR1, CR3 and CR4 in the opsonin-independent adherence process of legionellae to macrophages, a series of monoclonal antibodies to CR1 (CD 35), CR3 (CD 11b + CD 18) and CR4 (CD 11c + CD 18) were used both individually and in combination to block these potential binding sites. These monoclonal antibodies either individually or in combination failed to inhibit the binding of L. pneumophila to U-937 cells as compared with untreated control cultures. It would seem that opsonin-independent attachment of L. pneumophila to U-937 cells is mediated by host cell binding ligands other than CR components.
4.2 Introduction

The nature of *Legionella pneumophila* attachment to host cells is poorly understood. Moreover, the mechanism by which intracellular pathogens infect host cells has been shown to be critical for the establishment of infection. Initial investigations by Horwitz *et al.* (87) demonstrated that uptake of *L. pneumophila* by human monocytes was mediated by the deposition of complement onto the MOMP of *Legionella* organisms. This complex is then bound to the complement receptors CR1 and CR3 (12). In addition, the LPS of *L. pneumophila* has been shown to activate the classical complement cascade (119) and this observation supports the opsonin-dependent process of host cell infection. Work from our laboratory (76, and this report Section II) demonstrated that direct attachment of *L. pneumophila* to U-937 cells occurs independent of opsonins and that this process may be central to the initiation of disease (75, 76, 91).

Cell mediated immunity (CMI) in conjunction with humoral support is critical to the recovery from disease. Macrophages and polymorphonuclear leukocytes (PMNL) are the primary phagocytes involved in the eradication of these bacterial pathogens. The process by which these cells engulf and destroy bacteria is well documented; however, they fail to function adequately when the host is challenged by the facultative intracellular pathogen, *L. pneumophila*. Normally, macrophages first engulf, internalize and process invading microorganisms before destroying them. In functional terms, the phagocytic cell appears
to be an unattractive host for Legionella; despite this, Legionella have the ability to parasitize these cells. The processes by which these bacteria gain access to host phagocytes and then evade their microbicidal activities are diverse. The successful activation of such processes directly affect the survival of either the organisms or of the host cells in the ensuing struggle for supremacy. Two groups of bacterial attachment mechanisms to phagocytes have been identified and these include opsonin-dependent and opsonin-independent processes. Opsonin-dependent mechanisms of microorganism uptake have been elaborated in greater detail than those binding systems which occur via opsonin-independent processes.

Opsoninization involves two processes in which organisms pre-coated with specific antibody bind to phagocytic cells usually through Fc receptors. This binding induces cellular production of reactive oxygen metabolites (187) which are critical to the breakdown of foreign material. In contrast, those pathogens that bind to complement receptors (CR1 or CR3) via complement do not elicit an oxidative burst (182, 187). Organisms which use CR1 and CR3 for entry into the intracellular matrix of the host cell are afforded a potentially safer route for intracellular infection than those entering via Fc receptors.

Recently, the heterodimeric β2 integrin family of leukocyte cell surface adhesion molecules have been shown to be important for binding prokaryotic and eukaryotic pathogens, as well as bacterial surface-expressed components to host cells (4, 12, 162). This family of leukocyte
receptors includes LFA-1, CR3, and CR4 (p150,95), of which CR3 and CR4 are important mediators for binding the third component of complement (C3) as well as for mounting a functional immune response (157, 158, 159). As a group, these molecules comprise an α (CD11a, b, c) and β (CD18) subunit with the β subunit conserved by all members of the integrin group (167). The human histiocytic lymphoma, U-937 cell line, has a receptor profile similar to that of both monocytes (pre-phorbol ester activation) and macrophages (post activation) (161). Known opsonin receptors on the surface of activated U-937 cells include, fibronectin-binding protein, CR1, CR3, CR4 and Fc receptor for IgG (158, 161, 162, 167). The profile and distributions of these receptors is similar to that for human alveolar macrophages and make this cell an attractive model for the study of direct attachment of L. pneumophila via complement receptors (12).

4.3 Materials and Methods

4.3.1 Bacterial Growth and Maintenance

L. pneumophila strain Nottingham N7 was grown and maintained as previously described (Section 2.3.1).

4.3.2 U-937 Cell Cultivation

U-937 cells were grown and phorbol ester treated as previously described (Section 2.3.2).
Table 4.1: Monoclonal Antibodies used for Complement Receptor Inactivation

<table>
<thead>
<tr>
<th>Type</th>
<th>Raised Against</th>
<th>Concentration Used</th>
<th>Blocking Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Goat anti-Rabbit</td>
<td></td>
<td>100 µg/ml</td>
<td>Fc Receptor</td>
</tr>
<tr>
<td><strong>Clone Products:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1/70.15.11.5</td>
<td>CD 11b</td>
<td>50 µg/ml</td>
<td>CR3 (α subunit)</td>
</tr>
<tr>
<td>M18/2a.12.7</td>
<td>CD 18</td>
<td>50 µg/ml</td>
<td>CR3 and CR4 (β subunit)</td>
</tr>
<tr>
<td>YFC118.3</td>
<td>CD 18</td>
<td>50 µg/ml</td>
<td>CR3 and CR4 (β subunit)</td>
</tr>
<tr>
<td>E11</td>
<td>CD 35</td>
<td>50 µg/ml</td>
<td>CR1</td>
</tr>
<tr>
<td>Sp2/0-Ag14</td>
<td>CD 11c</td>
<td>50 µg/ml</td>
<td>CR4 (α subunit)</td>
</tr>
</tbody>
</table>

a Incubation conditions were 60 min at 37°C for all treatments

b Clones YFC 118.3, E11, and Sp2/0-Ag14 are of mouse origin and were obtained from Serotec International, Indianapolis, IN. Monoclonal antibodies from clones M1/70.15.11.5 and M18/2a.12.7 were rat anti-mouse and possessed cross-reactivity with human epitopes. These were kindly donated by Dr. A. Sloan.
Figure 4.1: Complement receptors responsible for binding C3b and C3bi. * Denotes those receptors responsible for opsonin-independent attachment of *L. pneumophila* to host cells. Adapted from Springer 1984.
4.3.3 Receptor Inhibition Studies Using Specific Monoclonal Antibodies

U-937 cells grown on coverslips in 6-well cell culture plates previously treated with PMA were washed 5 times with HBSS to remove RPMI-1640 and serum components (Appendix 1). The cells were resuspended in fresh HBSS and host cell Fc receptors were blocked by incubation at 37°C for 1 h with a non-specific rabbit antisera (generated in goat) used at a concentration of 100 μg/ml (Table 4.1). Following three washes to remove unbound globulin, monoclonal antibodies specific for CR1, the α and β components of CR3 and the α and β subunits of CR4 were added individually as well as in combination to block receptor activity on U-937 cells. The monoclonal antibodies used to block CR1 were of mouse origin and were directed against human epitopes. The CD 11b, CD 11c and CD 18 monomers of CR3 and CR4 on U-937 cells were either rat anti-mouse, mouse anti-human or rat anti-human in origin (Table 4.1). The rat anti-mouse sera were specific for human CD 11b and CD 18 glycoproteins and it is known that these monoclonal antibodies recognize identical moieties conserved between human and murine systems. Treated cell cultures were incubated for 1 h at 37°C, after which unbound antiserum was removed by washing three times with HBSS. *L. pneumophila* were added to these monoclonal antibody treated U-937 cells at a MOI of 100 in HBSS. Cultures were then incubated for 1 h at 37°C, washed three times with HBSS and assayed by VBCC counts.
4.4 Results

The treatment of U-937 cells with goat anti-rabbit IgG facilitated recognition of the monoclonal antibodies each to the appropriate ligand and prevent loss of monoclonal activity to Fc receptors. This pretreatment was not inhibitory to the binding of *L. pneumophila* (Fig. 4.2).

Phorbol ester treated U-937 cells express CR1, CR3 and CR4 (161). When added to U-937 cell cultures monoclonal antibody to the human CR1 (CD 35) did not inhibit the binding of the organism to U-937 cells (Fig. 4.2). Two separate sets of monoclonal antibodies were used to block CR3 (Table 4.2) the major structure responsible for uptake of the Legionnaires’ disease bacterium in the presence of complement. Data generated from these antibody blocking studies indicated that CR3 was not involved in organism binding. No inhibition of bacterial binding was recorded using these levels of monoclonal antibodies (Fig. 4.2).

Although, CR4 is weakly expressed on alveolar macrophages and U-937 cells it is structurally analogous to CR3 and is a potent receptor for C3bi (162). To neutralize the CR4 receptor monoclonal antibodies directed to human CD 11c and CD 18 (Table 4.1) were used separately or in combination to block either the individual subunits or the receptor complex. These antibodies failed to neutralize the binding of *L. pneumophila* to U-937 cells. It was clear that CR1, CR3 and CR4 and their substituant sub-units did not function as receptors for *L. pneumophila* in opsonin-independent adherence (Fig. 4.2).
Figure 4.2: Monoclonal antibody blocking of CR1 (CD 35), CR3 (CD 18 and/or CD 11b) and CR4 (CD 18 and/or CD 11c) on the surface of U-937 cells for inhibition of *L. pneumophila*-U-937 cell binding as measured by VBCC counts. Fc receptors were inactivated using a goat anti-rabbit antiserum prior to monoclonal antibody incubations. Each plotted data set is a mean of three trials.
4.5 Discussion and Conclusions

Bacterial attachment to host cells is the first interaction of a pathogen with the host. This adherence phenomenon is an essential step in intracellular infection, whether for phagocytic or other cells (11, 165). It would appear that the mechanisms used by phagocytes to take-up intracellular pathogens profoundly influences the development of cellular infection (11, 178, 182, 188).

*L. pneumophila* has been shown to infect an extensive array of cells including amoebae, epithelial cells, fibroblasts, macrophages and monocytes (59, 75, 87, 94, 111, 122, 134, 152). In opsonin-mediated adherence, the fragments C3b and C3bi of the complement system coat the organism prior to binding of legionellae to the surfaces of monocytes via CR3 and to a lesser extent CR1.

Recently, opsonin-independent binding of this organism to U-937 cells has been reported (75, 76, 91, 152, and Sections 2 and 3 of this report). Preliminary investigations to characterize receptor structures have proved inconclusive but suggest a glycolipid moiety as the adherence receptor for MRC-5, U-937 and GAM cells. The monoclonal antibody blocking studies offer some confirmation of these findings in that the glycoprotein receptors CR1, CR3 and CR4 were not involved in the direct binding of *L. pneumophila* to host cells.

Two of the three complement receptors CR3 and CR4 (p150.95) responsible for binding various C3 components on the surface of phagocytes are integrins while CR1 is not. These heterodimeric integrins
which bind C3bi consist of a unique α subunit and a common β subunit. The CR3 receptor is responsible for binding and uptake of complement coated legionellae (12, 162).

Infection of U-937 cells in the absence of complement was achieved by infecting these cells with legionellae in HBSS. The role of CR molecules during complement-independent adherence of L. pneumophila was assessed using a series of monoclonal antibodies. The CR1 receptor was shown to take-up legionellae in an indirect fashion, while CR3 directly bound L. pneumophila to monocytes. As all the monoclonal antibodies used in this study were of IgG origin, it was necessary to block the Fc receptor of the U-937 cells prior to the addition of monoclonal antibodies using goat anti-rabbit serum to U-937 cells. Binding of the organism was unaffected by the presence of this antibody at the host cell surface indicating that as expected, as expected the Fc receptor was involved in opsonin-independent binding.

The present data indicated that the CR molecules (CD 35 of CR1; CD 11b and CD 18 of CR3; CD 11c and CD 18 of CR4) were not involved for either attachment or uptake. As described in Section 3, the chemical nature of host cell receptors indicated that glycolipids (carbohydrate and/or lipid), rather than proteins or glycoprotein structures such as CR1, CR3 and CR4 were involved in the binding legionellae by this "alternate" non-complement mechanism. In addition, the bacterial adhesin would appear to be protein or glycoprotein-like in nature (Section 2).
During the first recognition of this organism by alveolar macrophages and prior to the initiation of disease, the environment of the lung and the alveoli contains few opsonins as shown by analysis of lavage fluids (145). In the present model, it is suggested that CR components are not the primary host cell receptors to which \textit{L. pneumophila} bind in the earliest phases of disease. Indeed, it would seem likely that this non-opsonic uptake would be of greater importance until cellular damage has occurred and opsonin levels increase in the lungs. Whether a given cell can take up \textit{Legionella} organisms by a non-opsonic process simultaneously with opsonin-mediated mechanisms is not known. However, unlike studies in which complement components are excluded and therefore only opsonin-independent processes are assessed, experiments conducted in the presence of complement do not exclude the possibility of opsonin-independent uptake from occurring also. Indeed, it seems probable that in experiments conducted with serum or complement present, organisms may enter the cell by both processes. Whether the intracellular fate of the organism differs under these circumstances is unknown, but it may be speculated that since a full cycle of cell infection occurs in the absence of opsonins that opsonin-independent uptake may be the major route leading to intracellular replication of the organism and eventual death of the cell.
SECTION V

CONCLUDING REMARKS

5.1 General Conclusions

*Legionella pneumophila* is a facultative intracellular bacterial pathogen responsible for the potentially fatal illness Legionnaires' disease. This organism has previously been shown to infect a wide variety of host cells including amoebae, epithelial cells, fibroblasts, macrophages and monocytes (59, 94, 99, 122, 134, 147, 187). However, following exposure and inhalation by humans, legionellae preferentially infect alveolar macrophages in the lung (183). The processes by which *L. pneumophila* infects macrophages and monocytes has been shown to be dependent on the deposition of complement on the MOMP of these organisms and the opsonized bacteria are taken-up via CR1 and CR3 (12). In the present work an alternative process for *Legionella* attachment to host cells have been identified and the molecules involved in adherence has been partially characterized. This process occurred in a fashion independent of complement, opsonization or complement receptors. Opsonin-independent attachment occurred independent of cytochalasin treatment of host cells and wash-resistant, suggesting that this type of binding occurred independent of phagocytosis and the MIP protein and that attachment has proceeded beyond the weak
erelectrostatic or Van der Waals forces responsible for early host cell-pathogen interaction. This attachment mechanism led to complete replication and release of organisms in the macrophage-like U-937 cell and was similar in to other published studies on the ultrastructural events and multiplication kinetics of \textit{L. pneumophila} in other host cells (75, 111, 152).

These studies describe an adhesive structure for \textit{L. pneumophila} that was responsible for direct cell infection and indicated that the molecule was a complex of protein and carbohydrate and/or lipid moieties and which possessed lectin-like activities. The host-cell receptor for binding \textit{L. pneumophila} was comprised of carbohydrate and lipid in the absence of proteins. As the complement receptors CR1 and CR3 are glycoproteins on macrophage surface membranes and are believed to be solely responsible for the docking \textit{L. pneumophila}, carbohydrate oxidation and proteolytic modification should inhibit organism binding. As this was not the case it suggested receptor structure other than complement receptors. Monoclonal antibodies specific for CR1, CR3, CR4 and their subunits demonstrated that these cell-expressed components were not involved in opsonin-independent binding to macrophages. These antibodies did not inhibit \textit{L. pneumophila} binding to U-937 cells.

It is postulated that such an alternative mechanism of binding is central to the initial establishment of cell infection in the human lung. Clinical evidence to support this suggestion indicates that the lung are essentially devoid of complement (145). If disease is to become established in the lung non-opsonin-mediated processes would need to
occur. The identification of alternative attachment mechanisms and the isolation and purification of the molecules responsible for attachment of \textit{L}. \textit{pneumophila} by macrophages may prove useful for the production of vaccines. Such vaccines would be of major advantage for augmenting current antimicrobial strategies especially since antibiotics unlike vaccines neither prevent infection nor reduce the mortality arising from Legionnaires’ disease.

5.2 Future Studies

Host infection with \textit{L. pneumophila} is a complex series of events and expression of bacterial virulence factors as well as the status of the immune system of the host are central to the initiation and development of this disease. Several interesting lines of inquiry emerge from the present study and these merit further investigation:

1- A panel of monoclonal antibodies should be raised against \textit{Legionella} surface proteins to challenge the organism-host cell binding event to define those structures as potential adhesins.

2- Identification of the genes responsible for the expression of adherence factors on \textit{L. pneumophila} should be conducted. The generation of isogenic strains of \textit{L. pneumophila} that do not express the adhesin should be undertaken and these mutant strains should be tested for binding potential.
3- The role of complement receptors in the establishment of disease requires clarification. Mutants of *L. pneumophila* carrying different genotypic markers but possessing similar binding and intracellular growth characteristics as parent strains should be developed and used to differentiate which receptors are preferentially used for uptake by this organism. In such studies, one genotype could be added to cells without opsonins and the different genotype could be added with complement and serum and the progeny assayed to determine which replicated intracellularly. These studies may resolve whether organisms taken-up by complement-mediated processes are destroyed or eventually lead to cell infection.

4- The role of cytokines and other immune function modulators should be assessed for their part in the bacterial infectious process at the cellular and molecular levels. It may prove feasible for those with inhibitory effects to be used in association with conventional therapy to augment antibiotic therapy.

5- The role of B and T cells in immunity is unclear. Data on the recognition events by T cells as well as the influence of specific antibody in long term immunity is required. These studies may profoundly influence vaccine development.

6- The interaction of legionellae with environmental amoebae
should be investigated to determine whether the binding processes characterized in the present study are conserved in nature. Such studies would assist with our understanding of human disease, as well as survival of the organism in the supernatant.

7- The part played by the major secretory protein of *L. pneumophila*, the zinc metaloprotease, as well as, other exotoxic products produced by the organism should be determined for the part these compounds play in virulence both at the cellular level and in the development of human Legionnaires' disease.
REFERENCES


Appendix 1: Biological Studies

A. Bacterial Cultivation

1.1. Storage of Bacterial Cultures

Legionella pneumophila serogroup 1 strain N7 is a virulent strain of this organism. Organisms were stored at -70°C throughout this study. Thawed aliquots grown on buffered charcoal yeast extract agar (BCYE-α) were periodically assessed for virulence using the fertile hens egg model (Tzianabos).

1.2. De-ionized water

All water used in these studies were obtained from a Milli-Q filtration system (Millipore). Water was collected from the outflow port after ten megaohm resistivity was reached. The water was passed through a 0.2 μm sterile filter and stored at 4°C in the dark.

1.3. Buffered Charcoal Yeast Extract Agar (BCYE-α)

The preferred agar for the cultivation of Legionella is BCYE-α agar. This media was purchased in dehydrated form from Becton Dickinson.
(Cockeysville, MD) and is resuspended in Milli-Q water. The formulation of this agar is as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>10.0</td>
</tr>
<tr>
<td>ACES Buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>Ferric Pyrophosphate</td>
<td>0.25</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
<tr>
<td>Activated Charcoal</td>
<td>2.0</td>
</tr>
</tbody>
</table>

To 800 ml of fresh Milli-Q water 38.3g of BCYE-α was added and mixed until dissolved. A volume of 10N KOH was added dropwise to the agar solution to adjust the pH to 6.9. The final volume was adjusted to 1 liter by adding Milli-Q water. The agar was sterilized by autoclave using 121°C at 12 psi for 20 min and the media was allowed to cool to 52°C. 4 ml of a 10% L-cysteine in Milli-Q water was added to each liter, was evenly distributed throughout the media and plates were poured. After the plates cooled, they were placed in an incubator at 37°C overnight to check for sterility and were stored at 4°C in a sealable plastic bag containing moist paper towels until needed.

1.4. Buffered Yeast Extract Broth (BYE-α)

BYEα- broth was used in this study to grow legionellae from plate cultures. This media was the standard broth for *L. pneumophila* cultivation and was not supplemented with antimicrobials. The formulation used was:
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast Extract</td>
<td>10.0</td>
</tr>
<tr>
<td>ACES Buffer</td>
<td>10.0</td>
</tr>
<tr>
<td>Ferric pyrophosphate</td>
<td>0.25</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>1.0</td>
</tr>
</tbody>
</table>

These components were added in sequence to 800 ml of Milli-Q water. The broth was then adjusted to pH of 6.9 with the dropwise addition of 10N KOH and the total volume was adjusted to 1 liter with fresh Milli-Q water. The BYE-α broth was filtered through a 0.45 μm filter and then through a sterile 0.22 μm filter for final sterilization. 4 ml of a 10% L-cysteine solution was added per liter and this was alliquoted into 5 ml batches and tested for sterility in a similar manner to BCYE-α agar.

1.5. Phosphate Buffered Saline (PBS)

This isotonic buffer was used in various studies for washing bacteria as well as host cells and was made in Milli-Q water. The formulation used was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.2</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.15</td>
</tr>
</tbody>
</table>

PBS was adjusted to a pH of 7.2 by addition of 10N NaOH and was sterilized by autoclaving. Solutions were stored at 4°C in 500 ml batches in the dark until needed.
1.6. 1% Peptone

1 g of peptone was dissolved in 100 ml of Milli-Q water. The solution was sterilized by autoclaving and was then aliquoted in 0.9 ml volumes with a cornwall syringe into sterilized standard type dilution blank test tubes. These 0.9 ml dilution blanks were stored overnight at 4°C prior to use.

B. Cell Culture

1.7. De-ionized water

See appendix 1.2 above.

1.8. Hank’s Balanced Salt Solution (HBSS)

Sterile batches of 500ml 10x concentrated HBSS with supplemented phenol red was purchased from Sigma (Sigma, St. Louis MO). To 450 ml of sterile Milli-Q water, 50 ml of sterile HBSS was added. The pH of this solution was adjusted to 7.2 with sterile 1N Na₂HCO₃. These batches were tested for sterility by placing the prepared bottles at 37°C for 24 h. Furthermore, a 0.1 ml sample of HBSS was plated on both nutrient and blood agar and these plates were checked for sterility following 24 h incubation at 37°C. Sterile HBSS was stored at 4°C until needed.

1.9. Glutamine

L-glutamine was purchased in sterile 100 ml batches at a concentration of 200 mM in water (Sigma) and was stored at -20°C until needed.
1.10. Fetal Bovine Serum

Sterile fetal bovine serum (Hybrimax) was purchased from Sigma in 500 ml batches. The contents of each bottle was thawed and aliquoted into 25 ml batches in sterile 50 ml conical tubes and stored at -20°C until needed.

1.12. Sodium bicarbonate

A 7.5% sodium bicarbonate solution was prepared by adding 7.5 g Na$_2$HCO$_3$ to 100 ml fresh Milli-Q water. This was filter sterilized by positive pressure filtration through a 0.22 μm membrane filter into a sterile 100 ml dilution bottle and was stored at 4°C until needed.

1.12. Sodium Hydroxide

A 1N NaOH was prepared by adding 4g NaOH pellets to 100ml distilled water and this solution was sterilized by autoclaving.

1.13. RPMI-1640

U-937 cells and guinea pig alveolar macrophages were cultivated in RPMI-1640 without antibiotics. Basal RPMI-1640 cell culture media containing phenol red was purchased as 10x stock solutions in 500 ml aliquots from Sigma and were stored at -20°C. For culture, the basal media was prepared in 500 ml batches as follows:
<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>377 ml</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>50 ml</td>
</tr>
<tr>
<td>FBS</td>
<td>50 ml</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>18 ml</td>
</tr>
<tr>
<td>7.5% Sodium bicarbonate</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

The RPMI-1640 medium was adjusted to pH 7.2 with NaOH. Samples of each media were batch tested for sterility by adding 0.1 ml aliquots of cell culture media to both nutrient and blood agar plates. These plates were incubated overnight at 37°C and examined the following morning. Sterile media was stored at 4°C.
Appendix 2: Electron Microscopy

A. Transmission Electron Microscopy

2.1. Grids

Copper grids of 400 mesh were purchased from Electron Microscopy Supplies (EMS) and were used for all studies.

2.2. Collodion

A stock solution collodion was purchased as 10% nitrocellulose dissolved in EM grade amyl acetate. This solution was diluted to 1% with EM grade amyl acetate and stored at room temperature in the dark.

2.3. Collodion Coating of Grids

3 drops of collodion solution were dropped onto the surface of a standard petri dish containing 20 ml Milli-Q water. The lid was placed over the top of the dish in canted fashion to prevent dust accumulation on the films and to permit evaporation of amyl acetate resulting in the production of ultra-thin collodion films. Copper grids were placed face down on the collodion films using forceps and were collected using dust-free parafilm. Collected copper grids were allowed to dry at room temperature overnight prior to use.
2.4. Cacodylate buffer

0.1M cacodylate buffer (Electron Microscopy Sciences) was made by adding 21.4 g sodium cacodylate-trihydrate to 1 L Milli-Q water. Then 10 mM MgSO₄ was added to this solution. Cacodylate buffer was adjusted to pH 7.4 and stored in the dark at 4°C until needed.

2.5. Glutaraldehyde

EM grade glutaraldehyde was purchased from EMS as a 50% solution and was stored refrigerated, in the dark. Prior to TEM and SEM studies, glutaraldehyde was diluted to 3% in cacodylate buffer and was used for specimen fixation. Glutaraldehyde was used in a fume hood.

2.6. Osmium Tetroxide

Osmium tetroxide was purchased as a solid from EMS in glass vials in vacuo and were stored at 4°C. Working strength osmium tetroxide was prepared by placing the contents of a 1 g vial in 50 ml cacodylate buffer to make a 2% (w/v) stock solution. Equal volumes of 2% osmium solution and cacodylate buffer were placed over each sample and they were fixed overnight at room temperature. OsO₄ was used in a fume hood and waste was disposed of in proper fashion according to University policy.

2.7. Graded Ethanol Series

Absolute ethanol was diluted with cacodylate buffer to create a series of 50%, 70%, 75%, 90% and 95% ethanol. Each dilution was stored in...
sealed bottles and was kept at room temperature.

2.8. Propylene Oxide

Propylene oxide was purchased from EMS ready for use and was stored tightly stoppered in a cool dark place.

2.9. Resins

An epon-aryldite resin mixture was used to embed specimens for thin sectioning. All resin components were purchased from EMS and were stored at room temperature in the dark. The recipe and proportions used in their studies were as follows:

<table>
<thead>
<tr>
<th>Resin</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epon 812</td>
<td>5 parts</td>
</tr>
<tr>
<td>Araldite</td>
<td>11 parts</td>
</tr>
<tr>
<td>Dodecyl succinic anhydride</td>
<td>3 parts</td>
</tr>
<tr>
<td>Dibutyl phthalate (plasticiser)</td>
<td>0.8 parts</td>
</tr>
<tr>
<td>DMP-30 (accelerator)</td>
<td>0.4 parts</td>
</tr>
</tbody>
</table>

All resins were measured using plastic tri-pour beakers or plastic syringes. The resins were added in sequence to a glass bottle (except for the DMP-30) and were shaken vigorously for 10-15 min. The resin mixture was divided into 4 equal parts and was complete by the addition of DMP-30 at the appropriate concentration (see TEM procedure 2.10). The complete resin was then shaken for an additional 3 min to facilitate even distribution of DMP-30. All manipulations were performed in a fume hood.
2.10. TEM Procedure

Cell cultures in T-75cm² flasks either infected with *Legionella* or non-infected were fixed in situ with 3% (w/v) glutaraldehyde for 2 h. Samples were not allowed to dry at any stage of the embedding process. Following fixation, cells were washed 10 times with cacodylate buffer and were scraped from the surface of the flask using a silicon rubber-coated pipette. The samples were lightly pelleted in an eppendorf tube and supernatants were decanted. Samples were then embedded in 2% nobel agar and fixed overnight in 1% (w/v) osmium tetroxide.

Washed, osmicated samples were dehydrated using a graded ethanol series of 50%, 70%, 75%, 90%, 95% at 5 min each to reach absolute ethanol followed by two changes of 10 min each in absolute ethanol. The samples were further dehydrated in propylene oxide with 2 changes of 10 min each. Following the preparation of the epon-aryldite resin mixture, a gradient resin series was made to facilitate impregnation of specimens. The samples were placed in each step of a gradient consisting of 1 part resin and 2 parts propylene oxide for 1 h and then 2 parts resin and 1 part propylene oxide for 1 h. Samples were then placed in 100% resin and then into 100% degassed resin for 1 h each.

Agar embedded, resin impregnated samples were placed at the bottom of a beam capsule, one specimen per capsule, using a wooden dowel trimmed to form a spatula-like end. In addition, paper labels marked with lead pencil were placed into each of the appropriate capsules. Samples were covered with complete degassed resin and were
polymerized in a 60°C oven for 24 h. Blocks were removed from capsules after 48 h and allowed to fully harden. Blocks were trimmed and ultra-thin 60-90 nm section cut with a LKB ultratome III ultramicrotome using a Diatom diamond knife. Sections were collected on 400 mesh copper grids. Sections were stained with uranyl acetate for 1 min., washed with 20 ml of boiled, cooled Milli-Q water and the stained with lead citrate for 30 sec., followed by 50 ml rinsing with boiled, cooled milli-Q water. After drying, stained grids were observed and photographed on a Hitachi scanning-transmission electron microscope in the transmission mode at an accelerating voltage of 75kV.

2.11. Uranyl acetate staining

Uranyl acetate was purchased from EMS and was stored at room temperature in the dark. A 5% stain solution was made by placing 1 g uranyl acetate in 20 ml sterile filtered Milli-Q water. After allowing overnight to dissolve, the stain was filtered through a 0.45 μm filter into a clean, dry, screw-top tube and was stored at room temperature covered with aluminum foil.

2.12. Lead citrate staining

A sodium hydroxide pellet was dissolved in 15 ml boiled, cooled Milli-Q water. A 0.5% lead citrate solution was made by placing 0.1 g lead citrate into the 15 ml solution and this was followed by the addition of 10 ml boiled, cooled Milli-Q water. After sitting overnight to fully dissolve, 1 ml
alliquots were centrifuged at 12,400 x g for 20 min and supernatants were stored at room temperature, in the dark, wrapped in foil until needed.

B. Scanning Electron Microscopy

2.13 Hexamethyldisilazane (HMDS)

HMDS was purchased from EMS in ready to use form. This highly volatile substance was stored tightly stoppered and stored in a cool dark place.

2.14 SEM Procedure

Samples in 6 well plates on coverslips were fixed with 3% glutaraldehyde for 2 h. Following fixation, samples were washed extensively with PBS and dried in a graded ethanol series in situ (50%, 70%, 75%, 90%, 95% and 100%) in similar fashion to TEM studies (Appendix 2.10). Specimens on coverslips were dried from hexamethyldisilazane (HMDS) were fixed on the top of a carbon coated stub and were stored at room temperature in a desiccated environment. The coverslips were placed in a sputter coater and were coated with 20 nm gold-palladium using a target to specimen distance of 5 cm and 15 mA for 4 min. Specimens were placed in an AMR-1000 scanning electron microscope with an accelerating voltage of 60kV.
Appendix 3: Guinea pig Care and Alveolar Macrophage Collection

All animal procedures were conducted according to NIH guidelines as governed by the University of New Hampshire, Animal Care and Use Committee. Experiments were performed under the licence 930803 and its predecessors issued to Dr. Frank G. Rodgers.

3.1. Animal Specifications

Hartley outbread guinea pigs (either sex) of 600-650 g were purchased from Charles River Laboratory. The animals were shipped in one day in filtered cartons. Upon arrival, each animal was checked for general appearance, sexed, weighed, fed as needed and placed into a separate cage to stabilize.

3.2. Animal Living Conditions

All cages conformed to Animal Care and Use Committee guidelines for size. In addition, each cage contained a generous amount of wood chips which were changed on an animal to animal need basis.

3.3. Food and Water

Guinea pig chow (Ralston Purina) and water were supplied to animals ad libitum.
3.4. Euthanization

Each animal was weighed and sacrificed prior to collection of alveolar macrophages with an intraperitoneal injection of nembutal (sodium pentobarbital). Animals were then bleed by laceration of the dorsal aorta and descending vena cava to limit the amount of blood in macrophage cultures. Following collection of macrophages, each animal was delivered to UNH animal facilities for proper disposal.

3.5. Collection of Alveolar Macrophages

Following exsanguination, the trachea was exposed and clamped-off using a hemostat to prevent descending contamination of cultures and to restrict fluid motion in the respiratory tract. A 10 ml syringe with an 18-gauge needle containing ice-cold HBSS (pH 7.2) was inserted into the trachea and the contents of the needle was slowly expelled into the lungs. Following a 1-2 min incubation, the fluid was removed by aspiration with the same syringe. This procedure was repeated a total of four times per animal. Collected samples were placed on ice until they were processed.