Winter 1995

Characterization of the adherence mechanisms of Listeria monocytogenes to host cells and their role in the pathogenesis of listeriosis

Marcia M. Pierce
University of New Hampshire, Durham

CHARACTERIZATION OF THE ADHERENCE MECHANISMS OF *LISTERIA MONOCYTOGENES* TO HOST CELLS AND THEIR ROLE IN THE PATHOGENESIS OF LISTERIOSIS

BY

Marcia M. Pierce
B.S., Texas A & M University, 1986
M.S., Baylor University, 1989

DISSERTATION

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

Doctor of Philosophy

in

Microbiology

December, 1995

This dissertation has been examined and approved.

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

Dr. Thomas Foxall, Associate Professor of Animal Science

Dr. Aaron B. Margolin, Associate Professor of Microbiology

Dr. Thomas G. Pistole, Professor of Microbiology

Dr. Robert M. Zsigray, Professor of Microbiology

9/29/95
Date

TO MY MOTHER, JOYCE FISHER PIERCE

THANK YOU FOR YOUR LOVE AND SUPPORT

TO MY FATHER, LT. COL. RAYMOND L. PIERCE

HERE IT IS, DAD

TO BRUCE AND CRAIG PIERCE

THANKS FOR EVERYTHING

ACKNOWLEDGEMENTS

I would like to thank the Graduate School for my Graduate School Teaching, Research, and Dissertation Fellowships. I would also like to thank the College of Life Science and Agriculture for three COLSA Summer Teaching Fellowships and an Outstanding Graduate Student Award.

I wish to thank my project advisor Dr. Frank G. Rodgers for his instruction and support during this project. In addition, I would like to extend thanks to my dissertation committee: Dr. Thomas Pistole, Dr. Robert Zsigray, Dr. Aaron Margolin, and especially Dr. Tom Foxall.

During my years here at UNH, I have been involved in projects with undergraduate researchers; I would like to thank all those who worked with me over these five years. However, I would especially like to thank those whose friendship has continued to support me following completion of their projects: Bob Gibson, Vasu ɔSid Maganti, Alex Hoffmaster (his twin), Patricia Daggett, Kevin Richard, and Diane Emery.

Many people have assisted me at various times during my sojourn at this school; two that stand out in my mind are Robert Mooney and Carol King. Thank you both for saving my sanity, especially during the final months of this project. Thanks also to all the graduate students who have given me little ɔboosts along the way: Holly Veno, for help with the mice and the time when I was ɔhomeless ; Norman Moore, for help with keeping on track; and Frank Gibson, for his sense of humor when I was stressed out. I d also like to thank Richard Melanson and Michael Esposito for their support along the way.

Lastly, I thank my parents Joyce Fisher Pierce and Lt. Col. Raymond L. Pierce (Rtd.), and my brothers Craig and Bruce Pierce for their support, love, and encouragement. Completion of my Ph.D. was my father s last wish; I am sure he will know that it is done.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

CHARACTERIZATION OF THE ADHERENCE MECHANISMS OF *LISTERIA MONOCYTOGENES* TO HOST CELLS AND THEIR ROLE IN THE PATHOGENESIS OF LISTERIOSIS

by

Marcia M. Pierce
University of New Hampshire, December, 1995.

Listeria monocytogenes is the facultative intracellular pathogen responsible for causing the foodborne illness known as listeriosis. Disease occurs following invasion of cells of the intestinal tract by the organism following consumption of contaminated foods. In this study, a mechanism of opsonin-independent attachment of *L. monocytogenes* to host cells was identified. A clinical isolate of *L. monocytogenes*, together with murine-derived primary peritoneal macrophages were used to study this adherence phenomenon.

Transmission and scanning electron microscopy were used to visualize the attachment and uptake of *L. monocytogenes* by murine macrophages, while organism enumeration by viable bacterial cell colony counts was used as a measure of intracellular replication in host cells. *L. monocytogenes* was

shown to adhere to host cells in the absence of complement and a complete infectious cycle for the organism was found. These data confirmed previously identified stages of host cell infection by *L. monocytogenes* in other cell types, including adherence, uptake, phagosomal escape, and intracellular replication.

The nature of the *L. monocytogenes* bacterial ɔadhesin and the host cell ɔreceptor in binding studies was investigated. The results from competitive binding studies indicated the involvement of N-acetyl neuraminic acid, a member of the sialic acid group, in the binding of *L. monocytogenes* to murine peritoneal macrophages. Identification of the host cell receptor was not possible, as most treatments were ineffective at preventing organism binding.

Monoclonal antibodies directed against complement receptor 3 were used in blocking studies and these indicated that CR3 is not involved in the recognition of *L. monocytogenes* by nonlistericidal, thioglycollate-elicited macrophages. These results indicated the importance of opsonin-independent binding mechanisms for *L. monocytogenes* and shed new light on our understanding of the infectious processes of this pathogen.

SECTION 1

HISTORICAL PERSPECTIVE OF *LISTERIA MONOCYTOGENES* AND FOODBORNE LISTERIOSIS

1.1 Background

Listeria monocytogenes is the causative agent of the often severe disease listeriosis. The disease occurs as sporadic cases or as epidemics of foodborne disease and constitutes a significant medical problem in pregnant and immunocompromised individuals. Clinical presentation of listeriosis ranges from mild, flu-like symptoms to severe complications such as meningitis and encephalitis, septic abortion, and septicemia (8).

L. monocytogenes has the ability to infect the central nervous system (CNS), leading to a severe disease which often requires prompt medical attention, with a high mortality rate of 20 to 50%. Infections of the CNS by this organism usually results in neurological sequelae among survivors (8). Predisposing factors which may contribute to the contraction of listeriosis include those that lower cell-mediated immunity, such as tissue transplants, lymphomas, and acquired immunodeficiency syndrome (AIDS) (7,8,27,54,87) .

1.2 Characteristics of *L. monocytogenes*

1.2.1 Morphology

Listeriae are gram-positive bacilli that appear microscopically as nonbranching, regular, short rods (0.4 to 0.5 μm in diameter by 0.5 to 2 μm in length) with rounded ends (8,44). In normally sterile body fluids such as cerebral spinal fluid (CSF) or amniotic fluid, listeriae may be confused with streptococci or corynebacteria when gram stained and directly examined (8,44). Smears from 15 to 24 h colonies show typical diphtheroid palisade formation and a few diplococcoid forms (44). Coccoid forms are encountered most often in smears from infected tissue or broth cultures (44).

L. monocytogenes has characteristic tumbling motility which is temperature dependent and occurs only when the organism is grown at temperatures between 20 and 25°C (44,81). Organisms possess one to five peritrichous flagella, and the individual monomeric flagellin proteins have a molecular mass of 29kD, as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (81). *L. monocytogenes* is serotyped according to possession of certain somatic or 'O' antigens together with flagellar or 'H' antigens as demonstrated using homologous and heterologous absorbed antisera (81).

1.2.2 Taxonomy and Nomenclature

Previously classified in the family *Corynebacteriaceae*, the genus

Listeria is now included with the staphylococci and streptococci in the *Clostridium-Lactobacillus-Bacillus* branch of the gram-positive phylogeny. This group shares such common features as a low G+C DNA content (<50%), lack of mycolic acids, and presence of lipoteichoic acids in the cell wall (8). Analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA, however, indicates that it is phylogenetically remote from the genus *Lactobacillus* and it should not be included in the extended family *Lactobacillaceae* (19). Based on these results, the genus *Listeria* is more closely related to the genera *Brochothrix*, a nonpathogenic group of gram-positive rods (8,19).

On the basis of DNA-DNA hybridization and 16S rRNA cataloging studies, the genus *Listeria* has been divided into seven species, comprising two genomically distinct groups (8). One group consists of *L. murrayi* and *L. grayi*, which are rarely isolated and are considered to be nonpathogenic (8). The second group comprises three hemolytic species, *L. monocytogenes*, *L. seeligeri*, and *L. ivanovii*, and two nonhemolytic species, *L. innocua* and *L. welshimeri*. Of these five species, only *L. monocytogenes*, and on rare occasions *L. ivanovii*, are human pathogens. *L. ivanovii*, however, is a serious pathogen in animals (8).

Serotyping of *Listeria* species is based on 5 heat-labile flagellar 'H' antigens and 14 heat-stable somatic 'O' antigens (8). There are 16 serovars

recognized, of which only one is species-specific. Eleven serotypes of *L. monocytogenes* have been identified based on these O and H antigens; these are 1/2a, 1/2b, 1/2c, 3a, 3b, 4a, 4b, 4c, 4d, and 4e (95). Non-pathogenic *Listeria* species share one or more common antigens with *L. monocytogenes*; this limits the usefulness of serotyping without complete species identification (8). However, three serotypes (1/2a, 1/2b, and 4b) represent more than 90% of those *L. monocytogenes* strains isolated from human and animal sources (8).

Lipoteichoic acid (LTA), a component of the bacterial cell wall of *L. monocytogenes*, has been recognized as an activator of mammalian cells in general and macrophages (MØs) in particular (57). LTA has been isolated and characterized in various serotypes of *L. monocytogenes*. Indeed, analysis by gas chromatography following acid hydrolysis indicated that glycerol, galactose, and glucose are found in LTA from all strains (91). Glucose is present in low quantities only, whereas glycerol and galactose appeared in substantial amounts (91). The LTA of *L. monocytogenes* exhibits structural analogies with the LTA from other bacteria, including *Lactobacillus fermenti*, and in streptococcal strains classified in the genus *Lactococcus*(91). However, the glycosyl portions of the lipid anchors of LTA differ in *L. monocytogenes* from those of *Staphylococcus aureus, Streptococcus*, and *Bacillus* (91). The structure of LTA is identical in different serotypes of *L. monocytogenes*, suggesting that they do not play a role in the serological differentiation of

Listeria strains (91). Instead, it has been proposed that the different O-antigens found in the genus *Listeria* are derived from other cell wall components such as the structurally diverse ribitol teichoic acids found in the cell walls of different strains (91).

1.2.3 Cultural Characteristics

L. monocytogenes is a non-fastidious microorganism capable of growth on a number of different culture media, including Trypticase soy (T-soy), blood agar (sheep, human, or horse), and nutrient agar. Clinical specimens obtained from normally sterile sites for *Listeria* isolation are usually inoculated directly onto blood agar or into broth such as T-soy, brain heart infusion, or thioglycollate broth (8). Treatment with a 0.25% trypsin solution has been used to assist in the release of intracellular *L. monocytogenes* from clinical samples, thereby decreasing the time required for culture of the organism (4). Cold enrichment techniques can also be used for isolation of *L. monocytogenes*, although this is not suitable for rapid diagnosis as the process requires several weeks of culture (8).

Following cultivation on blood agar, *L. monocytogenes* appears as small, round, smooth and translucent colonies which after 24 h of growth measure <1mm in diameter (8). A narrow zone of beta-hemolysis is visible when individual colonies are removed from the plate (8). This narrow zone is in marked contrast to *L. ivanovii*, which produces a marked zone of beta-

hemolysis surrounding the colonies. Continued growth to 48 h results in an increase in size to 1-2mm in diameter and a more opaque appearance.

Specimens obtained from nonsterile sites or from potentially contaminated foods may be processed by using an enrichment technique and the use of selective media which inhibits competing flora (8). The U.S. Department of Agriculture Food Safety and Inspection Service utilizes a two-stage enrichment procedure involving nalidixic acid and acriflavin as inhibitory agents to suppress growth of competitive flora (8). This procedure involves incubation of samples at 30°C for 24 h in a primary enrichment broth and subculturing to a secondary enrichment medium under identical conditions. Subsequently, the enrichment culture is streaked onto LPM agar, a highly selective medium containing phenylethanol and two inhibitory agents, lithium chloride and the antibiotic moxalactam (8,48). LPM agar is a modification of the McBride selective agar used for isolating *Listeria* species. After 1 to 2 d of growth on this or on other clear medium *Listeria* colonies can be recognized using the Henry illumination method (8,48). This illumination method involves oblique lighting of colonies set on a dissecting scope by a concave mirror placed at a 45° angle to the light source, resulting in the colonies appearing pale blue (young colonies) to white (older colonies) (8,61).

1.2.4 Biochemical Properties and Identification

The principle tests used to identify *Listeria* species are given in Table 1 (8). These tests include: 1) inoculation of the cultures onto blood agar for the detection of beta hemolysis; 2) the CAMP test utilizing *Staphylococcus aureus* and *Rhodococcus equi* to distinguish between *L. monocytogenes, L. innocua,* and *L. ivanovii*; 3) reduction of nitrate to distinguish between *L. murrayi* and the other members of the genus; and 4) fermentation of the carbohydrates L-rhamnose, D-xylose, alpha-methyl-D-mannoside, and mannitol (8,55). In clinical microbiology laboratories, presumptive identification of *L. monocytogenes* is usually made based on the following tests: tumbling motility of organisms in a wet mount preparation of a *Listeria*-like colony; coccobacilli staining gram-positive; catalase production; "umbrella"-type formation in the subsurface of agar when cells are grown in semisolid media such as sulfide-indole-motility (SIM) media incubated at 25 to 30°C; beta-hemolysis on sheep blood agar and a positive CAMP test with *S. aureus*; esculin hydrolysis; and acid production from rhamnose and alpha-methyl-D-mannoside but not from xylose (8,98). *L. monocytogenes* is most frequently misidentified as a *Streptococcus* species or as a diphtheroid contaminant; differentiation of *L. monocytogenes* must be based on motility, Gram stain morphology, and catalase production (8). Motility also distinguishes *L. monocytogenes* from *Corynebacterium* species.

Table 1. Biochemical identification of *Listeria* spp.

Species	Beta Hemolysis	Camp Test		Acid Production From				Nitrate Reduction
		S. aureus	*R. equi*	Rh	Xy	MDM	Man	
L. monocytogenes	+	+	-	+	-	+	-	-
L. ivanovii	++	-	+	-	+	-	-	-
L. seeligeri	-	+	-	-	+	-	-	-
L. innocua	-	-	-	(+)	-	+	-	-
L. welshimeri	-	-	-	(+)	+	+	-	-
L. murrayi	-	-	-	V	-		+	+
L. grayi	-	-	-	-	-		+	-

+ = positive reaction; - = negative reaction; (+) = slow or weak positive reaction; V = variable results; Rh = L-Rhamnose; Xy = D-Xylose; MDM = alpha-methyl-D-mannoside; Man = Mannitol

1.2.5 Genetics and Molecular Biology

Until recently, studies on the pathogenesis of *L. monocytogenes* were limited to investigations of bacterial effects on the host cell and interactions with the host immune system. Development of techniques to study the cell biology of this facultative intracellular pathogen and the bacterial determinants of pathogenicity has led to a greater understanding of the mechanisms by which *L. monocytogenes* infects and reproduces within host cells. These techniques include the development of primary and explant cells together with established cell lines for use as models of cellular infection. Techniques of molecular genetics have also been applied to *L. monocytogenes* in order to clarify aspects of pathogenicity. In particular the transposable elements Tn*1545*, Tn*916*, and derivatives of Tn*917* have been used to generate

mutants with reduced virulence in order to study aspects of the infectious process (15,39,84,104). Other techniques which have been used in the determination of virulence genes include the transformation of plasmid DNA; the use of vectors which permit complementation, allelic exchange and site-specific plasmid integration, and finally, the use of *Bacillus subtilis* and *L. innocua* as hosts for the expression of *L. monocytogenes* genes (82,84). Through the use of these tools, the molecular basis for the infectious processes of *L. monocytogenes* has been determined and the individual genes responsible for bacterial virulence have been identified (84).

Those major genes which have been identified from *L. monocytogenes* that are involved in pathogenesis include: *hly*, the gene coding for the toxin listeriolysin O; *plcA*, encoding a phosphatidylinositol-specific phospholipase C; *plcB*, a lecithinase gene; *mpl*, encoding a metalloprotease; *actA*, the gene encoding a protein located on the bacterial surface, necessary for *L. monocytogenes* actin assembly; *prfA*, encoding a protein which functions as a positive regulatory factor, upregulating transcription and translation of several proteins involved in virulence; *inlAB*, the operon responsible for production of internalin, a protein required for invasion of epithelial cells; and *iap*, encoding a 60kDa protein which is involved in separation of cells following replication (Fig. 1, Table 2) (84). It has been shown that, in a manner similar to other facultative intracellular

pathogens such as *Shigella* and *Yersinia* species, the induction of expression of virulence genes in *L. monocytogenes* is thermoregulated and occurs at temperatures above 30°C (63). Therefore, expression of these genes is limited under environmental conditions of 15-25°C but is upregulated at temperatures such as 37°C, as when the organism enters the host (63).

The *L. monocytogenes* hemolysin, listeriolysin O (LLO), is a sulfhydryl-activated, pore-forming cytolysin whose most likely role in virulence enhancement is the lysis of bacterium-containing phagosomes within infected cells (42,70,80,84). Listeriolysin O is a 60kDa protein which is secreted by the organism and shows maximum cytolytic activity towards erythrocytes at lower pH (~5.5) (40,42). The lytic activity of the hemolysin may therefore be increased inside the acidic microenvironment of the phagosome (22,40). Through gene complementation studies, the hemolysin has been shown to be essential for the virulence of the organism in murine listeriosis (22,89). Mutants which are LLO-negative are unable to escape the host phagosome and this results in an inability to replicate intracellularly (22,39,42,84). The gene *hly* was cloned into an asporogenic mutant of *B. subtilis*, which expressed and secreted LLO. After the clone had been internalized by the J774 mouse macrophage-like cell line, it lysed the phagosomal membrane and replicated freely in the host cell cytoplasm (84).

Table 2. Virulence genes of *L. monocytogenes*.

Gene	Gene Product (Protein)	Hypothesized Function	References
hly	Listeriolysin O	Lysis of phagosome	(15,22,39,70,84)
plcA	Phosphatidylinositol-specific phospholipase C	Escape from phagosomes	(14,16,79,84,96)
plcB	Lecithinase	Lysis of double-membrane vacuole	(84,108)
mpl	Metalloprotease	Processing of lecithinase	(28,69,84,85)
actA	Surface protein	Required for actin assembly	(10,60,78,84)
prfA	Positive regulatory protein	Upregulation of transcription of *hly*, *plcA*, *mpl*, and *plcB* genes	(9,17,29,35,36,64,101)
inlAB	Internalin	Ability to invade epithelial cells	(37,84)
iap	Protein p60	Separation of cells following division	(42,46,59,84,111)

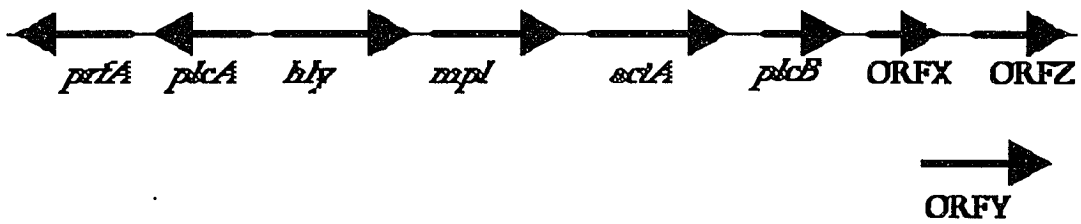

Figure 1: Virulence genes of *L. monocytogenes* listeriolysin gene (*hly*) and the two adjacent operons: the *plcA-prfA* operon and the lecithinase operon. *prfA* encodes a positive regulatory factor, *plcA* encodes a phosphatidylinositol-specific phospholipase C, *mpl* encodes a metalloprotease, *actA* encodes a surface protein necessary for actin assembly, and *plcB* encodes a lecithinase (84).

L. monocytogenes carries at least two genes coding for different phospholipase C enzymes: *plcA* and *plcB*. The first, *plcA*, encodes a phosphatidylinositol-specific phospholipase C which is involved in mediating escape of *L. monocytogenes* from phagosomes of primary murine MØs (16,43). Transposon mutagenesis within this gene was believed to cause loss of readthrough transcription from *plcA* into the downstream gene *prfA*, the positive regulatory gene (Fig. 1) (16,84). This in turn resulted in a lowered transcription of these virulence genes and subsequently a lowered virulence was seen in these mutants (16,84). However, construction of an in-frame deletion within *plcA* had no effect on expression of *prfA*, allowing the identification of the role the phosphatidylinositol-specific phospholipase C plays in pathogenesis (16,79). The gene *plcB* encodes two polypeptides each of 29 and 32kDa which hydrolyze phosphatidylcholine (lecithin) (84). It has been suggested that these lecithinases may be involved in lysis of the double membrane vacuole which is formed during cell-to-cell spread (84,108) .

The gene *mpl* encodes a protein which shares from 40 to 47% amino acid homology with metalloproteases produced by *Bacillus subtilis, B. thermoproteolyticus*, and *B. stearothermophilus* (28,69,84). Mutants with transposon insertions in *mpl* show both reduced virulence and reduced lecithinase production (84). It has been suggested that the metalloprotease may proteolytically process the lecithinase, as mutants in *mpl* produce only

the larger (32kDa) form of the lecithinase polypeptide, and the 29kDa polypeptide is not seen (84,85).

The gene *actA* encodes a 90kDa bacterial surface protein necessary for *L. monocytogenes* actin assembly (84). Mutations in this region resulting in ActA defective strains do not nucleate the polymerization of actin filaments and do not express lecithinase (84). The regulatory gene *prfA* is believed to encode a regulatory factor for *hly*, *plcA*, *mpl*, and *plcB*, as mutants with interruptions in the *prfA* gene or its promoter region were defective in expression of these gene products (9,17,35,64,84,101). This *prfA* gene can be expressed from either its own promoter in the *plcA-prfA* intergenic region or from the *plcA* promoter, suggesting that it regulates its own synthesis (9,17,30,35,36,84). However, loss of one of the two promoters through mutation did not result in lessened virulence in either an animal model or in cell culture models of infection, suggesting that the two *prfA* promoters are functionally redundant *in vivo* (35).

Internalin, the 80kDa protein encoded by the *inlA* gene, has been shown in cloning studies to confer the ability to invade epithelial cells upon the noninvasive species *L. innocua* (37,84). A second protein involved in invasion is p60, encoded by the *iap* gene (11,59,111). This protein is involved in the separation of cells following division, as mutants in this gene form long chains which possess double septa between the individual cells but

which are incapable of division (46,84,111). These mutants show a decrease in invasiveness as well (84,92).

1.3 Pathogenesis

The infectious processes of *L. monocytogenes* following entry of the organism into the host cell has been extensively investigated and has led to several breakthroughs not only in defining pathogenesis of the resultant infection but also in furthering our understanding of the eukaryotic cell and its functions during disease. The natural route of entry for *L. monocytogenes* is the gut; organisms are ingested, enter the intestinal tract, and adhere, invade, and replicate in Kupffer's cells in the host liver (68). Under normal circumstances circulating bacteria are cleared from the bloodstream within 15 min by resident MØs in sinusoidal organs, especially the spleen and liver (73).

Adherence of *L. monocytogenes* to a host cell is a necessary prelude to infection. Recent studies indicate that uptake through different cellular receptors results in very different intracellular fates for the organism (31,32). Two mechanisms of uptake of *L. monocytogenes* have been reported: opsonin-dependent via the complement receptor 3, resulting in bacterial killing, and opsonin-independent through a second unidentified receptor, resulting in parasitism of the host cell by the organism (31,32).

L. monocytogenes adheres to host cells via one of these two receptors and undergoes phagocytosis, escapes the phagosome by means of listeriolysin

O and phosphatidylinositol-specific phospholipase C, enters the cytoplasm, and initiates the polymerization of the cell actin through ActA (3,78,80). A "halo" of actin filaments surrounds the organism during replication and after the listeriae divide, the halo becomes asymmetric, forming a tail or "comet" at one end of the bacterium (78). As the tail elongates, the bacterium is pushed towards the MØ surface by the densely packed actin filaments at the tail of the comet (78). The ActA polypeptide is required for initiation of the actin accumulation but is not directly involved in the generation of the actin tail (78). Mutants in this gene show a reduction in virulence for animal and cell culture models (10). When the organism reaches the host membrane, a pseudopod-like extension is formed that touches an adjacent cell, triggering a phagocytic response and initiating uptake of the bacterium into a double-membraned vacuole. The organism then lyses the phagosome and escapes as described earlier, and the cycle commences again. In this manner, *L. monocytogenes* avoids contact with the humoral immune system of the infected host (Fig. 2).

Experimental murine listeriosis has served as an excellent model for the study of facultative intracellular organisms and cell-mediated immunity in infection (68). Three stages of murine infection are seen when *L. monocytogenes* is injected intravenously into mice: first, 90% of the inoculum is cleared from the blood by liver Kupffer cells and within 6 h,

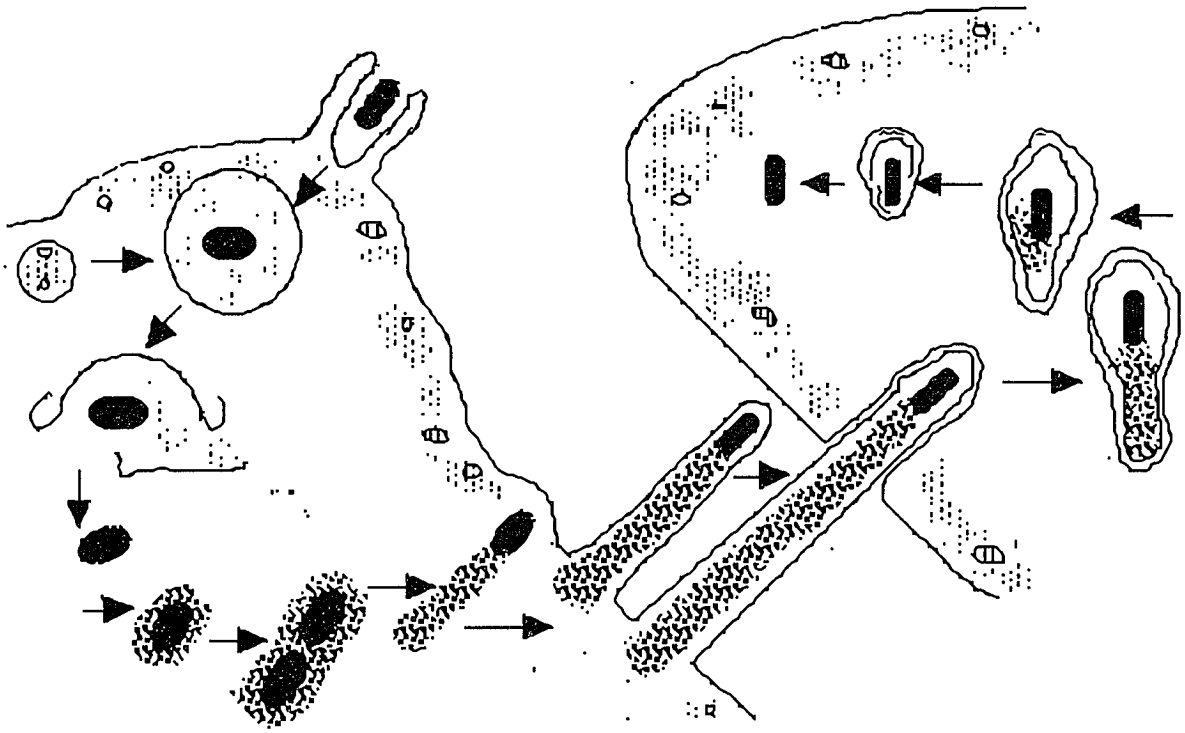

Figure 2: Pathogenesis of *L. monocytogenes* in host cells: stages in the entry, growth, movement, and spread of *Listeria* from one MØ to another (106).

90% of these bacteria are killed; and second, despite focal accumulations of polymorphonuclear granulocytes (PMNs) followed by MØs during the next 2-3 d, those residual organisms grow abundantly. Finally, quenching of the infection is triggered by T-cell activation of MØs (68). Detectable numbers of listeria-specific T cells become apparent on day 4 to 5 of the primary infection, and it would appear that a sustained release of tumor necrosis factor (TNF) and interferon-γ (IFN-γ) mediated by CD4+ cells is the focusing event stimulating MØ accumulation (73).

However, the eradication of bacteria that occurs during this time critically depends on CD8+ and/or CD4- CD8- Thy1+ cells (73). As yet, the effector functions of these T cells remain obscure, since cytokines have yet to be identified that will substitute for their presence (62,73). Additionally, the depletion of CD4+ T cells, resulting in a dramatic reduction of cytokines including interleukin-2 (IL-2), IL-3, IL-4, IFN-γ, and TNF, does not appear to have an effect on the animal's ability to eradicate high lethal doses of bacteria (62,73).

T-cell independent MØ activation has been shown to occur in mice with severe combined immunodeficiency syndrome (SCID); these mice have been shown to be relatively resistant to *L. monocytogenes* infection (6). This T cell-independent process involves macrophages and natural killer (NK)

cells, and the cytokines IL-1, TNF, and IFN-γ (6). MØs infected with *L. monocytogenes* release IL-1 and TNF, which in turn induce NK cells to release IFN-γ (6). The MØs respond to IFN-γ by producing large quantities of nitric oxide and thereby reducing the bacterial load (6). Both SCID and immunocompetent control mice showed increased mortality and spleen bacterial loads when the production of nitric oxide was inhibited *in vivo* (6). Therefore, the T cell-independent pathway of macrophage activation is essential for both the SCID and immunocompetent host to survive infection by *L. monocytogenes* (6).

1.4 Epidemiology of Listeriosis

1.4.1 Transmission

Listeriosis frequently occurs in pregnant individuals, both human and ruminants, in immunosuppressed individuals, and in those at the extremes of age (95). Outbreaks of this disease that occurred in the 1980s established that epidemic listeriosis is a foodborne disease (95). Recent studies suggest that a substantial portion of sporadic cases may be foodborne as well (95). Of the three major groups of individuals most susceptible to infection, disease has increased most rapidly in those having suppressed immune systems. This may reflect an increase in disease in these individuals or, more likely, has resulted from an increasing use of immunosuppressive drugs for the

treatment of cancers as well as to prevent rejection in organ transplantation recipients (95). Increase of disease has also increased concomitantly with the acquired immunodeficiency syndrome epidemic (7,27,54,95,99).

The number of cases of listeriosis which occur worldwide has been difficult to determine. In the United States, voluntary reporting to state health departments and analysis of hospital discharge data have been used to attempt to achieve an estimate of the incidence of disease (95). However, because listeriosis is not a notifiable disease in the U.S., these estimates are likely to be highly inaccurate and reflect a major underestimate of the true rate of disease. Table 3, adapted from the Bulletin of the World Health Organization, shows the reported incidence of listeriosis in 1990 in Europe and North America (87). From these results, the following conclusions were drawn: (1) human listeriosis is reported mainly in industrialized countries; (2) most of the data is obtained from voluntary reporting, which is likely to underestimate the true number of cases; (3) although uncommon, human listeriosis carries a high mortality rate, despite effective treatment (5-33%; mean 22%); and (4) there is a need for additional surveillance and epidemiological investigation into the sources of human listeriosis (87).

Table 3. Human listeriosis in 1990 in Europe and North America: number of cases recorded, incidence, mortality rate, and sex distribution

Country	No. of cases	Incidence (per 10^6 population)	Mortality rate (%)	Sex M	Sex F
Europe					
Belgium	25	2.5	-	17	8
Bulgaria	2	-	-	1	1
Czechoslovakia	4	-	-	-	4
Denmark	41	7.0	27	21	16
Finland	30	6.0	23	17	13
France	326	6.2	-	149	156
Germany					
(formerly GDR)[a]	71	4.4	-	27	43
(formerly FRG)[b]	24	-	-	-	-
Greece	0	-	-	-	-
Hungary	10	1.0	-	-	-
Italy	12	3.5	-	6	6
Netherlands	29	1.9	-	-	-
Spain	24	0.6	-	15	9
Sweden	38	-	5	18	20
Switzerland	14	2.1	21	7	6
Turkey	2	-	-	1	1
United Kingdom	131	2.2	33	58	73
Yugoslavia	35	1.4	12	4	28
North America					
Canada	50	1.8	8	25	25
USA[c]	147	7.4	19	75	72

[a] GDR = German Democratic Republic
[b] FRG = Federal Republic of Germany (only neonatal cases)
[c] Surveillance population = 19 million

1.4.2 Epidemics

An outbreak of listeriosis occurred in Nova Scotia in 1981 and this provided the first conclusive evidence for transmission of this disease through contaminated foods (Table 4) (95). Seven adult and 34 perinatal cases

of listeriosis occurred in this outbreak, including 5 spontaneous abortions and 4 stillbirths; the case fatality rate among liveborn infants was 27% (95). This outbreak was traced back to coleslaw which had been prepared from cabbages grown on land fertilized with raw sheep manure. The cabbages were stored over the winter which enhanced the growth of the organism through cold enrichment (12). The causal type was subsequently found to be *L. monocytogenes*, serotype 4b (12,95). The outbreak investigation documented

Table 4. Foodborne outbreaks of listeriosis.

Outbreak	Number of Cases	Case Fatality Rate	Food Implicated
Nova Scotia, 1981	41	27% (liveborn infants)	Coleslaw
Boston, 1979	20	NR	Raw vegetable
Massachusetts, 1983	40	29% (overall rate)	Milk
Los Angeles, CA, 1985	142	63% (neonatal) 37% (nonneonatal)	Soft cheese
Canton of Vaud, 1987	ND	ND	Soft cheese
Philadelphia, PA, 1987	36	44% (overall rate)	ND

NR=not reported; ND=not determined; (95,97)

that listeriosis is a foodborne disease and suggested potential problems associated with consumption of uncooked vegetables (95). Interestingly, nonpregnant adult patients in this outbreak had no evidence of underlying medical conditions or immunosuppression (95). Another outbreak of listeriosis which may have been due to consumption of uncooked vegetables occurred in Boston in 1979 and although no single source was determined for the outbreak, a raw-vegetable garnish consumed by patients was suspected to be the vehicle of transmission (95). The same serotype 4b was found in all cases (95).

A second large outbreak in Massachusetts occurred in 1983, with most cases occurring in nonpregnant adults who had received immuno-suppressive therapy (95). This outbreak was attributed to contaminated whole or 2% fat milk, which may have been contaminated post-pasteurization. The overall case fatality rate for this outbreak was 29% (95). Serotype 4b accounted for 32 of the 40 isolates that could be tested (12,95).

The largest epidemic of listeriosis in North America occurred in 1985 in Los Angeles, CA, and was caused by consumption of a Mexican-style soft cheese (18,95). Eighty-seven percent of pregancy-associated cases occurred in Hispanic women; serotype 4b was isolated from these patients or from their neonates (12,65,95). The same serotype was subsequently isolated from unopened packages of this cheese and from environmental samples taken

from the cheese factory (65,95). Most cases occurred in pregnant women or their immediate offspring. Perinatal cases included 87 infants either stillborn or with early-onset infections and 6 late-onset cases (18,95). The case fatality rate was 63% for early neonatal or fetal infections, and 37% for nonneonatal infections (18,95). For these investigations, the incubation period for listeriosis was determined to range from 11 to 70 d, with a median of 31 d, and a striking feature for this disease was that the interval between eating contaminated food and the onset of symptoms appeared to be much longer than for other foodborne diseases (18,95). A further outbreak due to serotype 4b in soft cheese occurred in Europe in 1987 and was centered in Switzerland in the Canton of Vaud (12,13,95). The cheese was a regionally produced food product which was distributed to several countries in Europe (13,95).

In the same year, an outbreak of listeriosis occcurred in Philadelphia, Pennsylvania, which was unusual in that multiple strains of *L. monocytogenes* were involved, and most patients were nonpregnant (32 of 36) (97). Investigation into the source of the outbreak indicated no single food product; patients were more likely to have eaten ice cream or salami prior to disease onset, but no single brand of either was implicated (95,97).

It is apparent, from the outbreaks described above, that listeriosis will continue to be a medical concern in the U.S. as well as in other industrialized countries where food is stored at low temperatures for long periods of time.

1.5 The Disease

1.5.1 Clinical Presentation

Invasive disease in nonpregnant adults is usually associated with some form of immunosuppression, including organ transplant cases, individuals with malignancies, the elderly, and AIDS patients (13,18,95,99). In a study of sporadic listeriosis cases occurring in 1986 to 1987 in a geographically diverse population in the U.S., 88% of nonperinatal cases occurred in individuals with one or more underlying diseases (95). Nonpregnant adults with listeriosis most often present with meningitis, meningoencephalitis, or sepsis (Table 5) (18,27,54,95,99). Additional syndromes include abscesses of the brain and spinal cord, endocarditis, endophthalmitis, osteomyelitis, and septic arthritis (7,95). Fever, seizures, depressed consciousness, and altered mental status are frequent symptoms of central nervous system listeriosis (7,95).

Listeriosis may develop at any time during pregnancy, although diagnosis occurs most often in the third trimester (18,95). Infections occurring earlier in pregancy may not be recognized if *L. monocytogenes* is not cultured; indeed, failure to culture the products of conception when spontaneous abortion or stillbirth occurs complicates the problem of estimating the proportion of fetal loss that may be attributable to listeriosis (95). Pregnant women infected with *L. monocytogenes* may experience flu-like symptoms, with fever, headache, and occasionally gastrointestinal symptoms (8,95).

Intrauterine infection can occur either from maternal bacteremia or from ascending spread from vaginal colonization with *Listeria* species (8,95). Amnionitis, preterm labor, spontaneous abortion, stillbirth, or early-onset infection of the neonate are associated with intrauterine infection (8,95). Antibiotic treatment during pregnancy can prevent neonatal illness (95).

Neonatal listeriosis occurs as two distinct syndromes, early-onset and late-onset disease, similar to infection of neonates by group B streptococci (95). Early-onset listeriosis results from intrauterine infection, causes clinical illness in the newborn at birth or shortly thereafter, and presents as sepsis or granulomatosis infantisepticum (95). Meningitis is rare in early-onset infection (95). Late-onset disease occurs several days to weeks after birth, and usually presents as meningitis (95). A higher fatality rate has been reported for early-onset than for late-onset listeriosis (95).

Table 5. Symptoms of listeriosis.

Individual with disease	Possible symptoms
Nonpregnant	Mild, flu-like symptoms Meningitis Encephalitis Septicemia Possible neurological sequelae in survivors
Pregnant	Mild, flu-like symptoms Influenzalike bacteremic illness Amnionitis and infection of fetus Abortion Stillbirth Premature birth of infected infant
Neonatal (Early-onset)	Sepsis Granulomatosis infantisepticum Meningitis (rare)
Neonatal (Late-onset)	Meningitis CNS infection

1.5.2 Diagnosis

Listeriosis is diagnosed when *L. monocytogenes* is cultured from blood, spinal fluid, or from another normally sterile body site (95). Direct detection of gram-positive rods in these specimens is of particular value in early diagnosis and treatment of disease; however, it is sometimes difficult to locate the organism in these samples (8,95). Therefore, cultures are necessary for

complete identification (8,95). Spinal fluid examination may show pleocytosis with predominantly PMNLs; the protein level is elevated, and the glucose level is usually at normal levels (95).

In perinatal cases, isolation of the organism from nonsterile specimens such as the placenta or amniotic fluid following birth or spontaneous abortion can be indicative of perinatal listeriosis (95). *L. monocytogenes* may be carried in the gastrointestinal tract; therefore, culture of the organism from stool samples is not particularly helpful in diagnosis (95). Additionally, serological tests do not contribute to diagnosis of listeriosis because patients with cultured *L. monocytogenes* often have undetectable levels of antibody against the organism (95).

1.5.3 Antimicrobial Chemotherapy

L. monocytogenes is susceptible to penicillin, ampicillin, gentamicin, erythromycin, tetracycline, and rifampicin *in vitro*; however, many of these antibiotics are bacteriostatic *in vivo* (8,50,66). This variability of antimicrobial effect upon listerial infections may be due in part to the intracellular residence of *Listeria* (50,72). Erythromycin produces only limited therapeutic effect in the normal mouse and practically no effect in nude mice, possibly due to storage in its inactive form in the lysosomes of the host MØ (50). Rifampicin has been shown to be highly active *in vivo* in both mouse models, as well as in a model of *Listeria* meningitis in rabbits (50).

Tetracyclines are concentrated within eukaryotic cells, but their therapeutic effect in infected mice is limited (50). Penicillin or ampicillin with or without an aminoglycoside is usually recommended for treatment of listeriosis (8,50). *In vivo* studies have shown that an aminoglycoside enhances the antimicrobial activity of penicillin to *L. monocytogenes* (8,66,71). For treatment to succeed, the antibiotic must reach high concentrations within phagocytic cells, it should cross the blood-brain barrier, and should be rapidly bactericidal (8,66). Cephalosporins have been shown to be ineffective and should not be administered if a listerial infection is suspected (8,50).

1.5.4 Comment on Current Status of Disease

Over the past 15 years, this once-obscure disease has received national and worldwide attention due to the dramatic outbreaks which have been documented. However, the incidence of disease can only be estimated, as countless cases go uncounted because this condition is not a notifiable disease in many countries. In addition, the high mortality rates which occur in spite of treatment indicate that rapid diagnosis or actual prevention of disease are vital in all cases.

1.6 Objectives of This Study

The purpose of this study was to investigate and evaluate the attachment of *Listeria* to host cells involving both opsonin-independent and opsonin-dependent processes. Preliminary experimental trials were carried

out to determine the suitability of murine peritoneal MØs as a host cell for the adherence and intracellular replication of *L. monocytogenes*. The MØs were then used as host cells in adherence studies of the organism. By identifying the means by which *L. monocytogenes* adheres to host cells, both in the presence and absence of opsonins, these studies add to the knowledge and understanding of the pathogen and may further assist in the development of new therapies for the treatment and prevention of listeriosis.

SECTION 2

CHARACTERIZATION OF A CLINICALLY ISOLATED STRAIN OF *LISTERIA MONOCYTOGENES*

2.1 Abstract

Listeria monocytogenes is a facultative intracellular pathogen and is the major cause of listeriosis, a severe foodborne disease especially prevalent in pregnant women and immunocompromised individuals. Of sixteen different serovars, three are responsible for 90% of clinical infections: 1/2a, 1/2b, and 4b. As a consequence, careful characterization of clinical isolates which are to be used for research purposes on the pathogenic mechanisms involved in the development of disease is therefore imperative. The strain of *L. monocytogenes* used in the present study was isolated from a clinical case of listeriosis in Rochester, New York. Identification of the isolate as *Listeria monocytogenes* was carried out using the following tests: catalase, bile esculin, hemolysis on blood agar, the CAMP test using *S. aureus*, and acid production from rhamnose, xylose, and mannitol. The serotype of this isolate was determined to be 1/2b by the Centers for Disease Control and Prevention in Atlanta, Georgia. Finally, antimicrobial susceptibilities and biochemical characteristics were determined by New Hampshire Medical Labs

in Manchester, NH as confirmation of results seen at UNH. Growth curve studies were carried out in our laboratory to determine the replication rate of this strain in broth at 37°C. The virulence of the strain was also determined by lethal dose $_{50}$ (LD_{50}) studies in fertile hens' eggs assays.

2.2 Introduction

L. monocytogenes is a pathogenic microorganism which is capable of causing fatality in 30% of all cases of listeriosis. It has been demonstrated that this organism replicates within host cells. During multiplication, the organism moves within the cytoplasm of infected cells using polymerized actin as the means of locomotion and this allows the organism to spread from cell to cell without leaving the confines of the cytoplasm (38,56,106). This ability is critical for the development of disease in humans and cattle, and aids organism survival by allowing it to evade the host's humoral immune system. As a consequence, cell-mediated immunity is vital in the defense mechanisms against this pathogen and in eventual recovery from disease.

Listeriae are ubiquitous in the environment and have been found in soil, sewage, silage, plants, water, and decaying vegetation (8). In addition, soil is believed to be the natural reservoir for the organism. This microorganism has also been isolated in low numbers from raw or processed foods, including dairy products, meat, vegetables, and seafood, as well as from the food-processing environment (8). Identification of the causative agent of

clinical disease is of importance both for the patient as well as for epidemiological investigations for the tracking of the source of disease. Characterization of clinically isolated pathogens is critical to research on pathogenesis carried out using these organisms.

2.3 Materials and Methods

2.3.1 Organism Cultivation

A fully virulent clinical isolate of *L. monocytogenes* was isolated on blood agar, subcultured once on Tryptic soy (T-soy) agar, and stored as stock cultures frozen at -70°C in 1% serum-sorbitol. Thawed samples were plated on agar and incubated overnight. Colonies were harvested, resuspended in T-soy broth to give 51 Klett units using a Klett-Summerson photoelectric colorimeter. 51 Klett units yielded a suspension of listeriae which when serially diluted and plated out gave a count of approximately 4×10^8 colony forming units (CFU)/mL for use as a standardized inoculum.

2.3.2 Biochemical Characterization

Preliminary testing of the isolate was done in the laboratory to determine the presence or absence of these characteristics: catalase, bile esculin, growth on blood agar, CAMP test using *S. aureus*, and acid fermentation from rhamnose, xylose, and mannitol. These tests were carried out as followed:

(1) Catalase: colonies were removed from T-soy agar plates using a

loop, placed on a glass coverslip, and 3% H_2O_2 added. The presence of catalase was indicated by bubbling.

(2) Bile esculin slants were streaked with the organism and incubated overnight; hydrolysis of esculin was indicated by a black growth on the slant.

(3) Hemolysis on blood agar was shown after 24 h of growth; *L. monocytogenes* produces a narrow zone which is best seen by removing the colony from the plate.

(4) The CAMP test was carried out by placing a vertical streak of *S. aureus* on a blood agar plate and then streaking the unknown isolate at a right angle (but not touching) to the *S. aureus*. The hemolysis of *L. monocytogenes* becomes enhanced in the area closest to the *S. aureus* streak, resulting in a characteristic "arrowhead" appearance.

(5) Fermentation from rhamnose, xylose, and mannitol was carried out by inoculation of tubes containing Bacto Phenol Red Broth base (prepared with the appropriate sugars) with the isolate and incubating for 24 h. A positive reaction showed yellow, indicating a change in the pH (by phenol red pH indicator). All assays were performed as outlined in the Manual of Clinical Microbiology, 5th edition (8).

The strain was subsequently sent to the New Hampshire State Health Department Laboratory for determination of its biochemical characteristics

and to the Centers for Disease Control and Prevention (CDC) for serotyping. Antimicrobial susceptibilities of the strain were also determined by New Hampshire Medical Labs in Manchester, NH.

2.3.3 Growth Curves

L. monocytogenes was inoculated from a culture at 51 Klett units to give a concentration of approximately 5 x 10^5 CFU/mL into tubes containing T-soy broth. Tubes were placed on an orbital shaker set at 200 rpm, and incubated at 37°C. Tubes were removed from the shaker at 1 h intervals over an 11 h period. One hundred microliters was removed from the tube, serially diluted in 1% peptone, and 50µL plated in duplicate on T-soy agar. Turbidity of cultures was measured simultaneously on the Klett colorimeter.

2.3.4 Lethal Dose $_{50}$ Studies

Fertile white Leghorn chicken eggs (UNH Poultry Farm) were maintained at 37°C in a humid atmosphere. *L. monocytogenes* was inoculated at approximately 5 x 10^5 CFU/mL into T-soy broth tubes and placed on an orbital shaker at 37°C. Organisms were harvested after 8.5 h, washed three times by centrifugation in 1% peptone, and serial dilutions from 10^{-1} to 10^{-8} prepared. Aliquots of 0.1mL of each dilution was injected into seven-day-old fertile hens' eggs by the yolk sac route (Fig. 3). Experiments were performed using a minimum of 10 eggs per dilution and the results of six separate experiments were averaged (Appendix 7.2) (107). Control eggs were

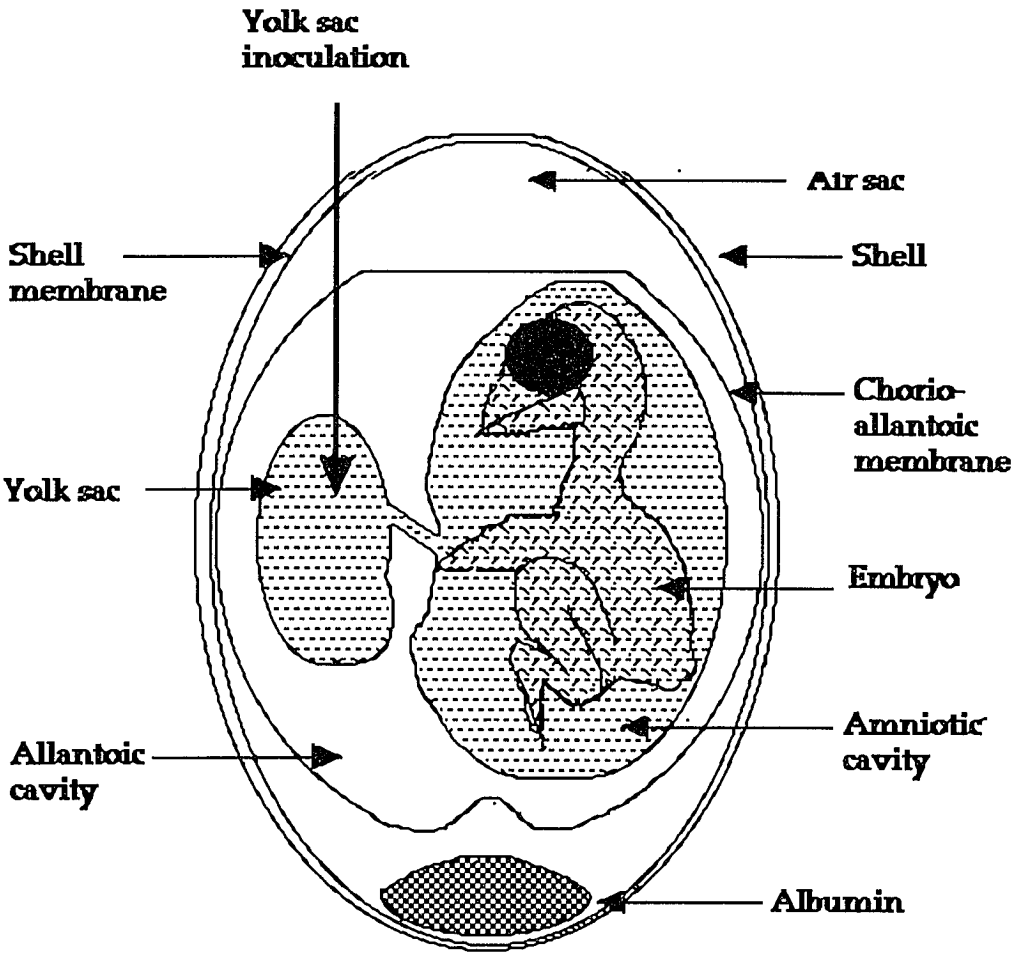

Figure 3: Diagrammatic representation illustrating the various inoculation sites for the fertile hens' egg. The yolk sac route of inoculation was used to determine and monitor virulence of *L. monocytogenes* in this study. Drawing adapted from that made by Dr. Frank C. Gibson, III.

injected with 1% peptone only and maintained up to 8 d post-inoculation. The original CFU/mL for each trial was determined by serial dilution and plating onto T-soy agar plates. Eggs were candled twice daily to determine viability. The lethal dose $_{50}$ (LD_{50}) was calculated by the method of Reed and Muench (1938).

2.4 Results

Biochemical tests performed in our laboratory on the strain isolated in Rochester, NY, from a clinical case of listeriosis, were positive for esculin, catalase, rhamnose but not xylose or mannitol, and showed narrow zones of beta hemolysis on blood agar (Table 6). It was also positive for the CAMP test using *S. aureus.*

Table 6. Biochemical characteristics of the Rochester, NY clinical isolate of *L. monocytogenes.*

Biochemical test	Reaction
Bile esculin	+
Catalase	+
Acid from:	
Rhamnose	+
Xylose	-
Mannitol	-
Beta hemolysis	+ (narrow)
CAMP test with *S. aureus*	+

*All tests were performed in the laboratory at UNH Dept. of Microbiology.

The isolate was determined by the CDC to be *L. monocytogenes* serotype 1/2b. Results from biochemical and antibiotic susceptibility assays as determined by the New Hampshire Medical Labs are shown in Table 7. The strain showed resistance to Ceftizoxime, Cefuroxime, and Clindamycin, while intermediate resistance was expressed to Cefotaxime and Ceftriaxone.

Table 7. Biochemical characteristics and antimicrobial susceptibilities of Rochester, NY clinical isolate of *L. monocytogenes*

Biochemical characteristics (NH State Labs)		Antimicrobial resistance (NH Medical Labs)	
Beta hemolysis on blood agar	+	Amikacin	S
		Ampicillin	S
Catalase	+	Cefamandole	S
		Cefazolin	S
Motility	+	Cefotaxime	MS
		Ceftizoxime	R
Acid reaction from sugars:		Ceftriaxone	MS
Glucose	1	Cefuroxime	R
D-Xylose	NR	Cephalothin	S
Mannitol	NR	Chloramphenicol	S
Lactose	1	Ciprofloxacin	S
Sucrose	2	Clindamycin	R
Maltose	1	Erythromycin	S
L-Rhamnose	1	Gentamicin	S
alpha-methyl-D-mannoside	1	Imipenem	S
		Nitrofurantoin	S
		Norfloxacin	S
		Penicillin	S
		Rifampin	S
		Tetracycline	S
		Vancomycin	S

Acid reaction: 1 = acid in 1-2 d; 2 = acid in 3-7 d; NR = no reaction
Antimicrobial susceptibilities: S = susceptible; MS = moderately susceptible; R = resistant
Results shown in this table were determined by NH State and NH Medical Labs.

The growth rate of *L. monocytogenes* as measured by CFUs is shown in Figure 4. Increasing turbidity of the cultures was measured by Klett units and is shown in Figure 5. This data indicated that the lag phase extended for 6 h post-inoculation and thereafter exponential growth developed. Active growth lasted between 6 and 9 h after inoculation and stationary phase was reached by 10 h. Similar results were obtained when growth was assayed by measuring turbidity of the cultures.

The lethal dose $_{50}$ values for *L. monocytogenes* are shown in Table 8. Calculations from which these figures derive are given in Appendix 7.2. The results of six trials were averaged and the LD_{50} value determined to be approximately 21 CFU per egg for this organism.

Table 8. Lethal dose $_{50}$ values for *L. monocytogenes* inoculated into chicken embryo yolk sac.

Trial number	LD_{50}Endpoints (CFU/mL)
1	8.8
2	6.3
3	35.2
4	37.9
5	2.2
6	33.0

Ten eggs were inoculated per dilution with 0.1mL samples and LD_{50} data were calculated for each experiment. Control eggs were injected with 1% peptone; no death occurred in this group up to eight d post inoculation. The data obtained were averaged to give a mean LD_{50} of approximately 21 CFU/egg.

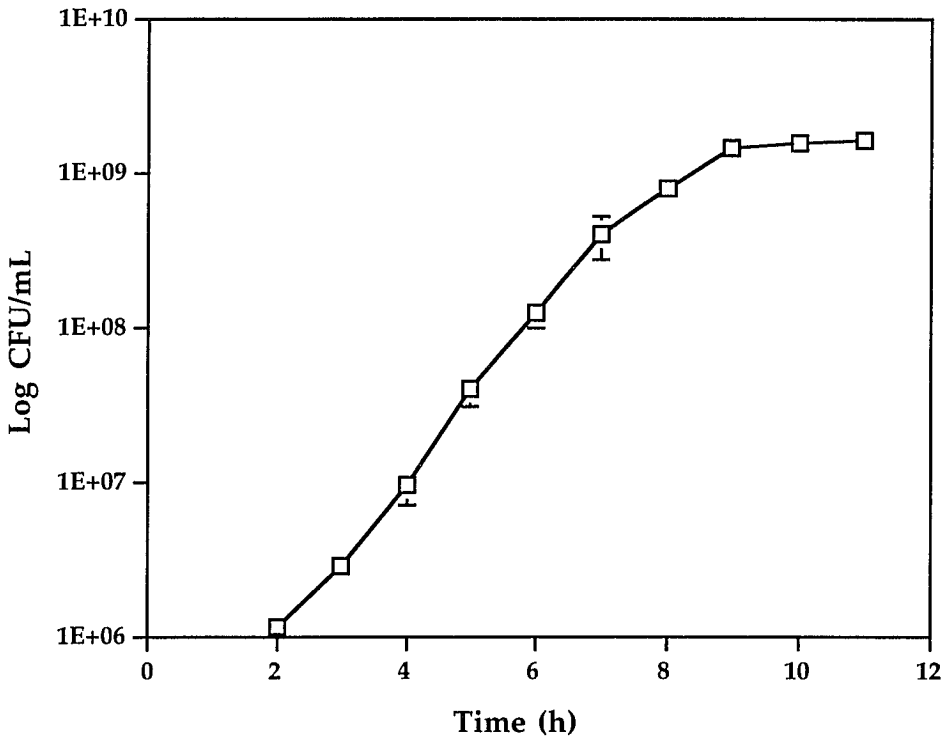

Figure 4: Replication of *L. monocytogenes* in T-soy broth at 37°C as measured by CFUs. Assays were the average of three trials and bars represent standard deviation of the mean.

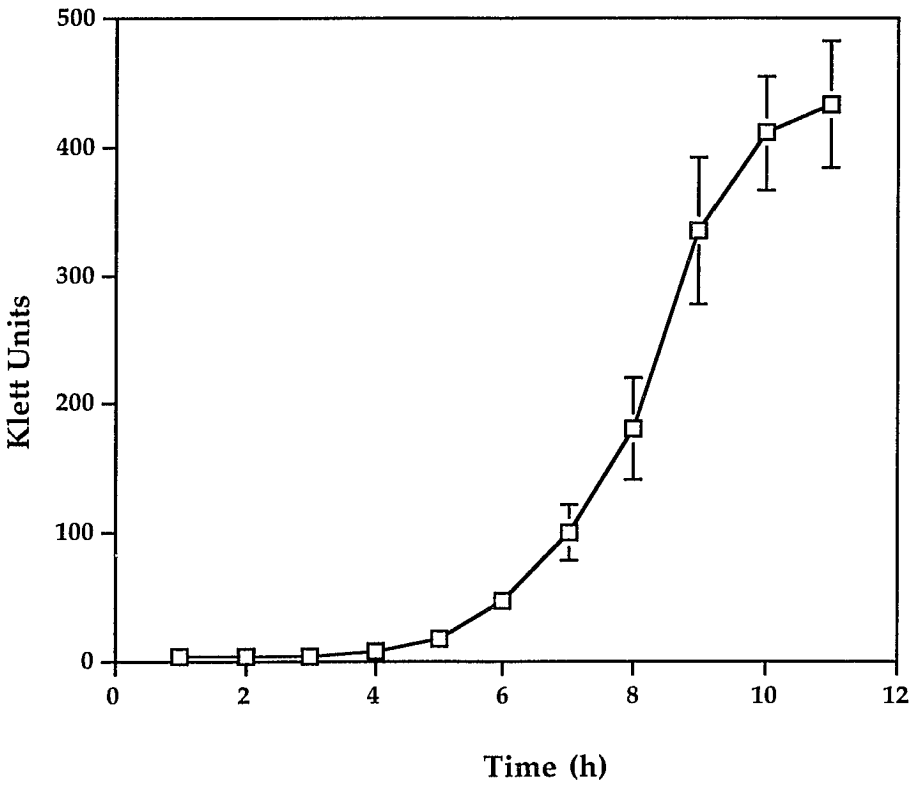

Figure 5: Replication of *L. monocytogenes* in T-soy broth at 37°C as measured in Klett units. Assays were the average of three trials and bars represent standard deviation of the mean.

2.5 Discussion and Conclusions

The work outlined in this section was performed to define the properties of this strain of *L. monocytogenes* prior to its use in more specific aspects of pathogenicity research. These results confirmed that the isolate was *L. monocytogenes*, serotype 1/2b, and defined its characteristics in terms of antimicrobial susceptibilities and biochemical properties. Growth rates of the organism in T-soy broth indicated that exponential growth was reached at 6 h and stationary phase at about 9.5 h. Accordingly, for subsequent experiments cultures were grown for 8.5 h before harvesting. This allowed use of the organism grown under reproducible conditions and giving bacteria at a consistent physiological state, which occurs during the balanced growth of the exponential stage (76). Balanced growth refers to the average behavior of cells in a population and, during this stage, the mean cell size remains constant (76). Therefore, the population is in its most homogeneous state (76). This state may not, however, correspond to that of the infectious organism. The organism may be growing at a slower rate and as a much more heterogeneous population in a refrigerated food source prior to its ingestion by the host. However, the ability to utilize a known number of *Listeria* at a specified time greatly facilitated further experiments.

Virulence of the organism was confirmed and further defined through LD_{50} studies in fertile hens' egg assays. Using the yolk sac route of

inoculation, the organism had a mean LD_{50} of 21 CFU per chick embryo. In contrast, the LD_{50} endpoint for *Legionella pneumophila* N_7 was 6.6×10^2 CFU per egg as measured by Dr. Arthur Tzianabos in our laboratory (107). It was apparent that the clinical isolate of *L. monocytogenes* remained a virulent microorganism during its subculturing onto T-soy agar and that it was, therefore, a highly suitable strain for conducting research on the pathogenesis of *Listeria*. These experiments identified the characteristics of the clinical isolate of *L. monocytogenes* and documented its virulence using the fertile hens' egg assay. Culture of the organism for future studies was defined, and the effectiveness of this strain for the study of the pathogenesis of *L. monocytogenes* was established.

SECTION 3

CHARACTERIZATION OF OPSONIN-INDEPENDENT ADHERENCE, PHAGOCYTOSIS, AND INTRACELLULAR REPLICATION OF *LISTERIA MONOCYTOGENES* TO MURINE PERITONEAL MACROPHAGES

(The work outlined in this Section has been published in abstract form: **Pierce, M.,** F. G. Rodgers, D. M. Slater, and R. E. Gibson. 1993. Early events in the phagocytosis and intracellular replication of *Listeria monocytogenes* in murine peritoneal macrophages. Abst. Gen. Meeting ASM Atlanta, GA: D-49: p. 24.)

3.1 Abstract

Listeria monocytogenes is an environmental bacterium which can also function as a facultative intracellular pathogen that multiplies in cells of the intestinal tract, as well as in peritoneal macrophages (MØs). Recent studies show that the intracellular fate of this organism depends upon the mechanism by which it adheres and is phagocytosed by the host cell. An opsonin-independent process appears to be involved in uptake by nonlistericidal, permissive MØs, whereas an opsonin-dependent process is involved in uptake by listericidal, nonpermissive MØs. In this study, *L. monocytogenes* adhered to and multiplied intracellularly in murine

peritoneal MØs in the absence of opsonins. The infectious process in these cells was evaluated by CFU counts of intracellular organisms and documented by transmission and scanning electron microscopy. Adherence of listeriae to MØs involved surface interactions of the prokaryotic cell surface and eukaryotic cell membranes. Subsequent phagocytosis was seen to occur through a process in which host cell-derived pseudopodia surrounded and engulfed organisms leaving them within phagosomes in the cytoplasm of infected cells. This process of uptake of *L. monocytogenes* by MØs occurred at 4°C. Following invasion of the cell, partial escape of *L. monocytogenes* from the phagosome into the cytoplasm occurred as early as 10 min into the infectious process. Intracellular multiplication of bacteria continued for 8 h after inoculation at which point loss of adherent MØs due to the infectious process was evident. The cellular and ultrastructural events of *L. monocytogenes* adherence to and phagocytosis by murine MØs in the absence of antibody or complement were defined.

3.2 Introduction

Investigation of the processes of intracellular infection by *L. monocytogenes* has served to illuminate the cell-mediated immune response and the processes of inflammation in murine hosts. Establishment of listeriosis in infected individuals is brought about by an initial recognition or attachment event between binding molecules or adhesins on the organism

and receptors located at the host cell membrane surfaces. The process eventually leads to internalization of the pathogen by affected cells. For other pathogens such as *Legionella pneumophila*, this recognition event is initiated by either the host cell or bacterial cell (88). In experimental murine infections, *Listeria* organisms were shown to accumulate primarily in the liver and spleen, replicating within cells of the mononuclear phagocytic system (67) as well as in nonprofessionally phagocytic cells including hepatocytes (20). Intracellular growth of this organism in host cells has been characterized using the transformed human enterocyte-like cell line Caco-2 (75) and J774 macrophage-like cells (84).

Attachment and entry of the bacteria occurs through their apical surfaces and these processes do not appear to be actin dependent (56). After adherence to host cells, bacteria are taken into membrane-bound phagosomes. Subsequently, the organism escapes the phagosome and enters the cytoplasm, where the bacteria replicate (26). Following escape from the phagocytic vacuole, the organism becomes coated with actin filaments forming a large tail or comet-like structure up to 5μm long (106). This organism-actin complex is critical for the intracellular movement of *L. monocytogenes* in that as the actin polymerizes, the pathogen is "pushed" through the host cell cytoplasm. In addition, this phenomenon is essential to cell-to-cell spread of the organism in that as bacteria, under the action of polymerized actin, arrive

at the surface of the host cell, a cytoplasmic pseudopod projection is produced with *Listeria* at the tip (106). This pseudopod touches a neighboring cell that engulfs it, enclosing the organism in a double-membraned vacuole (21,25,106). The organism then escapes from the vacuole and repeats this cycle to initiate further infection of neighboring cells. In this fashion, organisms remain cell-associated throughout the infectious process and thereby avoid attack by the humoral immune system of the host (26).

Mounting a successful host defense against this pathogen requires a patent cell-mediated immune system with MØ killing of internalized bacteria (32). However, it has been shown that mononuclear phagocytes are heterogeneous with regard to listericidal activity. Drevets & Campbell demonstrated that mouse peritoneal MØs produced by stimulation with thioglycollate permit intracellular growth of *L. monocytogenes*, whereas MØs stimulated by proteose-peptone are listericidal (32). In addition, these workers showed that thioglycollate-stimulated MØs utilized complement receptor 3 (CR3) as a minor binding molecule for listeriae. This suggested that an alternative host cell receptor was responsible for binding the majority of organisms to MØs in nonlistericidal fashion. In the present study, the binding of *L. monocytogenes* to host cells in the absence of opsonins was investigated to determine the potential for uptake mechanisms other than those previously studied. Bacterial uptake in an opsonin-deficient

environment may occur at the very earliest stages of disease and prior to the induction of antibody or increased levels of complement. Such opsonin-independent adherence mechanisms may offer intracellular survival advantages for *Listeria*.

3.3 Materials and Methods

3.3.1 Organism Cultivation

A fully virulent clinical isolate of *L. monocytogenes* serotype 1/2b was isolated on blood agar, subcultured once on T-soy agar, and stored as stock cultures frozen at -70°C in 1% serum-sorbitol. Virulence was assayed using fertile hens' eggs (5) and over the course of this study the lethal dose 50 (LD_{50}) for this isolate remained unchanged at 21 CFUs. Thawed samples were plated on agar and incubated overnight. Colonies were harvested, resuspended in T-soy broth to give 51 Klett units (equivalent to approximately 4×10^8 CFU/mL). This suspension was diluted 1:10 and 62.5µL added to tubes containing 5mL T-soy broth. These cultures were incubated for 8.5 h to mid-exponential growth phase in a shaking incubator at 37°C. Cells were collected by centrifugation at 5000 X g, washed 3 times with Hanks' balanced salt solution (HBSS) (Sigma, St. Louis, MO.), and resuspended in HBSS to give 1×10^9 CFU/mL prior to inoculation of MØs.

3.3.2 Production of Murine Peritoneal Macrophages

Balb/c mice of both sexes were used at 3-6 m. Animals were housed at

the University of New Hampshire animal maintenance facility according to Animal Care and Use Committee guidelines (HHS/NIH publication # 85-23) and were given feed and water ad libitum.

Murine peritoneal MØs were elicited by intraperitoneal injection of 1.5mL thioglycollate per mouse. Mice were sacrificed by carbon dioxide asphyxiation 2.5 to 3.5 d later, and peritoneal exudate cells were extracted by three 10mL peritoneal cavity lavages of HBSS. These cell extracts were pooled and collected by centrifugation at 220 X g for 10 min. Red blood cells were lysed by addition of 9mL of sterile distilled water followed by 1mL of 10X HBSS. MØ-enriched cell suspensions were then washed twice with HBSS, and the total number determined by hemacytometer.

3.3.3 Transmission Electron Microscopy

For transmission electron microscopy (TEM), cells were added to 50mL centrifuge tubes at a concentration of 1 x 10^7 cells/tube. MØs and bacteria were held separately on ice for 10 min prior to mixing. Bacteria were added to suspensions of MØs at a multiplicity of infection (MOI) of 100 bacteria per host cell in 1.5mL HBSS. The inoculated cell preparations were centrifuged at 220 X g for 10 min to pellet the MØs and then at 850 X g for 10 min to deposit the bacteria onto the cells by a method outlined previously (51). During these manipulations the suspensions of bacteria and MØs were maintained on ice (4°C). The tubes were then immediately warmed by gentle rotation in a water

bath at 37°C to facilitate the phagocytic process. Samples were collected after 1, 2, 3, 3.5, 5, 10, 15, 20, 30, 40, and 60 min intervals. For each time point, a sample was placed on ice and the supernatant fluid removed. The harvested pellets were fixed using prewarmed 5% glutaraldehyde in 0.05M cacodylate buffer containing 10mM $MgSO_4$ pH 7.2 (CB). A 0 min sample was collected immediately following the centrifugation steps and this was fixed in glutaraldehyde in CB precooled to 4°C.

Samples were washed 10 times with CB, and pre-embedded in molten 2% noble agar at 55°C. Agar was rapidly cooled by placing samples at 4°C for 2-3 min and cells in agar were cut into approximately 1-mm^3 blocks, and post-fixed in 1% OsO_4 in CB for 36 h. Samples were then dehydrated in an ethanol series to propylene oxide and embedded in an epon-aryldite resin mixture. After polymerization at 60°C for 24 h, blocks were trimmed and sectioned on an LKB ultramicrotome III. Sections of approximately 60nm thickness were stained for 1 min each with 5% uranyl acetate and 0.4% lead citrate, and examined at 75kV in the transmission mode of a Hitachi H-600 scanning-transmission electron microscope.

3.3.4 Scanning Electron Microscopy

For scanning electron microscopy (SEM), MØs were harvested as described, and 3mL of RPMI-1640 medium with 10% fetal bovine serum (FBS) containing $1x10^6$ cells were seeded into wells of 6-well culture plates

(Corning-Costar, Cambridge, Mass.) each containing a sterile 22 mm diameter glass coverslip. The MØs were then incubated at 37°C for 3 h. The MØs were washed three times with HBSS to remove unbound cells and cell culture media, and 2mL HBSS was placed on the cells. Suspensions of *L. monocytogenes* were added in HBSS at an MOI of 100 bacteria per host cell ($1x10^8$ CFU/well). The plates were then incubated at 37°C in the presence of 5% CO_2 and samples taken at 5 min intervals for 1 h.

Nonadherent bacteria were removed by washing the cell cultures three times with HBSS and the cells fixed in 5% glutaraldehyde in CB for 24 h. Samples were then washed 10 times with CB *in situ*, and dehydrated using a graded ethanol series followed by drying from hexamethyldisalizane (Electron Microscopy Sciences, Fort Washington, Pa.). Monolayers were then sputter coated with 20nm of gold-palladium in a Hummer V sputter coater and observed in an AMR 1000 scanning electron microscope at 60kV.

3.3.5 Intracellular Replication of *L. monocytogenes*

To further define the binding, uptake and intracellular replication of *L. monocytogenes* in peritoneal MØs, growth curves as measured by CFUs were used. MØs were placed in 6-well plates at $1x10^6$ cells/well and allowed to adhere for 8 h. Cultures were washed three times with HBSS to remove nonadherent cells and serum. *Listeria* were suspended in HBSS and added to the wells at an MOI of 1. Bacteria were allowed to adhere to and penetrate

MØs for 1 h at 37°C. The cells were washed three times to remove nonadherent bacteria. RPMI-1640 medium supplemented with 10% FBS and 6μg/mL gentamicin (10 times the minimum inhibitory concentration (MIC) of the antibiotic for this organism) was added to the cell monolayers. Samples were collected each h for 9 h by washing individual wells to remove the antibiotic. At the 9 h time point appreciable loss of adherent MØs was noted. Adherent cells were lysed in 2mL sterile water and disrupted by agitation following aspiration in a Pasteur pipette. For each time period, a 100μL aliquot was removed from the lysate samples and serially diluted in 1% peptone and 50μL plated in duplicate on T-soy agar plates incubated at 37°C for 24 h for enumeration of intracellular listeriae.

3.3.6 Intracellular Replication: Impact of Gentamicin

The impact of 6μg/mL gentamicin on intracellular growth was also evaluated using CFU assays. Culture plates were set up as described in the previous section and gentamicin was added after removal of unbound listeriae. Following incubation for 2 h, gentamicin was removed from 1/2 the wells (controls) by washing 3X with HBSS. Samples were collected each h for 5 h by washing a control and an antibiotic-treated well 3X with HBSS. Cells were then lysed in 2mL sterile water, and enumerated as described above.

3.3.7 Adherence and Uptake of *L. monocytogenes* at 4°C

The potential for uptake of *L. monocytogenes* by MØs at 4°C was

investigated using CFU assays. Culture plates were prepared as described in section 3.3.5. Washed listeriae and culture plates with adherent MØs were separately chilled to 4°C on ice. MØs were infected for 1 h with listeriae, with one culture plate maintained on ice and a second maintained at 37°C throughout the assay. Unbound bacteria were then removed by washing 3X with cold HBSS, and 2mL of HBSS containing 6μg/mL gentamicin added to selected test wells. Gentamicin was allowed to saturate the cells for 30 min; subsequently, the plate held at 4°C was placed at 37°C for 1 h. Wells were washed 3X with HBSS to remove gentamicin. Cells were then lysed in 2mL sterile water, and enumerated as described above. To confirm the effectiveness of the antimicrobial to kill *Listeria* in these assays, the gentamicin was additionally tested on *L. monocytogenes* in culture at 4°C and 37°C for 1.5 h.

3.4 Results

Adherence of *L. monocytogenes* to mouse peritoneal MØs in the absence of antibody or complement was observed in the study. SEM examination of monolayers showed numerous listeriae adherent to filopodia extruded from MØs (Fig. 6A) as well as to the surface membranes of these cells (Fig. 6B); in either case with few host cell changes. However, these monolayers were not centrifuged to bring the listeriae into rapid contact with the MØs. Observation of TEM thin-sections showed electron-dense material

"streaming" between the cell wall of the organism and the host cell surface (Fig. 6C and inset). This material may have been involved in the bacterial binding process. Alternatively, the material may have been due to membrane degradation. This degradation may have occurred through the action of surface-associated listeriolysin O. Extracellular bacteria apparently bound to the MØs in all stages of infection involved single bacteria or organisms in the process of binary fission.

Examination of peritoneal MØs by TEM revealed internalized listeriae in all samples, including those cells taken at zero min post-centrifugation. Additional studies carried out investigating this phenomenon confirmed the intracellular presence of the organism at 4°C. Internalized bacteria were present in membrane-bound phagosomes throughout the first hour of infection. Escape of the bacterium from the phagosome appeared to begin approximately 10 min after the infectious cycle was initiated (Fig. 6D). This phenomenon confirmed and extended reports on the infectious process of *L. monocytogenes* in the mouse MØ-like cell line J774 in which listeriae escaped and were seen free in the cytoplasm after 30 min of incubation (106).

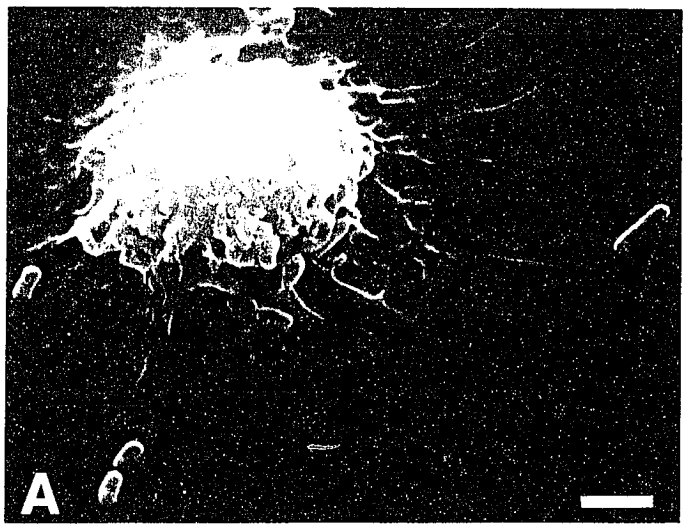

Figure 6A: Scanning electron micrograph of a macrophage with multiple listeriae shown bound to host cell filopodia. Magnification X 5,000. Scale bar = 2μm.

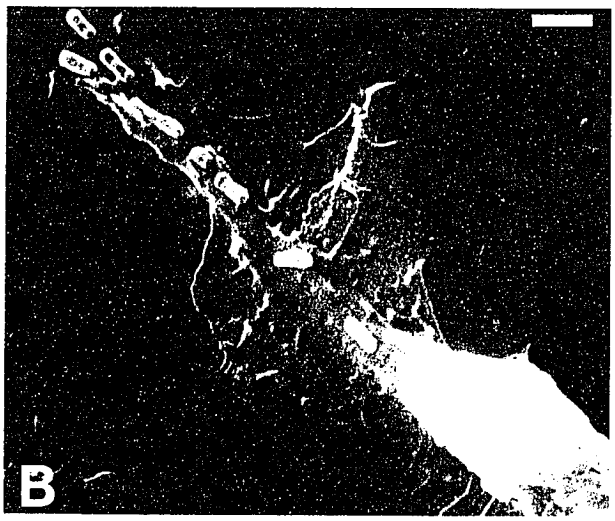

Figure 6B: Scanning electron micrograph of a macrophage with numerous listeriae bound directly to the cytoplasmic membrane. Magnification X 4,000. Scale bar = 2μm.

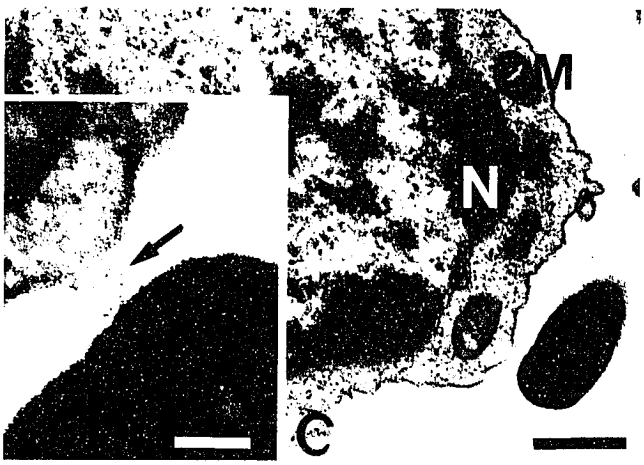

Figure 6C: Transmission electron micrograph of a macrophage with a single bacterium undergoing wash-resistant attachment at 5 min post-inoculation. Magnification X 25,500. Scale bar = 0.5µm. N = nucleus; M = mitochondrion.
Inset: Close association of organism surface with host cell plasma membrane is evident. Note streaming material between organism and host cell membrane (arrow). Magnification X 104,000. Scale bar = 0.1µm.

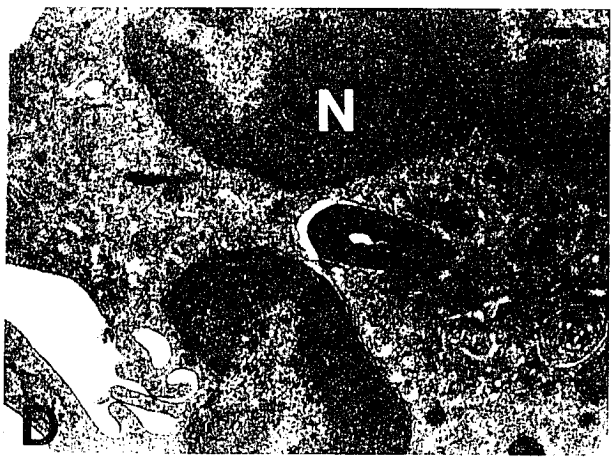

Figure 6D: Internalized organism showing partial escape of the bacterium from the phagosome occurring by 10 min post-infection. Magnification X 19,500. Scale bar = 0.5μm. N = nucleus.

L. monocytogenes was taken up by "classic" mechanisms of phagocytosis (in which two pseudopod arms surround the bacterium) rather than by coiling phagocytosis (Fig. 6E) (51). This coiling phenomenon may occur only in the presence of opsonins, as coiling phagosomes were not observed during initial infection of the human monocyte/MØ cell line U-937 with *L. pneumophila* in the absence of opsonins (88). The significance of these findings remain to be established. As early as 1 min post-inoculation, numerous intracellular bacteria were seen within phagosomes by TEM (Fig. 6F).

The presence of 10 or more listeriae bound to individual MØs was noted to induce MØ lysis in the absence of infection. This destruction was confirmed during growth studies of MØs infected with *L. monocytogenes* at an MOI of 100 as the cells were rapidly lost due to lysis. By 4 h of intracellular replication greater than 90% of the MØs were nonadherent. Heavily infected Caco-2 cells have also been reported to lyse in a similar time frame (38). CFUs showed that approximately 2.3×10^5 *Listeria* attached to 1×10^6 MØs at 1 h, thus indicating that approximately 14% of the bacterial inoculum had bound to the cell monolayers. By 2 h after the addition of gentamicin, the CFUs measured from harvested cells had declined to 7% of the originally adherent listeriae, indicating death of extracellular *Listeria*. Therefore it is apparent that more listeriae bind to the MØs than are taken up.

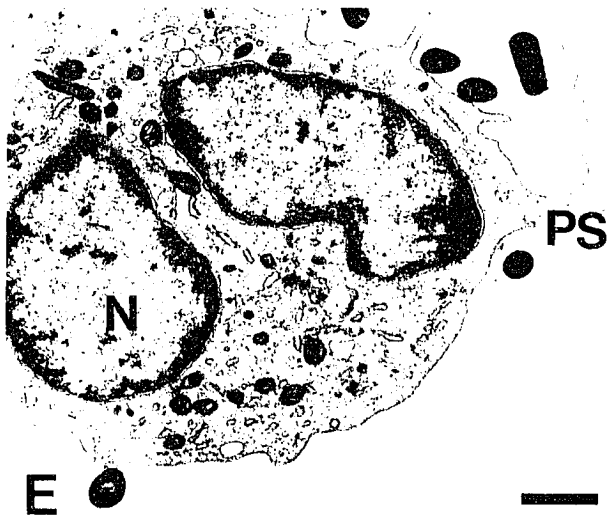

Figure 6E: Transmission electron micrograph of organisms undergoing phagocytosis 3 min after inoculation. Note pseudopodia engulfing organisms. Magnification X 10,400. Scale bar = 1μm. N = nucleus; PS = pseudopod.

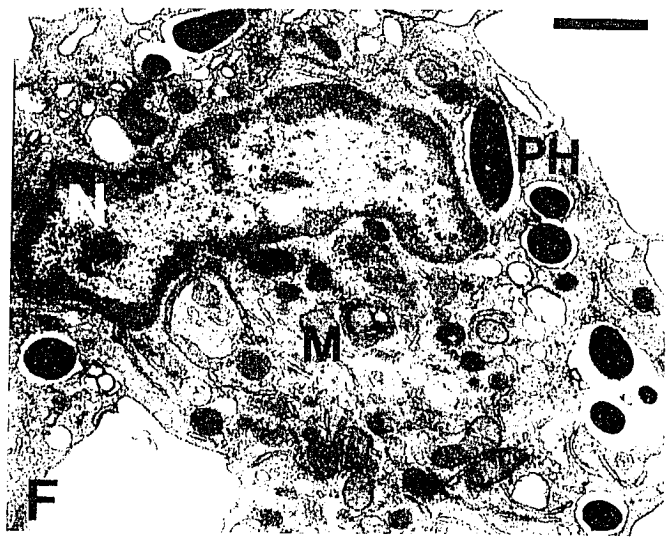

Figure 6F: Transmission electron micrograph of macrophage showing multiple internalized listeriae at 1 min post-inoculation. Magnification X 13,000. Scale bar = 1μm. N = nucleus; M = mitochondrion; PH = phagosome.

Growth curves of *L. monocytogenes* indicated that the population of listeriae did not replicate in HBSS over a 3 h period (Fig. 7); in addition, the minimum inhibitory concentration (MIC) of gentamicin in RPMI-1640 with 10% FBS was determined to be 0.6μg/mL for this strain. The mean generation time during most rapid intracellular growth in these MØs was calculated to be approximately 46 min (Fig. 8). At 9 h, the monolayers were significantly disrupted by the infectious process, with greater than 95% of the MØs detached or lysed. The presence of gentamicin added to inoculated cells for the intracellular growth studies appeared to have no effect on the growth rate of the organism (Fig. 9).

CFU assays investigating the possible adherence and uptake of *L. monocytogenes* by MØs at 4°C appeared to indicate that some form of protection for the organism occurred in the presence of MØs (Fig. 10). A log-decrease in organisms bound or inside MØs occurred at 4°C; however, a decrease in the number of organisms did not occur in the presence of gentamicin at 4°C. Figure 11 indicates the efficacy of identical gentamicin treatment on *Listeria* grown in the presence of gentamicin and the absence of MØs. This would appear to indicate that the organism is somehow protected from the deleterious effect of gentamicin, either through close association with the host cell or alternatively through uptake and intracellularization by the MØ.

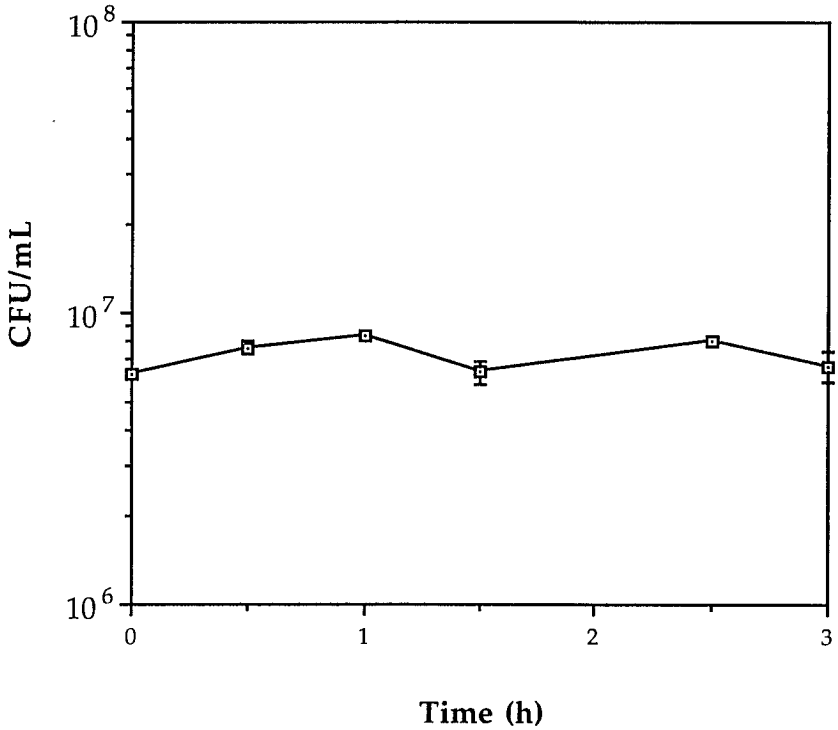

Figure 7: Lack of replication of *L. monocytogenes* in HBSS over 3 h. An inoculum of 6 x 10^8 CFU/mL was added to 3mL of HBSS at time zero and the cultures incubated at 37°C with 5% CO_2. Samples were taken at 30 min intervals. Assays were the average of two trials and bars represent standard deviation of the mean.

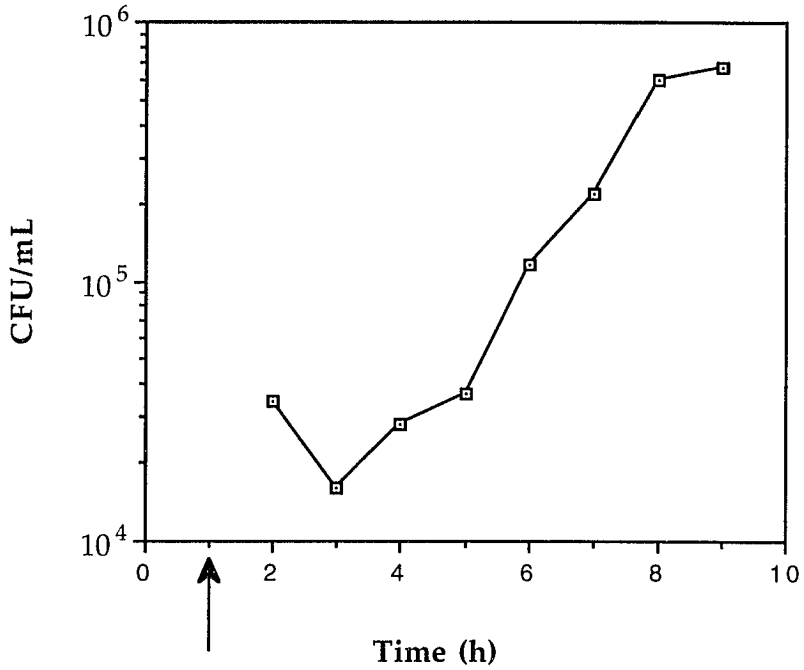

Figure 8: Intracellular growth of *L. monocytogenes* in murine peritoneal macrophages. An inoculum of 1.6X10^6 CFU was added to macrophages (MOI of 1) at time 0, incubated for 1 h to allow adherence and penetration to occur and washed to remove unbound organisms. Approximately 15% of the bacterial inoculum adhered to macrophages. Gentamicin was added at 1 h (arrow) and samples were collected and assayed for bacteria by CFU counts. Mean generation time for *Listeria*, assayed between 3 and 8 h after inoculation, was approximately 46 min. Data represent the typical growth curve observed during 3 trials.

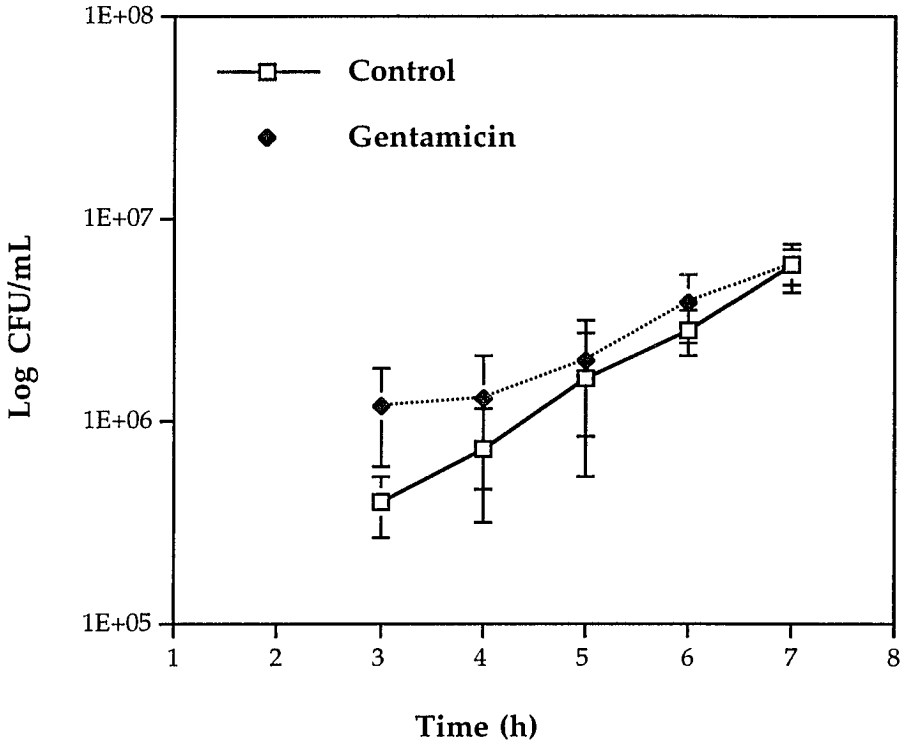

Figure 9: Intracellular replication of *L. monocytogenes* in the presence and absence of 6μg/mL gentamicin. Assays were the mean of two trials and bars represent standard deviation of the mean.

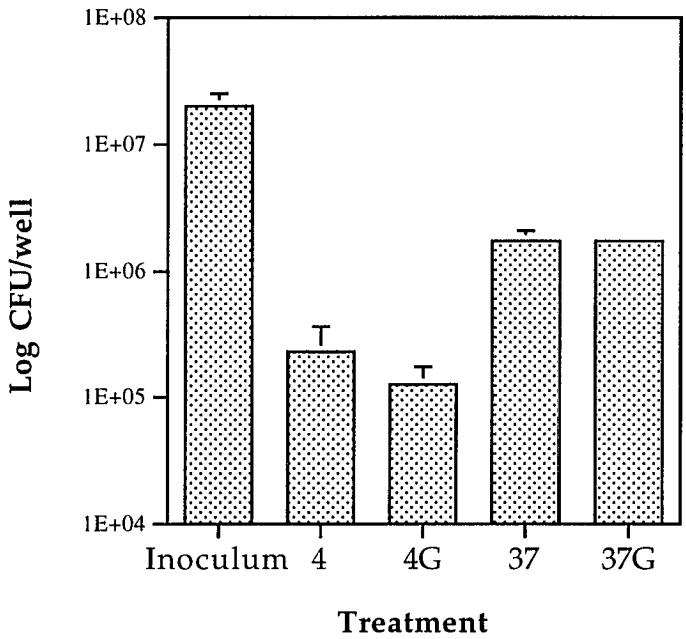

Figure 10: Apparent uptake of *L. monocytogenes* at 4°C. Treatments shown are: 4 = 4°C; 37 = 37°C; G = gentamicin. Assays were the mean of four trials and bars represent standard deviation of the mean. The standard deviation for 37G was extremely low and did not register on the graph program.

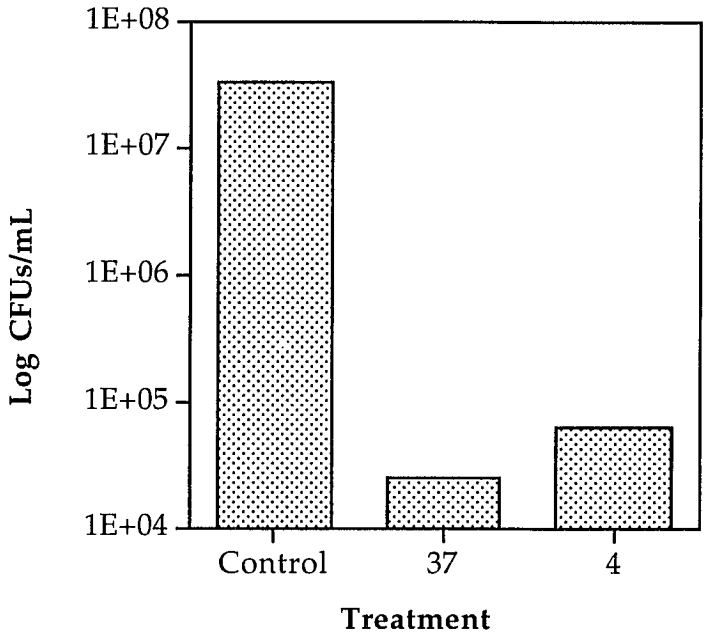

Figure 11: Effect of 6μg/mL gentamicin on *L. monocytogenes*. No gentamicin was used on control bacteria; bacteria at 37°C were treated for 1.5 h at that temperature; bacteria at 4°C were treated for 30 min at 4°C followed by 1 h at 37°C. Gentamicin was washed away and serial dilutions of each test made and plated in duplicate. Results are the mean of duplicate plates from 1 trial.

3.5 Discussion and Conclusions

From these studies it is clear that successful adherence and infection of peritoneal MØs by *Listeria* occurs in the absence of opsonins. It has been postulated that the receptor by which intracellular pathogens are recognized by MØs may define their intracellular fate (100). A study on the uptake of *Mycobacterium tuberculosis* showed that bacterial entry mediated by Fc receptors resulted in lysosome-associated vesicles, whereas entry via CR3 receptors resulted in lysosome-free vesicles (100). It is possible that such differences exist in the intracellular fate of *L. monocytogenes* taken up by opsonin-dependent and opsonin-independent mechanisms. The presence of intracellular *Listeria* taken up by cells at 4°C was indicated both by TEM and in CFU assays. The organism appeared to be located within cell membranes within the cytoplasm when examined by TEM, and some protection from the effect of gentamicin was afforded by the MØs. However, this protection may have resulted from close association of the bacteria with the host cell. It is also possible that the presence of organisms inside MØs at 4°C may be due to host cell stresses caused by centrifugation or to invagination of the membrane around the organism.

The intracellular replication of *L. monocytogenes* in murine peritoneal MØs was established through CFU assays. The mean generation time in these MØs was approximately 46 min; this was comparable to a 40 min

generation time seen in bone marrow-derived murine macrophages (26). In contrast, the mean generation time seen in Caco-2 cells was in the order of 90 min (38). Gentamicin has been shown to have an adverse effect on intracellular replication of *L. monocytogenes* at concentrations of 50-100μg/mL but not at 0-5μg/mL (33). In keeping with this, data from the present study showed that the presence of 6μg/mL gentamicin had no effect on the intracellular replication of the organism. Additionally, it was shown that high ratios of bacteria to host cells (high MOI) resulted in lysis of the host cells incubated over extended periods of time. Subsequent experiments using high MOIs therefore employed a 1 h period of infection.

The process of infection has been illustrated in this work, from initial adherence of *L. monocytogenes* to escape of the organism from the phagosome. Adherence was shown to occur in a wash-resistant manner in the absence of opsonins. An electron-dense "streaming" material was seen flowing between the organism and the host cell; the origin of this material is difficult to determine. It is possible, however, that this streaming resulted from loss of MØ membrane integrity due to specific action of the listeriolysin on the host cell membrane at the point of attachment. However, "streaming" material was not evident at all points of attachment between listeriae and host cells. Additionally, control cells showed no loss of membrane integrity. Phagocytosis occurs via two pseudopod arms encircling the organism; this

mechanism is known as "classic" phagocytosis (51). Following a very brief period of infection, several listeriae are seen within the host cells, indicating that binding of the organism occurs in multiple numbers. Partial escape of the organism occurred at 10 min, which confirmed reports of the infectious process of listeriae in other cell types (106). Investigation of the subsequent replication and dissemination to other host cells has been described previously by others (21,25,58,75,93,105,106).

This work demonstrated an efficient adherence and uptake mechanism for *L. monocytogenes* in the absence of opsonins. It has been shown that thioglycollate-elicited, nonlistericidal MØs take up *Listeria* in a CR3-mediated fashion only 33% of the time (32). Nonlistericidal MØs also phagocytose *Listeria* more efficiently than do proteose-peptone-elicited, listericidal MØs. In contrast, CR3 mediated approximately 66% of the uptake of serum-opsonized *Listeria* by listericidal MØs (32). Bacterial escape in these studies was defined by the presence of cytoplasmic, actin-coated listeriae in the MØs (32). Similar numbers of listeriae were phagocytosed by both populations of MØs but the number of actin-coated organisms present after 2 h was dramatically higher (130-fold) in thioglycollate-elicited, permissive MØs than in the listericidal MØs (32). Successful binding of this pathogen to permissive (as opposed to listericidal) MØs facilitates replication and enhances infection of the host. It seems certain, therefore, that these opsonin-independent

binding processes play an important role in the successful infection of *Listeria*-permissive host MØs, as uptake through an opsonin-dependent mechanism results in bacterial killing.

SECTION 4

THE ROLE OF SIALIC ACID IN THE OPSONIN-INDEPENDENT AND OPSONIN-DEPENDENT ADHERENCE OF *LISTERIA MONOCYTOGENES* TO MURINE PERITONEAL MACROPHAGES

(The work outlined in this Section has been published in abstract form: **Pierce, M.**, S. Maganti, A. Hoffmaster, and F.G. Rodgers. 1995. Opsonin-independent adherence of *Listeria monocytogenes* is blocked in a competitive fashion by N-acetyl neuraminic acid. Abst. Gen. Meeting ASM., Washington, D.C.: B-389)

4.1 Abstract

Adherence of *L. monocytogenes* to host cells occurred in both the presence and absence of opsonins. Murine peritoneal MØs were used to study and characterize the opsonin-independent and opsonin-dependent adherence mechanisms for *L. monocytogenes*. "Adhesin" and "receptor" characterization studies were performed using indirect immunofluorescence assays (IFA) and enzyme-linked immunosorbent assays (ELISA). Observations on the rate of adherence of *L. monocytogenes* to host cell receptors indicated that at an MOI of 100, approximately 19% of the non-

opsonized bacteria were adherent. At an MOI of 1000, the number of organisms bound to the MØs increased to approximately 70 organisms per host cell; however, the number of bacteria attached nonspecifically to the plastic surface was such that determination of host cell-bound bacteria was difficult. Therefore, a relationship of one bacterial "adhesin" per host cell "receptor" for *L. monocytogenes* could not be established.

N-acetyl neuraminic acid (NAcNeu) significantly blocked binding of *L. monocytogenes* in competitive binding assays as measured by IFA and ELISA. None of the remaining monosaccharides examined affected binding. This inhibition was also seen in the presence of opsonized *L. monocytogenes*. The presence of decreasing concentrations of NAcNeu had a decreasing effect on inhibition of opsonin-independent binding. NAcNeu showed similar inhibitory effects on *L. monocytogenes* binding in the presence of opsonins. In addition, the lectins from *Maackia amurensis* and *Triticum vulgaris* (wheat germ agglutinin) inhibited *L. monocytogenes* binding.

The binding structures of both *L. monocytogenes* and murine peritoneal MØs were modified using chemical agents and enzymes to determine the nature of the bacterial adhesins as well as the host cell receptors. These surface bacterial and host cell membrane treatments were performed to determine the interruption of binding due to the enzymatic loss or destruction of particular groups of molecules. Treatment of *L.*

monocytogenes with these agents, with the exception of glutaraldehyde, had no effect on the viability of the organism. Treatment of the organism with the enzyme neuraminidase reduced binding by 40% as measured by IFA; this reduction was not, however, confirmed by ELISA. The results derived from glutaraldehyde treatment of *L. monocytogenes* were contradictory, with a reduction in binding being shown by ELISA while by IFA an increase in binding was indicated. The reduced binding seen by ELISA, however, may be due to a dampening effect on the horseradish peroxidase indicator enzyme caused by the glutaraldehyde-treated listeriae.

Treatment of the host cell surface with either formaldehyde or glutaraldehyde decreased binding of the organism; however, other enzyme treatments of both bacterial and host cell surfaces were ineffective in inhibiting binding. Use of a monoclonal antibody (M1/70) specific for the opsonin-dependent receptor CR3 had no measurable effect on the binding of either opsonized or nonopsonized *L. monocytogenes*. It is proposed, therefore, that NAcNeu is involved in the opsonin-independent adherence of *L. monocytogenes* to nonlistericidal, permissive host cells. This process appears to occur through some receptor other than CR3, and permits the intracellular replication of the organism inside host cells, resulting in disease.

4.2 Introduction

Adherence is the necessary first step in the infectious process of many intracellular pathogens. This adherence and subsequent phagocytosis occurs either through an opsonin-dependent or an opsonin-independent process. Opsonin-driven mechanisms involve complement or antibody and their appropriate receptors on the host cell, while opsonin-independent processes involve bacterial adhesin recognition and attachment of the organism to specific host cell receptors. Opsonin-mediated adherence and phagocytosis has been documented in the pathogenesis of leprosy (94); antibody present in nonimmune serum mediates C3 fixation to the surface of *Mycobacterium leprae*, resulting in phagocytosis by human mononuclear phagocytes (94). Complement receptor 4 was shown to be the primary receptor involved in uptake of *M. tuberculosis* by human alveolar MØs; uptake by blood monocytes, the precursor of alveolar MØs, occurred mainly through CR3 and CR1 (49). However, uptake of *M. tuberculosis* can also occur through Fc receptor interaction with specific antibody (49). The intracellular fate of this pathogen is dependent upon the mechanism by which it is taken up: uptake through the Fc receptor results in acidification of the bacterial phagosome and bacterial killing, whereas uptake through CR3 receptors did not cause a subsequent acidification of the phagosome (100). This would appear to indicate that there are differential signalling pathways between the two

different mechanisms of uptake (100).

Bacterial adhesins can be present as adhesive pili, as found in many gram-negative bacteria, or as nonpilus adhesins which cannot be visualized by standard electron microscopy techniques (52). These nonpilus adhesins are most probably linked to the cell surface as monomers or simple oligomers (52). The interaction between bacterial adhesins and the host cell receptor structures can be highly specific, allowing for selective interactions with the host (52). In some cases, the adherence process may involve two different bacterial adhesins or a cofactor may be involved as seen with *Pseudomonas aeruginosa* (102). This gram-negative rod appears to require the presence of glucose to bind to alveolar MØs using a mechanism independent of complement receptor 3 (102). However, the glucose appears to act upon the MØ, as phagocytosis of killed *P. aeruginosa* is glucose dependent (102). Adhesion of the gram-positive cocci *Streptococcus sanguis* involves multiple, interacting sites (47). At least six different mechanisms of adhesion have been proposed for *S. pyogenes*, a pathogen which colonizes the pharynx and tonsils of humans (47).

However, the receptors involved in opsonin-dependent adherence may also be involved in opsonin-independent adherence, as in the case of Group B streptococci, which recognizes and binds to complement receptor type 3 (CR3) in the absence of opsonins (2). Direct bacterial attachment

between adhesins and host cell receptors has also been reported for *Legionella pneumophila* (41,88), *Listeria monocytogenes* (32), and *Pseudomonas aeruginosa* (74,102).

L. monocytogenes is the causative agent of foodborne listeriosis, a disease which primarily affects pregnant women and neonates. Clinical manifestations range from mild, flu-like symptoms to meningoencephalitis and septic abortion. In human listeriosis, the initial site of entry into the host is presumably the intestine after consumption of *Listeria*-contaminated food (30). It has long been recognized as a facultative intracellular pathogen capable of infecting a great variety of cells including fibroblasts, epithelial cells, hepatocytes, and cells from the mononuclear phagocyte system (1,20,38,109) . It has been shown that mononuclear phagocytes constitute the major effector cells of immunity in experimental listeriosis infections (1,67) . *L. monocytogenes* has been shown to induce the deposition of C3b and its cleavage products iC3b and C3d through ester and amide linkages; this subsequently results in the activation of the alternative pathway of human complement (24). The ability of this organism to invade host cells appears to depend on the presence of the surface-bound protein internalin; however, the involvement of this protein in actual adherence of the organism to the host cell has not been demonstrated (30).

Recent studies indicate that complement receptor 3 (CR3) mediates

phagocytosis of *L. monocytogenes* in the presence of opsonins by a population of listericidal macrophages (31,32). However, the same studies demonstrated that nonlistericidal macrophages utilized CR3 as a minor binding molecule for listeriae (32). Use of a monoclonal antibody directed against CR3 (CD11b/CD18) inhibited killing of *L. monocytogenes* in a dose-dependent manner for listericidal, proteose-peptone-elicited macrophages, and cells became permissive hosts at high doses (34). In contrast, use of anti-CR3 to block adherence and phagocytosis by nonlistericidal, permissive macrophages was largely ineffectual. This appears to indicate two possible mechanisms of adherence and uptake for this pathogen: an opsonin-dependent mechanism through CR3 in which the organism is killed by the host cell, and an opsonin-independent mechanism through some receptor other than CR3 in which the organism parasitizes the host cell.

In the studies presented here, the potential adhesive mechanisms for *L. monocytogenes* in both the presence and absence of opsonins were defined. Three main questions were addressed. (i) How avidly does *L. monocytogenes* bind to host cells in the absence of opsonins, and at what point does this binding become saturated? (ii) What possible bacterial adhesins are involved in the attachment of the organism to host cells? (iii) Are these adhesins involved in both opsonin-dependent and independent adherence of *L. monocytogenes* to host cells?

4.3 Materials and Methods

4.3.1 Bacterial Growth and Maintenance

A fully virulent clinical strain of *L. monocytogenes* serotype 1/2b was grown and maintained as described in Section 3.3.1.

4.3.2 Murine Peritoneal Macrophage Preparation

Murine peritoneal macrophages (MØs) were elicited by intraperitoneal injection of 1.5mL thioglycollate per mouse. Mice were sacrificed by carbon dioxide asphyxiation 2.5 to 3.5 d later, and peritoneal exudate cells were extracted by three 10mL peritoneal cavity lavages of HBSS. These cell extracts were pooled and collected by centrifugation at 220 Xg for 10 min. The cells were subsequently resuspended in RPMI-1640 (Sigma, St. Louis, Mo.) medium containing 10% heat-inactivated Fetal Bovine Serum (HI-FBS) (Sigma), and the total number determined by hemocytometer. For assay by indirect immunofluorescence assay (IFA), cells were seeded at 1×10^6 MØs/well in 3mL of RPM-1640 with 10% HI-FBS into 6-well plates containing sterile, washed coverslips. For evaluation by enzyme-linked immunosorbent assay (ELISA), cells were seeded into 96-well plates at 1×10^5 MØs/well in 200 µL of RPMI-1640 with 10% HI-FBS. The MØs were incubated at 37°C for 6-8 h in a 5% CO_2 atmosphere.

4.3.3 M1/70 Monoclonal Antibody

The anti-CR3 MAb is a rat IgG2b antibody specific for mouse and

human CR3 and blocks binding of iC3b-coated targets to CR3 (34). The B-cell hybridoma M1/70 was cultured in RPMI 1640 (Sigma) with 10% FBS and the supernatant fluid collected and concentrated using Centriprep-10 ultrafiltration units (Amicon, Beverly, MA). Hybridoma supernatant fluid was passed over a protein G-Sepharose 4 Fast Flow column (Pharmacia Fine Chemicals, Piscataway, N.J.) and eluted with 0.2M glycine (pH 2.0). Fractions were collected, concentrated using a Centriprep-10, and the protein concentration of the collected fractions was determined using the bicinchoninic acid assay (BCA) (Pierce, Rockford, IL).

4.3.4 Bacterial and Host Cell Treatments and Competitive Binding

All treatment reagents were prepared at the time of use in HBSS, adjusted to pH 7.2, and filter sterilized through a 0.22μm filter (41). Bacteria were exposed to modifying agents, washed with HBSS, and added to untreated host cells prior to assay for adherence. The concentrations, treatment times, and mode of action of the bacteria-modifying agents are outlined in Table 9 as outlined by Gibson et al. (41,83). To confirm enzyme treatment results, some enzyme concentrations were doubled to increase action on bacterial surfaces. Host cells were similarly treated with membrane-modifying agents as described in Table 10 (41). Cells were washed and inoculated with untreated listeriae. The action of all enzymes used for prokaryotic or eukaryotic surface treatments are shown in Table 11.

In addition, the monosaccharides fucose, galactose, glucose, mannose, N-acetylglucosamine, N-acetylgalactosamine, N-acetylneuraminic acid (Sigma) were tested individually at a concentration of 100mM and were added to cell cultures 1 h prior to the inoculation of listeriae (Table 12) (23,41). In these competitive inhibition assays, the lectins from *Limulus polyphemus* (E-Y Laboratories, San Mateo, CA), *Maackia amurensis* (E-Y Laboratories), *Triticum vulgaris* or wheat germ agglutinin (WGA) (Sigma), and concanavalin A (Con A) (Sigma) were similarly used to compete for binding sites on host cells but at 100μg/mL (Table 12) (23,41). Bacterial attachment to host cells was determined in the presence of the sugars and lectins in a competitive fashion.

Table 9. Treatment protocol for the surface expressed adhesins of *Listeria monocytogenes*.*

Agent	Activity	Treatment time (min)	Function
NAc-Neuraminic acid aldolase	10 units/mL	60	Degradation of protein-,sugar- and lipid-containing moieties on the surface of *L. monocytogenes*
ß-Galactosidase	100 units/mL	60	
Chymotrypsin	500 units/mL	60	
Lipase	100 units/mL	60	
Neuraminidase	20 units/mL	60	
Protease	10 units/mL	60	
Trypsin	500 units/mL	60	
Glutaraldehyde	0.1%	10	Immobilization of protein moieties
Sodium metaperiodate	10mM	60	Oxidation of carbohydrate moieties

*Note: Agents were added to bacterial cells for the specified time and then washed to remove excess.

Table 10. Treatment protocol for the modification of surface expressed receptors on host cells.*

Agent	Activity	Treatment time (min)	Function
Chymotrypsin	0.1 units/mL	30	Degradation of sensitive host cell receptors
Lipase	100 units/mL	30	
Pepsin	100 units/mL	30	
Protease	0.005 units/mL	30	
Trypsin	50 units	30	
Formaldehyde	1.0%	10	Immobilization of protein moieties
Glutaraldehyde	0.1%	10	
Nonidet P-40	0.005%	60	Oxidation of lipids
Sodium metaperiodate	5mM	10	Oxidation of carbohydrate moieties

*Note: Agents were added to bacterial cells for the specified time and then washed to remove excess.

Table 11. Specific action of surface treatments for *L. monocytogenes* and host cells.*

NAc-Neuraminic acid aldolase	Cleaves pyruvate from N-acetyl neuraminic acid.
β-Galactosidase	Hydrolyzes Lactose-->Galactose + Glucose.
Chymotrypsin	Cleaves proteins on the carboxyl side of the aromatic side chains tyrosine, tryptophan, and phenylalanine as well as large hydrophobic residues such as methionine; hydrolyzes ester bonds.
Lipase	Hydrolyzes Triacylglycerol-->Glycerol + Fatty acids
Neuraminidase	Cleaves N-acetyl neuraminic acid.
Pepsin	Acid protease; optimum pH between 2 and 3; cleaves proteins on the nitrogen terminal of tyrosine, phenylalanine, and tryptophan.
Protease	Crude extract containing many different proteases with different specific activities.
Trypsin	Cleaves proteins on the carboxyl side of arginine and lysine residues.

*Sources: (86,103) and Sigma Chemical Co., St. Louis, Mo.

Table 12. Competitive binding experiments: monosaccharides and lectin treatments.*

Agent	Concentration	Function
Monosaccharides		
N-acetyl-D-glucosamine (NAcGlu)	100mM	
N-acetylgalactosamine (NAcGal)	100mM	Competitive binding
N-acetylneuraminic acid (NAcNeu)	100mM	
α-D(-) Fucose	100mM	
β-D(+) Glucose	100mM	
D(+) Mannose	100mM	
D(+) Galactose	100mM	
Lectins		Binding Affinity[t]
Limulus polyphemus	100μg/mL	NAcNeu NAcGal, NAcGlu
Maackia amurensis	100μg/mL	NAcNeu (α2,3)Gal
Triticum vulgaris	100μg/mL	β-NAcGlu, NAcNeu
Concanavalin A	100μg/mL	α-mannose, α-glucose

*Each of the select treatments were added to the host cells 1 h prior to infection.
[t]E-Y Laboratories, San Mateo, CA.

4.3.5 Infection Assay

The MØs were washed three times with HBSS to remove unbound cells and serum opsonins from cell culture media, and serum-free RPMI-1640 media with 0.5μg/mL Cytochalasin D added to uncouple adherence from uptake. The plates were then incubated for 1 h at 37°C in the presence of 5% CO_2 prior to modification of either the prokaryotic or eukaryotic cell membranes or treatment with competitive binding substances or monoclonal antibodies. MØs were inoculated with *L. monocytogenes* at a multiplicity of infection (MOI) of 100 bacteria per host cell. Following each treatment

regimen, listeriae were allowed to adhere 1 h, washed three times with HBSS to remove nonadherent bacteria, and the number of bound listeriae assayed by IFA and ELISA.

4.3.6 Detection: Immunofluorescent Assay

After 1 h of incubation, host cells were washed three times to remove nonadherent bacteria and fixed in 10% formalin in HBSS, pH 7.2, for 30 min at 25°C. The cultures were then washed three times with phosphate-buffered saline, pH 7.2, and treated with rabbit polyclonal anti-*L. monocytogenes* serum for 1 h at 37°C. Unbound globulin was removed, and goat anti-rabbit fluorescein isothiocyanate-conjugated antibody (Boehringer Mannheim, Indianapolis, IN) was added for 1 h at 37°C. After multiple washing with PBS, cells were stained with 0.01% propidium iodide in PBS for 30 min, washed, air dried, and mounted in glycerol containing 1% 1,4-diazobicyclo (2,2,2) octane. IFA results were averages of three trials enumerating the number of adherent bacteria on the first 100 cells per trial counted.

4.3.7 Detection: Enzyme-Linked Immunosorbent Assay

After 1 h of incubation, host cells were washed three times to remove nonadherent bacteria and fixed with pure methanol for 10 min at 25°C. The methanol was then removed and the plates placed at 37°C until dry. The plates were then washed three times with PBS containing 0.05% Tween-20, pH 7.2, followed by incubation with 1% gelatin in PBS at 37°C for 1 h. After

multiple washing with PBS-Tween 20, cells were treated with rabbit polyclonal anti-*L. monocytogenes* for 1 h at 37°C. Unbound globulin was removed, and goat anti-rabbit horseradish peroxidase-conjugated antibody (Boehringer Mannheim) was added and the cells incubated for 1 h at 37°C. The cells were subsequently washed with PBS only, and the chromogen tetramethylbenzidine (Sigma) added. Development was halted at 10 min by addition of 2M H_2SO_4, and the plate read at 405 nm on an EIA reader model MA310 (Whittacker M.A. Bioproducts). ELISA results were the average of three trials enumerating the number of adherent bacteria, based on a previously established standard curve (Fig. 12).

4.3.8 Criteria for Assessing Data

ELISA data were an average of at least 3 trials, each performed using a minimum of 10 wells on a 96-well plate. The number of bacteria bound to either control cells or to cells following the various treatment regimes was determined by comparison of absorbance at 405nm with a standard curve previously established (Fig. 12). IFA and ELISA data were graphed using CA-Cricket Graph III 1.5.1 (Computer Associates International, Inc., Islandia, NY) on a Macintosh LC III microcomputer. Adherence was expressed as a percentage of control cells for ELISA data. One-way analysis of variance (ANOVA) was carried out on ELISA absorbances data using Statview SE + Graphics v. 1.02 (Abacus Concepts, Berkeley, CA) (Appendix 7.5). IFA results

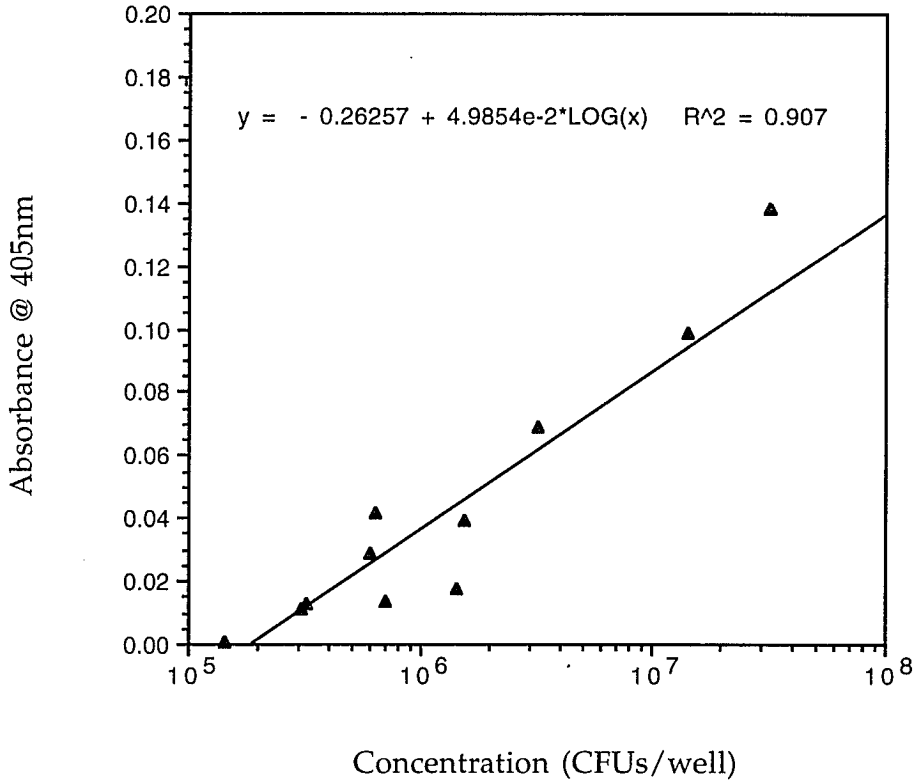

Figure 12: Standard curve for determination of ELISA data. *L. monocytogenes* was suspended in sterile 1% bovine serum albumin in PBS and aliquoted into wells. Plates were allowed to dry overnight at 37°C and probed using the standardized ELISA system outlined in 4.3.7. Background absorbance was subtracted from the data obtained and the results plotted as shown.

were the average of at least 3 trials enumerating adherent bacteria on the first 100 cells observed per trial. Data were expressed as the mean number of bacteria bound to each host cell. Standard deviation bars were provided for all IFA data and in certain cases were small enough that they did not appear on the actual graph.

4.3.9 Adherence Rate of *L. monocytogenes* to Host Cell Receptors

Wells containing 1 x 10^6 MØs in 6-well culture plates were washed three times with HBSS to remove unbound cells and cell culture media, and 2mL RPMI-1640 medium with 0.5µg/mL Cytochalasin D was added to uncouple adherence from uptake. Fresh HBSS was added to each well containing MØs, and the cells were infected with varying multiplicity of infections (MOIs). Following a 1 h incubation period at 37°C, unbound bacteria were removed by multiple HBSS washings and infected MØs were assayed by IFA to determine the point at which host cell receptors become saturated with *L. monocytogenes*.

4.3.10 Opsonin-Dependent Adherence Studies

The MØ cultures were washed three times with HBSS to remove nonadherent cells and cell culture medium, and 2mL RPMI-1640 medium with 0.5µg/mL Cytochalasin D was added. This medium also contained 10µg/mL goat IgG in order to block Fc receptors from non-specifically binding antibody, thus removing the influence of Fc-mediated uptake from the data.

The MØs were incubated at 37°C for 1 h in the presence of 5% CO_2, followed by a 1 h treatment with NAcNeu or a 30 min treatment with the anti-CR3 monoclonal antibody, M1/70 as used in previous studies (31,32,34). For these studies, *L. monocytogenes* was grown in T-cell broth, and cells were harvested at 8.5 h, were washed three times with HBSS, and were opsonized for 30 min in 5% normal mouse serum (NMS) in HBSS (Sigma) at 37°C. Organisms were subsequently added to MØs, allowed to adhere 1 h, and then nonadherent bacteria were removed by washing with HBSS. Plates were then assayed by IFA and ELISA.

4.4 Results

4.4.1 Adherence Rate of *L. monocytogenes* to Host Cell Receptors

Listeria monocytogenes bound to MØs in the absence of either complement or specific antibody in a wash-resistant manner. The data indicated that at an MOI greater than 200, host cell receptors were saturated with virulent *L. monocytogenes* (Fig. 13). However, at this MOI the amount of nonspecific binding of listeriae to the coverslips was unacceptably high, resulting in an inability to distinguish *Listeria* attached to host cells from those attached to the plastic. Therefore, an MOI of 100 was used for subsequent studies. After the 1 h incubation period, IFA data showed that host cells bound approximately 19% of the bacterial inoculum at this MOI, and the level of background bacteria attached to the plastic was minimal.

4.4.2 Bacterial Surface Treatment

Results from modification of adhesins using those treatments listed in Table 9 are shown in Figures 14 and 15. IFA data showed a decrease in binding following treatment of the surface of the organism with sodium metaperiodate, neuraminidase, lipase, and β–galactosidase. Binding increased dramatically after treatment with glutaraldehyde (Fig. 14). Contrasting results were seen by ELISA following treatment with glutaraldehyde, where binding appeared to decrease to approximately 30% of the control (Fig. 15). Decreases were also apparent following treatment with

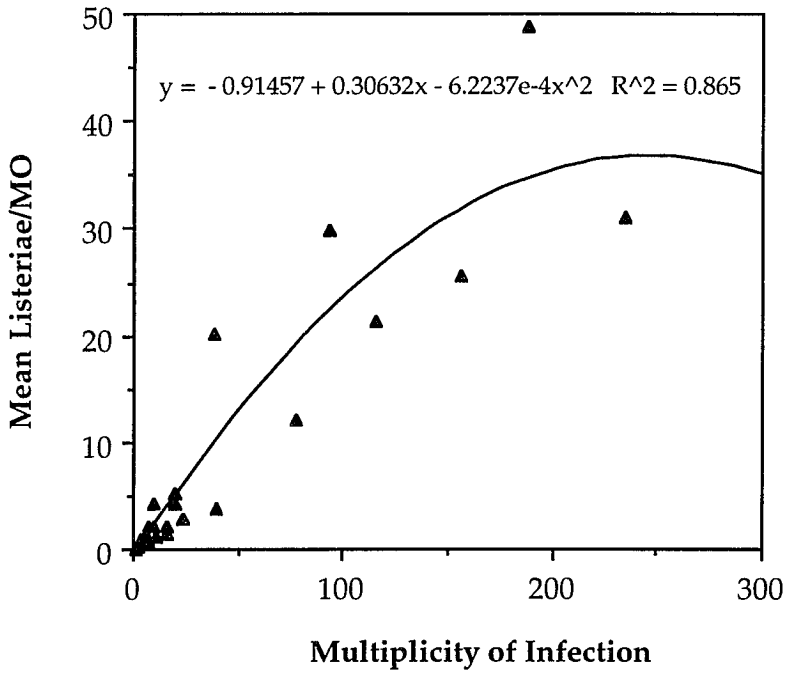

Figure 13: Adherence rate of *L. monocytogenes* to host cell receptors using varying multiplicities of infection of *L. monocytogenes*. Data represent points taken from four trials measuring the number of bacteria bound to the MØs following the 1h infection period. An MOI of 100 was used for subsequent experiments.

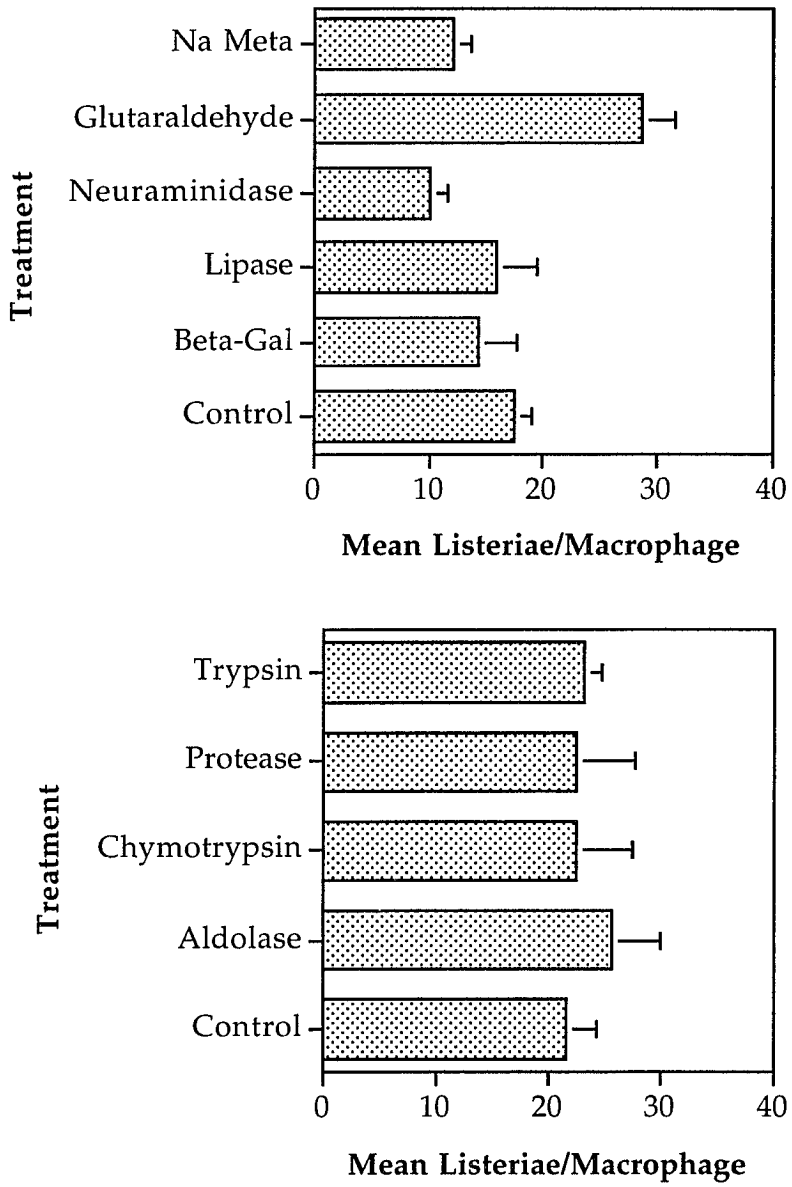

Figure 14: Adherence of *L. monocytogenes* to MØs following surface treatment of the organism as measured by IFA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

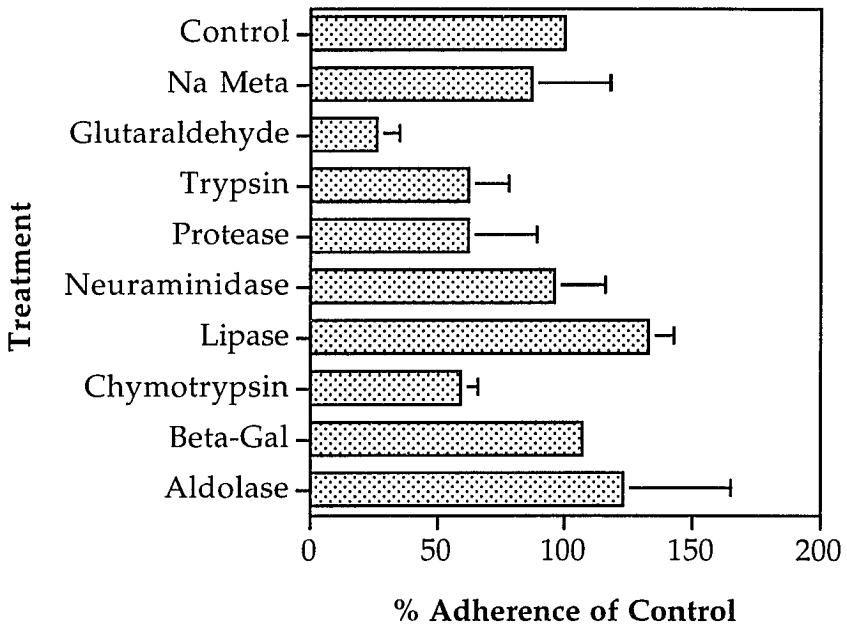

Figure 15: Adherence of *L. monocytogenes* to MØs following surface treatment of the organism as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

sodium metaperiodate (10%), trypsin (40%), protease (40%), neuraminidase (5%), and chymotrypsin (45%). When analyzed by ANOVA, however, only the trials using β-galactosidase, lipase, and neuraminidase were statistically significant with a P value below 0.05 (Appendix 7.5). Of these treatments, β-galactosidase and lipase actually showed a significant increase in binding whereas the effect of neuraminidase was not significant at 95% by the Scheffe F-test. With the exception of glutaraldehyde, none of these adhesin-modifying agents was shown to decrease the viability of the bacterial population as determined by CFU counts (Fig. 16A). Additionally, these prokaryotic treatments did not affect the sensitivity of the ELISA and IFA detection systems used (Fig. 16B), although the presence of glutaraldehyde-treated listeriae produced a dampening effect on the ELISA detection system (Fig. 17).

4.4.3 Host Cell Surface Modification

Results from the host cell surface modification treatment assays are summarized in Figures 18 and 19. Modification of host cell surfaces by enzyme or fixative treatment, with the exception of glutaraldehyde and formaldehyde, appeared to have little or no effect on bacterial binding when assayed by IFA (Fig. 18). When analyzed by ANOVA, the data from ELISA trials was statistically significant, with a P value of 0.0001 (Fig. 19 and

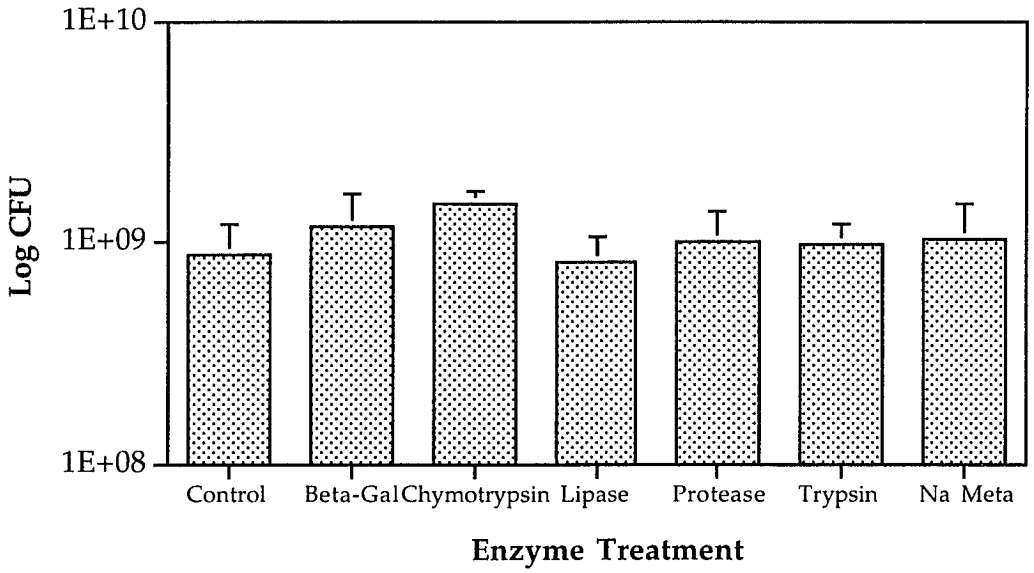

Figure 16A: Effect of prokaryotic treatment on viability of the organism by CFU assay. Data represent the mean of at least 3 trials. Bars represent standard deviation of the mean.

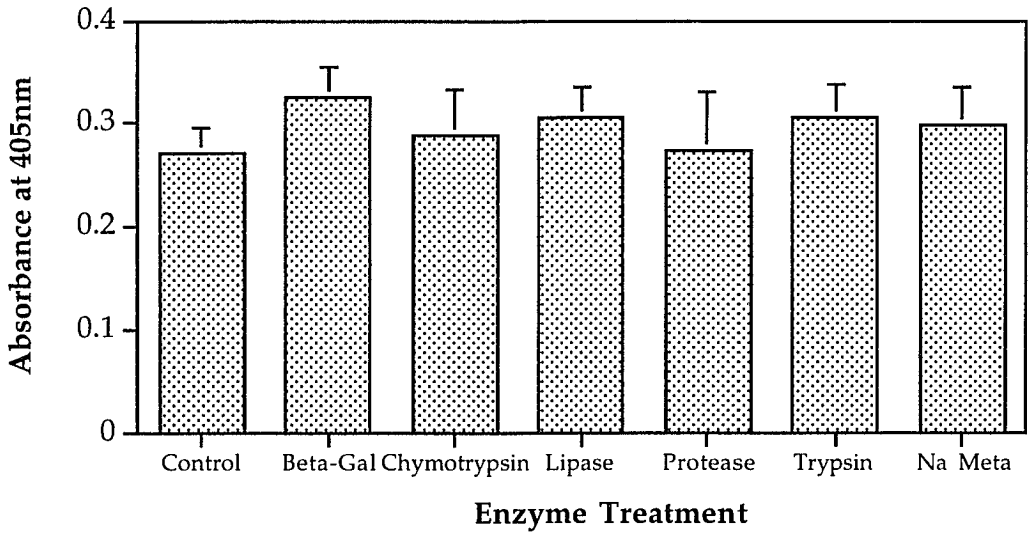

Figure 16B: Effect of prokaryotic treatment on detection of the organism by ELISA. Data represent the mean of at least 3 trials. Bars represent standard deviation of the mean.

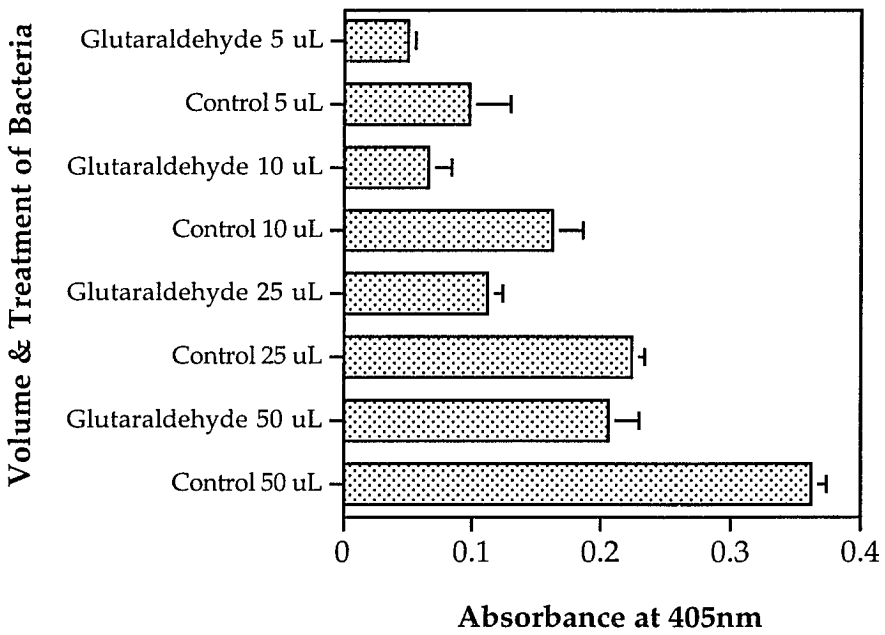

Figure 17: Effect of glutaraldehyde-treated bacteria on the ELISA detection system. Organisms were harvested, washed 3X with HBSS, and treated with glutaraldehyde or left in HBSS (controls) for 10 min. The listeriae were then washed 3X with HBSS and identical amounts pipetted into wells on a 96-well plate. Plates were dried at 37°C overnight and processed as described in section 4.3.7. Results are the average of six wells per volume shown, and bars represent standard deviation of the mean.

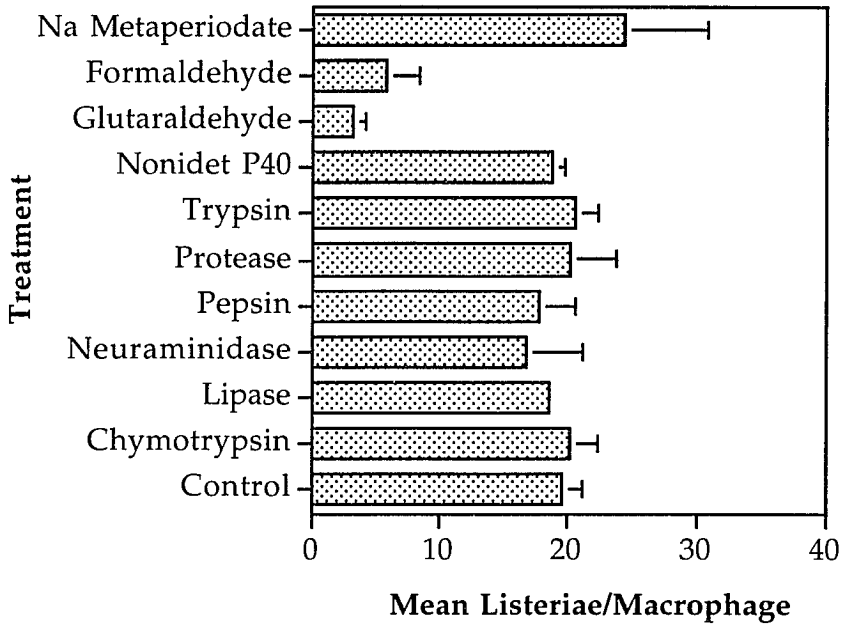

Figure 18: Adherence of *L. monocytogenes* to MØs following treatment of surface membrane receptors on MØs as measured by IFA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

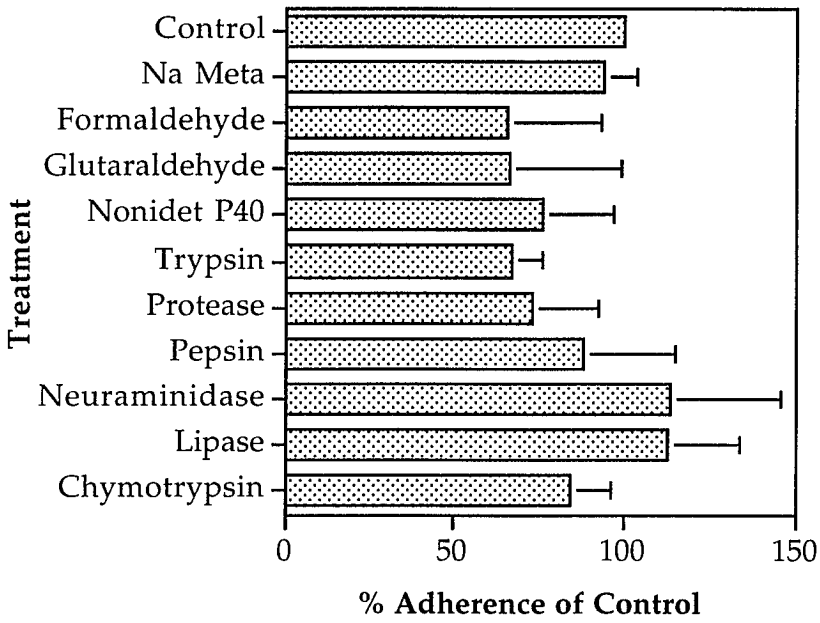

Figure 19: Adherence of *L. monocytogenes* to MØs following treatment of surface membrane receptors on MØs as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

Appendix 7.5). Using the Scheffe F test, however, only formaldehyde was significantly different at 95% from the control wells.

4.4.4 Competitive Binding Treatments

Coincubation of the saccharides with *L. monocytogenes* in the presence of host cells indicated that N-acetylneuraminic acid (NAcNeu) dramatically lowered binding of the organism to receptor sites on the host cells (Figures 20 and 21). The remaining saccharides did not appear to compete for binding sites on the host cell. ANOVA analysis of the ELISA data from the sugar assays produced significant P values, with glucose, mannose, NAcGal, NAcGlu, and NAcNeu statistically significant at 95%. The addition of the lectin *Maackia amurensis* caused a strong reduction in binding of the organism by both IFA and ELISA (Figures 22 and 23). WGA also had an adverse effect on binding. The lectins Concanavalin A and *Limulus polyphemus* did not reduce binding of the organism to host cells. *Maackia amurensis* has binding specificity for oligosaccharides with a terminal NAcNeu α(2,3)Gal linkage, while wheat germ agglutinin (*Triticum vulgaris*) has binding specificity for both NAcGlu and NAcNeu. ANOVA analysis of the ELISA data from the lectin assays indicated that the data was not significant, as the P value was greater than 0.05.

The competitive binding effect of NAcNeu at decreasing concentrations is shown in Figures 24 and 25. The greatest effect was seen at a

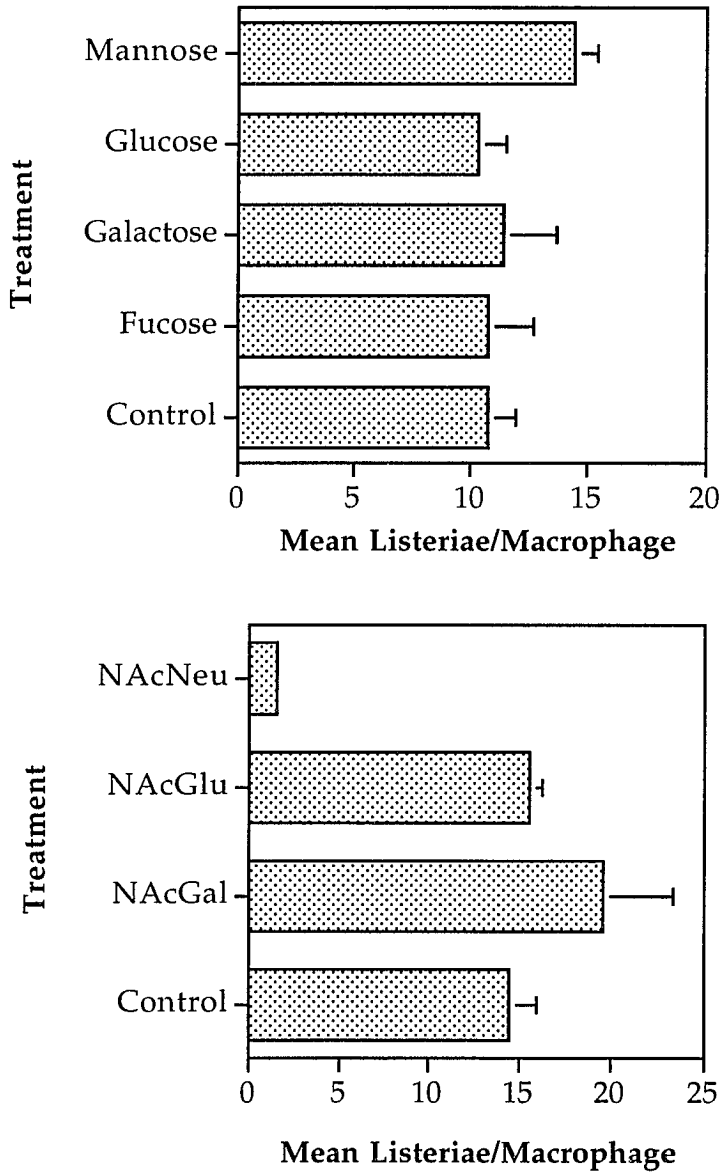

Figure 20: Adherence of *L. monocytogenes* to MØs following treatment with competitive binding sugars as measured by IFA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

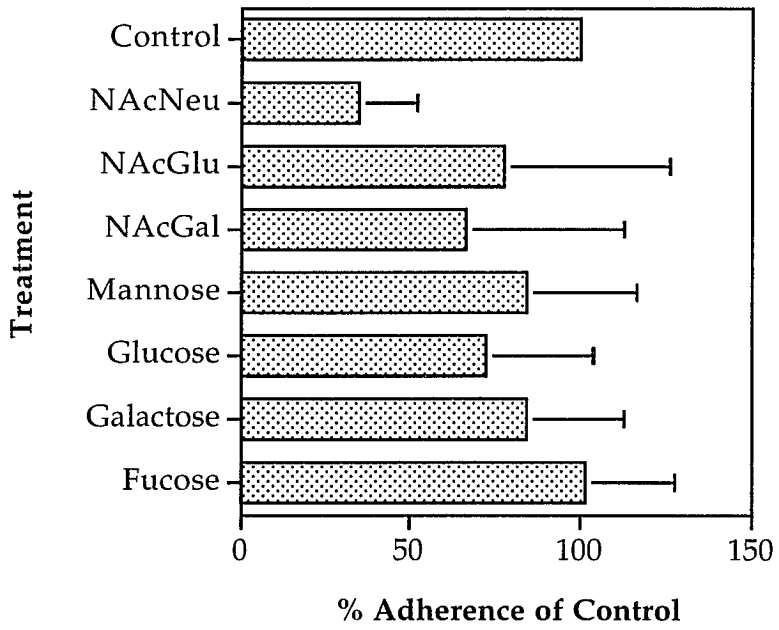

Figure 21: Adherence of *L. monocytogenes* to MØs following treatment with competitive binding sugars as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

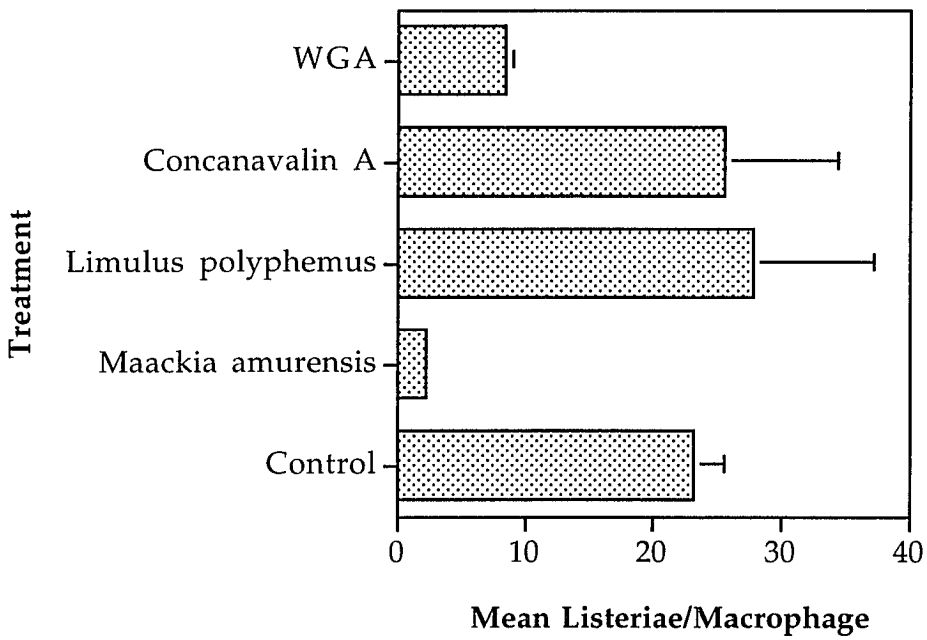

Figure 22: Adherence of *L. monocytogenes* to MØs following treatment with lectins as measured by IFA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

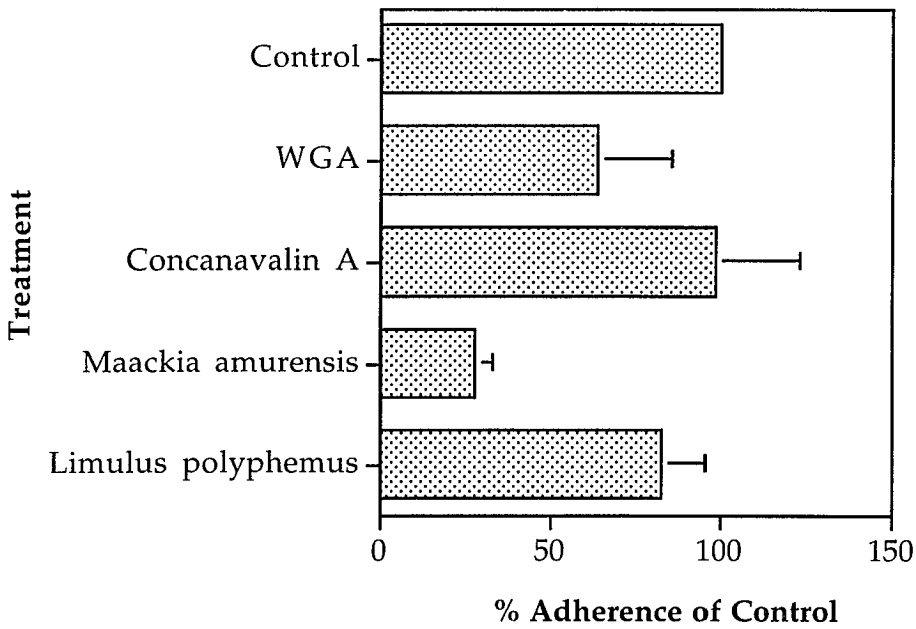

Figure 23: Adherence of *L. monocytogenes* to MØs following treatment with lectins as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

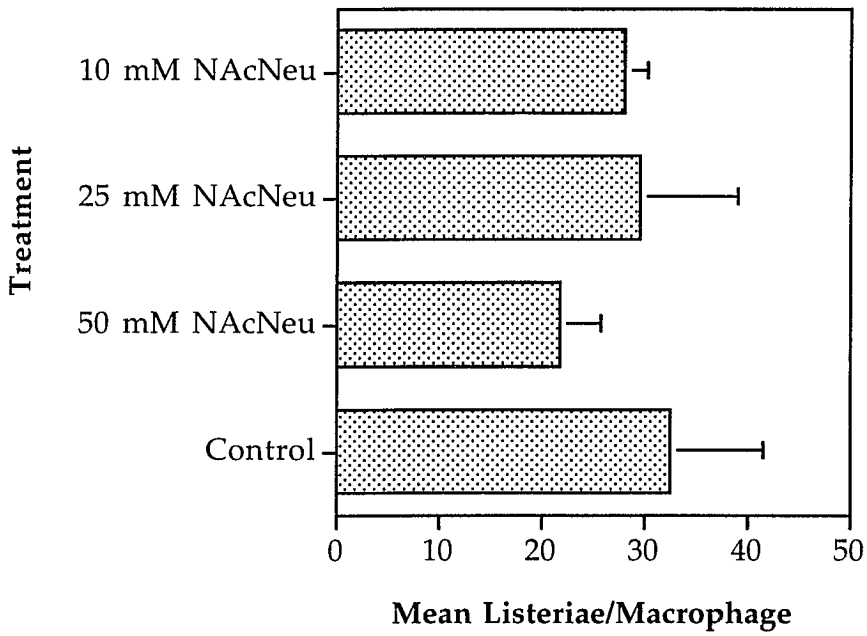

Figure 24: Adherence of *L. monocytogenes* to MØs following treatment with decreasing concentrations of N-acetylneuraminic acid as measured by IFA. Data represent the mean of 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

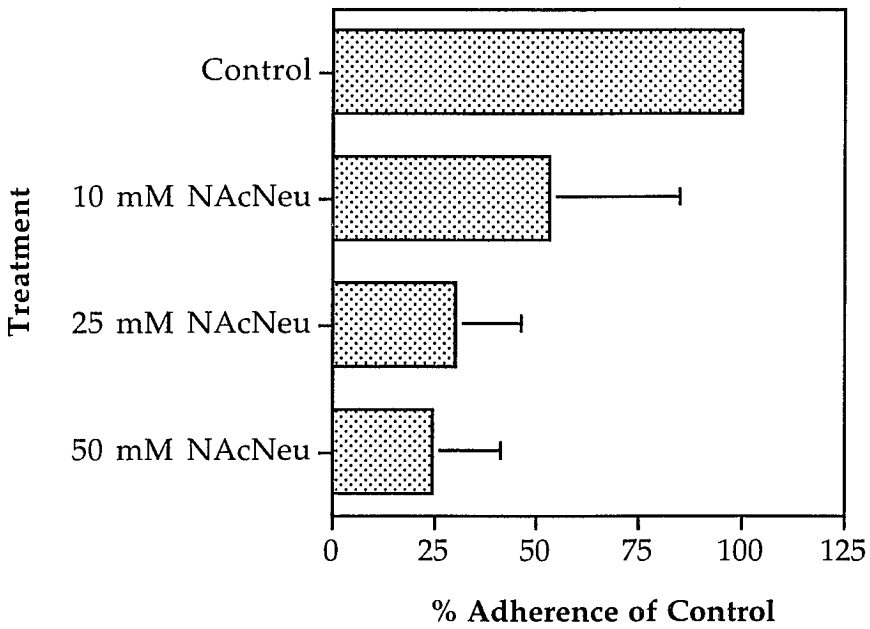

Figure 25: Adherence of *L. monocytogenes* to MØs following treatment with decreasing concentrations of N-acetylneuraminic acid as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

concentration of 50mM NAcNeu, with less effect seen at 25 and 10mM concentrations. Analysis of ELISA data by ANOVA was statistically insignificant, with a P value greater than 0.05.

4.4.5 Opsonin-Dependent Adherence Studies: N-Acetyl Neuraminic Acid

NAcNeu produced a dramatic inhibitory effect on binding of *L. monocytogenes* opsonized with 5% NMS (Fig. 26). A large discrepancy was noted between binding of the opsonized control bacteria as measured by ELISA and IFA (Figures 26 and 27). When analyzed by ANOVA, the ELISA data was statistically valid, with a P value of 0.0012. However, in contrast to the marked effect seen by IFA, the ELISA data indicated that NAcNeu was not statistically significant at 95%.

4.4.6 M1/70 Monoclonal Antibody Studies

Use of the M1/70 monoclonal antibody to the CR3 receptor produced a slight decrease in the binding of opsonized *L. monocytogenes* as measured by IFA (Fig. 28). Actual increases in binding occurred in ELISA trials; however, analysis of ELISA data by ANOVA indicated that the data was not statistically significant, with a P value greater than 0.05 (Fig. 29 and Appendix 7.5). M1/70 also had little or no effect on the binding of nonopsonized *L. monocytogenes* as seen by IFA (Fig. 30). Following analysis, the ELISA data produced only one concentration of M1/70 which was significant at 95% (Fig. 31 and Appendix 7.5).

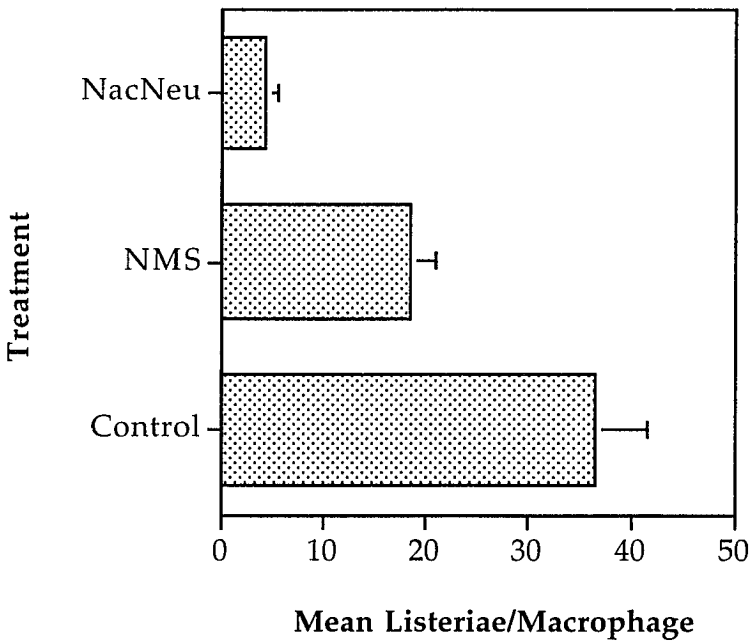

Figure 26: Adherence of opsonized *L. monocytogenes* to MØs following treatment with NAcNeu as measured by IFA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

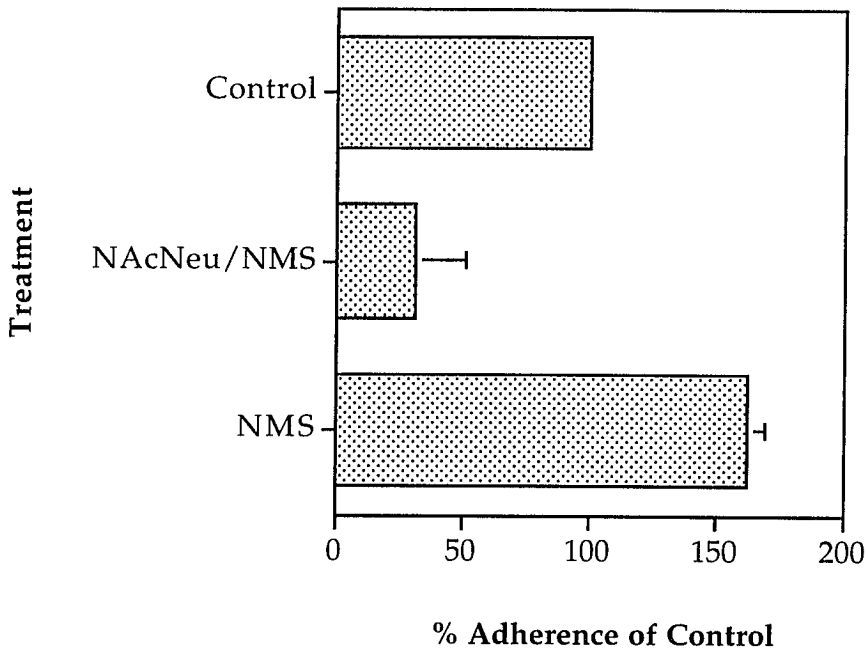

Figure 27: Adherence of opsonized *L. monocytogenes* to MØs following treatment with NAcNeu as measured by ELISA. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

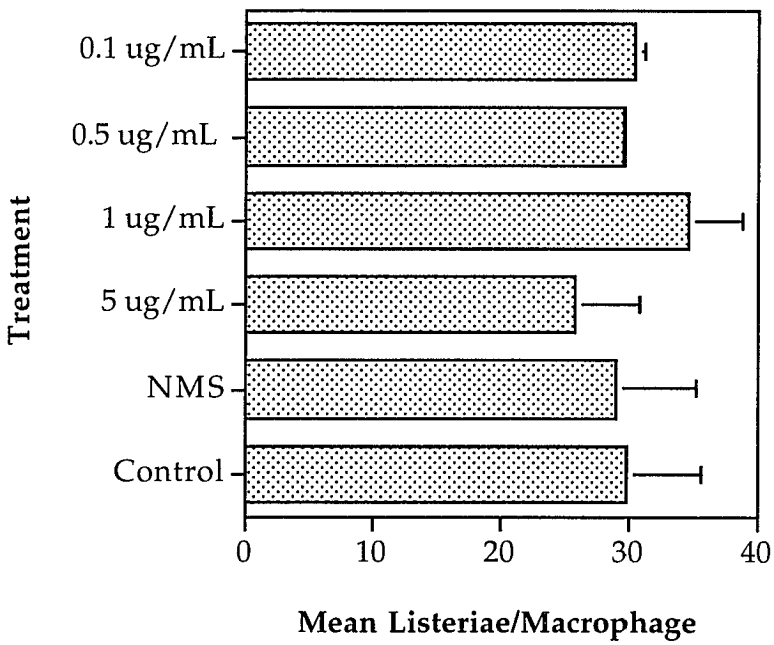

Figure 28: Adherence of opsonized *L. monocytogenes* to MØs following specific M1/70 MAb blocking treatments of CR3 receptors on host cells as measured by IFA. Goat anti-rabbit IgG was used in all samples to saturate Fc receptors on the surface of MØs prior to addition of complement receptor-specific MAb at the concentrations shown. Data represent the mean of three trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

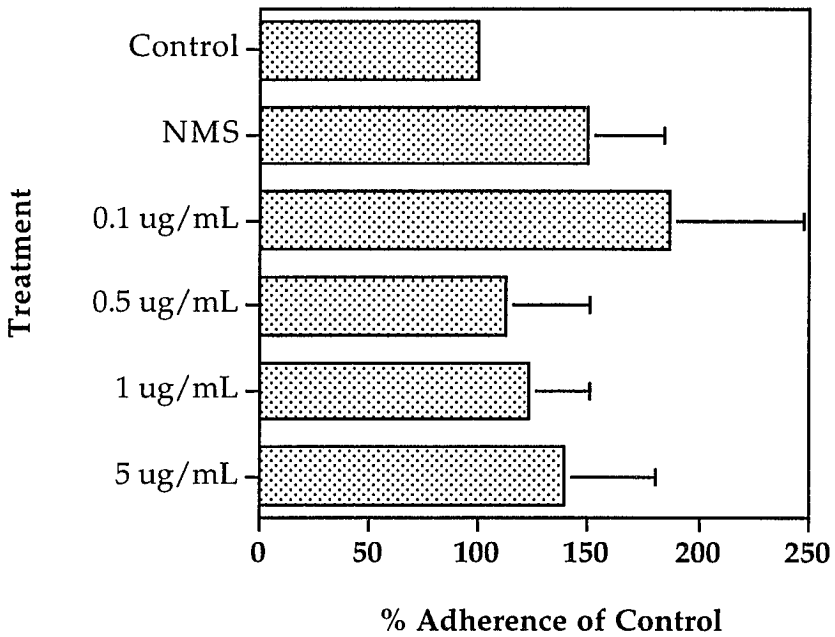

Figure 29: Adherence of opsonized *L. monocytogenes* to MØs following specific M1/70 MAb blocking treatments of CR3 receptors on host cells as measured by ELISA. Goat anti-rabbit IgG was used in all samples to saturate Fc receptors on the surface of MØs prior to addition of complement receptor-specific MAb at the concentrations shown. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

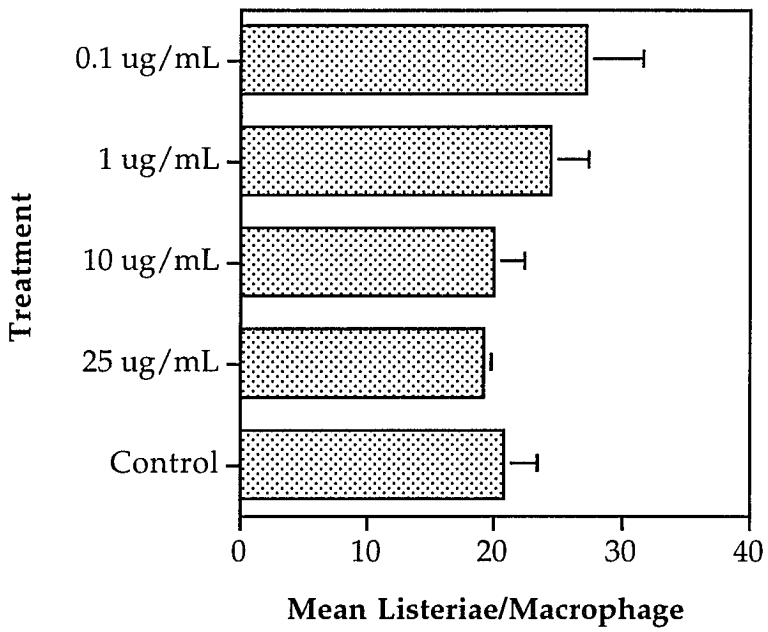

Figure 30: Adherence of nonopsonized *L. monocytogenes* to MØs following specific M1/70 MAb blocking treatments of CR3 receptors on host cells as measured by IFA. Goat anti-rabbit IgG was used in all samples to saturate Fc receptors on the surface of MØs prior to addition of complement receptor-specific MAb at the concentrations shown. Data represent the mean of three trials measuring the number of *L. monocytogenes* bound to MØs. Bars represent standard deviation of the mean.

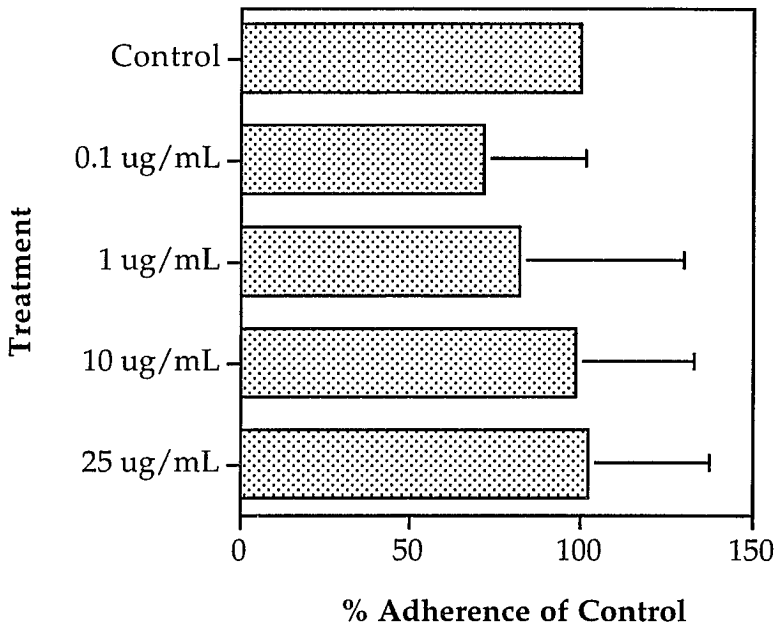

Figure 31: Adherence of nonopsonized *L. monocytogenes* to MØs following specific M1/70 MAb blocking treatments of CR3 receptors on host cells as measured by ELISA. Goat anti-rabbit IgG was used in all samples to saturate Fc receptors on the surface of MØs prior to addition of complement receptor-specific MAb at the concentrations shown. Data represent the mean of at least 3 trials measuring the number of *L. monocytogenes* bound to MØs. Controls were normalized for each trial to 100%. Bars represent standard deviation of the mean.

4.5 Discussion and Conclusions

Adherence to host cell surfaces is a necessary first step in infection by intracellular bacterial pathogens such as *L. monocytogenes*. However, it has been shown that this initial step can take place by two different mechanisms: an opsonin-dependent process, in which antibody and/or complement proteins become involved in the complex interaction between bacteria and host cell; and an opsonin-independent process, in which adhesins present on the bacterial cell surface recognize host cell receptors. These two processes may also result in very different reactions by the host cell to the bacterium; for *L. monocytogenes*, uptake by listericidal macrophages via the CR3 receptor resulted in death of the organism, whereas uptake by nonlistericidal macrophages occurred through some other recognition factor and resulted in intracellular replication of the organism (32). These varied responses may be due to a signalling process which occurs following binding of the organism to the CR3, inducing the MØ to kill the organism. Alternatively, this response does not occur following binding of *Listeria* to another, opsonin-independent receptor. Adherence of *L. monocytogenes* to host cell membrane associated structures is therefore a complex interaction.

Following preliminary studies which established the ability of *L. monocytogenes* to bind to MØs in a wash-resistant manner, the adherence rate of *L. monocytogenes* to host cells and its ability to saturate host cell

receptors was investigated. At an MOI greater than 200, the level of nonspecific binding of *L. monocytogenes* to culture plates as determined by IFA proved problematic in ELISA assays. Saturation of host cell receptors could not be determined, as the background bacteria could not be distinguished from host-cell-bound organisms at this inoculum. An MOI of 100 gave acceptable receptor saturation with reduced background binding while affording the advantages of greater significance to a reduction in binding as a result of a particular treatment. The number of listeriae per cell averaged from 20-25; a 50% reduction due to enzyme treatment or competitive binding substances would reduce this to a mean of 10-12 organisms per host cell. In this study the presence of an opsonin-independent attachment mechanism for *L. monocytogenes* to murine peritoneal MØs has been established.

The monosaccharides used in competitive binding studies were selected based on previous reports of adherence studies on *L. monocytogenes* and other organisms (23,41). As *L. monocytogenes* is known to utilize glucose and galactose as nutrient sources, it is possible that such sugars may be utilized on the cell wall surface as portions of adhesin structures. In addition, NAcGlu, together with muramic acid, makes up the bacterial cell peptidoglycan (86). Group B streptococci produce a capsule made up of NAcNeu in chains; as *Listeria* are related to the streptococci, this sugar was

chosen for competitive binding studies (83). Clearly, the number of mono-, di-, or oligosaccharides other than those used may have also functioned to block binding. However, our experiments produced distinct inhibition of adherence in the presence of NAcNeu, suggesting a role for this monosaccharide in the binding of *L. monocytogenes* to MØs. It was subsequently decided to pursue this discovery with related treatments of the bacterial and MØ surfaces.

A strong inhibition of attachment was seen in the presence of 100 mM NAcNeu by both ELISA and IFA. NAcGlu, NAcGal, mannose, and glucose had a statistically significant effect by ELISA, although this was not confirmed by IFA data. These results contrast with an earlier report on the binding of *L. monocytogenes* to the human liver hepatocarcinoma cell line HepG2 (ATCC) (23). These workers observed a reduction in binding of the organism in the presence of D-galactose, and a corresponding reduction in binding after pretreatment of the host cell with neuraminidase to render the host cell galactose receptor functionally inactive (23). In the studies reported here, the presence of D-galactose or pretreatment of the host cell with neuraminidase had no effect on binding. The contradiction here may be due to the different host cell types used (liver carcinoma versus murine macrophage) or a difference in the isolate used for the studies. Interestingly, it has been shown that the adherence of two different species of *Shigella*, an organism which has

an intracellular growth cycle analogous to that of *L. monocytogenes*, is inhibited by NAcNeu (45).

Results from the use of lectins in similar binding assays strongly support the involvement of NAcNeu. The lectins selected for these studies were chosen for their ability to bind specific sugars or linkages of sugars (41). The lectins from *Maackia amurensis* (MA), *Limulus polyphemus*(LP), and *Triticum vulgaris* (WGA) have affinities for different linkages containing NAcNeu (Table 12). Concanavalin A does not have an affinity for this sugar, and was therefore used as a negative control. The MA lectin showed similar inhibitory effects to NAcNeu on binding of *L. monocytogenes*; this lectin has an affinity for binding specificity for oligosaccharides with a terminal NAcNeu α (2,3)Gal linkage. WGA reduced binding of *L. monocytogenes* by almost 50%; this lectin has binding specificity for both NAcGlu and NAcNeu.

The LP lectin is also known to bind NAcNeu and N-glycolyl-neuraminic acid. However, this lectin is extremely large, with an aggregate molecular weight of between 35-50kDa, with four major protein bands seen on SDS-PAGE whereas the MA lectin is a single polypeptide of 8-9kDa on SDS-PAGE. Steric hindrance may therefore be a factor in the prevention of competition of the LP lectin with the adhesin of the organism. Additionally, this lectin requires the addition of calcium for binding to NAcNeu in either glycopeptide or the free monomeric form. Use of this lectin in HBSS without

calcium or magnesium would therefore appear to be inhibitory to its ability to compete with the organism for binding sites.

The prokaryotic surface treatments were chosen either for a broad range of action, such as protease or lipase, or for specific action, such as chymotrypsin, neuraminidase, pepsin, and trypsin (Table 11) (23,41). These enzymatic or detergent treatments of the bacterial surface were used to determine whether a glycoprotein or glycolipid containing NAcNeu was the adhesin molecule. Bacterial surface treatments produced a decrease (as seen by IFA) in the binding of the organism following treatment with neuraminidase. ELISA data did not confirm these results when analyzed by ANOVA. However, the treatment was carried out at pH 7.0, whereas the optimal pH for the functioning of neuraminidase is pH 5.0. Due to the effect of the lowered pH on bacterial viability, such treatment was not carried out.

Treatment with glutaraldehyde appeared to increase binding by 60% as measured by IFA. This may be due to cross-linking of surface proteins between individual organisms and subsequent binding of these linked listeriae to the cell surface. In contrast, ELISA data for treatment with glutaraldehyde showed a statistically significant decrease in binding. However, tests performed using glutaraldehyde-treated listeriae in the absence of MØs showed a distinct decrease in absorbance in the presence of these bacteria as compared to untreated controls. It is probable, therefore, that

the indicator enzyme HRP (horse-radish peroxidase) used in the ELISA system was inhibited by residual amounts of glutaraldehyde present in the washed listeriae.

Treatment with sodium metaperiodate appeared to inhibit binding when assayed by IFA; however, the effect of this agent on adherence was statistically insignificant when assayed by ELISA. Sodium metaperiodate oxidizes carbohydrate moieties which should adversely affect NAcNeu residues present on the organism or host cell surface. However, as oxidation of the host cell surface produced less inhibition than did treatment of the prokaryotic surface, the results from this treatment remain ambiguous. Treatment of the bacterial surface with enzymes other than neuraminidase showed a detectable inhibition following treatment with β–galactosidase, an enzyme which hydrolyzes lactose to galactose and glucose. Lactose was not used in competitive binding assays, however, as the inhibition from β–galactosidase treatment was not as dramatic as that seen with the monosaccharide, NAcNeu.

Lipase treatments resulted in somewhat contradictory results, with a slight decrease by IFA and an increase by ELISA (significant in ANOVA to 95%). Use of the proteolytic enzymes with both specific and broad ranges of action did not significantly inhibit binding of the organism. Additional trials

were carried out using increased concentrations of enzymes without noticeable change of the adherence of the organism.

Receptor modification studies showed decreased adherence of *L. monocytogenes* following treatment of the host cell surface structures with formaldehyde and glutaraldehyde. Enzyme treatment of the host cell membrane had little or no effect on the adherence of the organism. Additionally, treatment with nonidet p40 and sodium metaperiodate proved ineffective at reducing adherence by degrading lipids (nonidet p40) or oxidizing carbohydrates (sodium metaperiodate) on the host cell surface. The results would appear to indicate that the receptor or receptors involved are protein in nature but either sterically protected from the effects of the proteolytic enzymes or present in sufficient numbers to allow binding to occur at numbers similar to the control cells. It is possible that an increase in the concentration of these treatments would result in a loss of bacterial adherence; however, due to possible adverse effects on the host cell such increases were not considered to be a viable alternative. It is interesting to note that formaldehyde and glutaraldehyde, agents that cross-link proteins, have such a pronounced effect on binding but little or no effect is seen following treatment with proteolytic enzymes.

Recent studies have indicated the involvement of complement receptor 3 (CR3) in the opsonin-mediated phagocytosis of *L. monocytogenes*

by listericidal MØs; CR3 is not, however, involved in the phagocytosis of the organism by permissive, nonlistericidal MØs (32,34). It was determined by the same workers that the complement receptors 1 and 4 were not involved in the adherence process (31,77,90,110). Accordingly, the effect of the anti-CR3 MAb on adherence of *L. monocytogenes* as measured were determined using the assays described here. The presence of the MAb appeared to have little or no inhibitory effect on the binding of *L. monocytogenes* to MØs in the presence or absence of normal mouse serum. These results are similar to those reported by Drevets and Campbell (1991), in which reduction of binding of *L. monocytogenes* by anti-CR3 had a limited effect on nonlistericidal, thioglycollate-elicited MØs (32). However, in contrast to their reported results, the presence or absence of NMS in our system did not appear to have an effect on binding and approximately the same number of listeriae bound to both groups of control cells (32). The results reported here appear to confirm that adherence and uptake in permissive MØs occurred through some mechanism other than CR3. Significantly, NAcNeu at a concentration of 100mM was inhibitory to the binding of opsonized *L. monocytogenes.*

In contrast to group B streptococci, *L. monocytogenes* do not appear to utilize CR3 as a means of attachment to nonlistericidal, permissive MØs (2,32) . However, the adhesin utilized by listeriae to attach to these host cells appeared to be identical. Attachment in both the presence and absence of

opsonins was strongly inhibited by NAcNeu. Based on these results, it is proposed that NAcNeu, a member of the sialic acid group, is involved in the attachment of *L. monocytogenes* to nonlistericidal, permissive host cells. This process of attachment appears to occur through some receptor other than CR3, and permits the intracellular replication of the organism inside host cells, resulting in disease. Understanding the nature of this adherence mechanism in both the presence and absence of opsonins is critical to elucidating the nature of the intracellular parasitism of this organism and may prove to be an important factor in the development of a vaccine for the prevention of listeriosis.

SECTION 5

CONCLUDING REMARKS

5.1 General Conclusions

Listeria monocytogenes is the source of approximately 1700 cases of listeriosis in the U.S. annually, associated with a high mortality rate in humans of up to 40-50% (53,97). Interest in the pathogenesis of *L. monocytogenes* has increased due to a number of major epidemic outbreaks of this organism during the 1980s as well as due to an evident increase in the incidence of sporadic cases of listeriosis (99). The infectious process by which *L. monocytogenes* causes disease has therefore been investigated extensively during the past several years.

Adherence of *L. monocytogenes* is a necessary first step in the infectious process of facultative intracellular pathogens; work done in this area has suggested that the mechanism by which the organism adheres and is taken up by host cells plays a major role in determining its intracellular fate. In the present work, an opsonin-independent process of adherence has been identified and the bacterial adhesin involved has been partially characterized. Complement, opsonization, and complement receptors were not seen to be involved in this process. Cytochalasin treatment did not affect the binding of

the organism to host cells; this binding was highly wash-resistant. Higher numbers of bacteria were seen attached to MØs than were taken up by these cells. The attachment mechanism led to intracellular replication of the organism in the MØs. Rates of replication observed were equivalent to those seen in cell lines of similar lineage but faster than those seen in nonprofessional phagocytes (38).

In these studies, NAcNeu was identified as being involved in the infection of murine peritoneal MØs by *L. monocytogenes*. This molecule appeared to function in both opsonin-dependent and opsonin-independent binding, as the presence of NAcNeu had an inhibitory effect on attachment of the organism under either test condition. The host-cell receptor for binding *L. monocytogenes* was more difficult to identify; it appeared to be at least partially protein in nature since aldehyde treatment prevented attachment of organisms but treatment with specific proteolytic enzymes had no effect on binding. Additionally, complement receptor 3 did not appear to be involved as a receptor by nonlistericidal MØs in either an opsonin-dependent or opsonin-independent system, as monoclonal antibodies directed against CR3 had no effect on *Listeria* binding. As CR3 is glycoprotein in nature, it is possible that oxidation of host cell surfaces using sodium metaperiodate should have an influence on the receptor if it is involved in bacterial adherence (77,110). However, inhibition of organism binding was not seen

following this treatment.

An opsonin-dependent process involving deposition of complement component C3 and complement receptor 3 functions during uptake of *L. monocytogenes* by listericidal MØs (32). This mechanism does not, however, appear to be involved during uptake by nonlistericidal, permissive MØs. Indeed, such an alternative method of binding may be central to the initial establishment of disease in the host (32). The identification of alternative attachment mechanisms may prove useful in the search for vaccines for use as preventative treatments for listeriosis. Such vaccines would be of particular use in the prophylactic treatment of those individuals at highest risk for this disease, namely, pregnant women and the immunocompromised.

5.2 Future Studies

Host infection with *L. monocytogenes* results from the expression of a complex series of virulence factors which are initiated upon adherence to and entry of the organism into the host cell. Several lines of inquiry emerge from the present study which merit further investigation:

1- Studies carried out using organisms grown at 4°C to simulate the growth conditions found in refrigerated food products to determine whether NAcNeu is expressed and utilized at this temperature.

2- Monoclonal antibodies raised against the surface glycoproteins of *L.*

monocytogenes should be produced to challenge the organism-host cell binding; those effective in binding could subsequently be used in an animal model to test their effectiveness at blocking binding and preventing disease in *vivo*.

3- Identification of the genes responsible for the expression of the adherence factors on *L. monocytogenes* should be conducted. The generation of isogenic strains by transposon mutagenesis should be carried out to isolate mutants that do not express the adhesin and are of low binding potential. The genes so isolated could be cloned into nonbinding strains of *E. coli* and the adherence of these clones measured and compared to the parent *Listeria* In addition, from the DNA sequence, the protein sequence could be inferred and pure adhesins generated by protein synthesis. Such adhesins could then be evaluated for their role in adherence both at the cellular level and in an animal model.

4- Clearance of listeriosis by the host is highly dependent upon recognition and activation by the cell-mediated immune system, i.e., T cells and Natural Killer cells. Data on the interactions of *Listeria*-infected MØs with $CD8^+$ T cells may profoundly influence development of a vaccine, as the influence of the humoral immune system upon this pathogen is minimal.

5- The host cell receptors involved in the initial attachment under opsonin-independent conditions should be identified by isolating the cell

membrane and electrophoresing those receptors using a nondenaturing gel. These could subsequently be transferred by Western blot and probed with *L. monocytogenes*.

REFERENCES

1. **Alvarez, Dominguez,C., E. Carrasco-Marin, and F. Leyva-Cobian.** 1993. Role of complement C1q in phagocytosis of *Listeria monocytogenes* by murine macrophage-like cell lines. Infect. Immun. **61**:3665-3672.

2. **Antal, J. M., J. V. Cunningham, and K. J. Goodrum.** 1992. Opsonin-independent phagocytosis of group B streptococci: role of complement receptor type three. Infect. Immun. **60**:1114-1121.

3. **Barclay, R., D. R. Threlfall, and I. Leighton.** 1989. Hemolysins and extracellular enzymes of *Listeria monocytogenes* and *L. ivanovii*. J. Med. Microbiol. **30**:111-118.

4. **Basher, H. A., D. R. Fowler, F. G. Rodgers, A. Seaman, and M. Woodbine.** 1984. Pathogenesis and growth of *Listeria monocytogenes* in fertile hens' eggs. Zbl. Bakt. Hyg. A **256**:497-509.

5. **Basher, H. A., A. Seaman, and M. Woodbine.** 1983. Infection, hemorrhagia, and death of chick embryos experimentally inoculated with *Listeria monocytogenes* by the intra-allantoic route. Zbl. Bakt. Hyg. A **255**:239-246.

6. **Beckerman, K. P., H. W. Rogers, J. A. Corbett, R. D. Schreiber, M. L. McDaniel, and E. R. Unanue.** 1993. Release of nitric oxide during the T cell-independent pathway of macrophage activation: its role in resistance to *Listeria monocytogenes*. J. Immunol. **150**:888-895.

7. **Berenguer, J., J. Solera, M. D. Diaz, S. Moreno, J. A. Lopez-Herce, and E. Bouza.** 1991. Listeriosis in patients infected with human immunodeficiency virus. Rev. Inf. Dis. **13**:115-119.

8. **Bille, J. and M. P. Doyle.** 1991. *Listeria* and *Erysipelothrix*, p. 287-295. In A. Balows (ed.), Manual of Clinical Microbiology. American Society for Microbiology, Washington, D.C..

9. **Bohne, J., Z. Sokolovic, and W. Goebel.** 1994. Transcriptional regulation of *prfA* andPrfA-regulated virulence genes in *Listeria monocytogenes*. Mol. Microbiol. **11**:1141-1150.

10. **Brundage, R. A., G. A. Smith, A. Camilli, J. A. Theriot, and D. A. Portnoy.** 1993. Expression and phosphorylation of the *Listeria monocytogenes* ActA protein in mammalian cells. Proc. Natl. Acad. Sci. USA **90**:11890-11894.

11. **Bubert, A., M. Kuhn, W. Goebel, and S. Kohler.** 1992. Structural and function properties of the p60 proteins from different *Listeria* species. J. Bact. **174**:8166-8171.

12. **Buchrieser, C., R. Brosch, B. Catimel, and J. Rocourt.** 1993. Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. Can. J. Microbiol. **39**:395-401.

13. **Bula, C. J., J. Bille, and M. P. Glauser.** 1995. An epidemic of food-borne listeriosis in Western Switzerland: description of 57 cases involving adults. Clin. Infect. Dis. **20**:66-72.

14. **Camilli, A., H. Goldfine, and D. A. Portnoy.** 1991. *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. J. Exp. Med. **173**:751-754.

15. **Camilli, A., D. A. Portnoy, and P. Youngman.** 1990. Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn*917* derivative that allows direct cloning of DNA flanking transposon insertions. J. Bact. **172**:3738-3744.

16. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8**:143-157.

17. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bact. **174**:568-574.

18. **Cherubin, C. E., M. D. Appleman, P. N. R. Heseltine, W. Khayr, and C. W. Stratton.** 1991. Epidemiological spectrum and current treatment of listeriosis. Rev. Infect. Dis. **13**:1108-1114.

19. **Collins, M. D., S. Wallbanks, D. J. Lane, J. Shah, R. Nietupski, J. Smida, M. Dorsch, and E. Stackebrandt.** 1991. Phylogenetic analysis of the genus *Listeria* based on reverse transcriptase sequencing of 16S rRNA. Int. J. Syst. Bact. **41**:240-246.

20. **Conlan, J. W., P. L. Dunn, and R. J. North.** 1993. Lekocyte-mediated lysis of infected hepatocytes during listeriosis occurs in mice depleted of NK cells or CD4+ CD8+ Thy1.2+ T cells. Infect. Immun. **61**:2703-2707.

21. **Cossart, P. and C. Kocks.** 1994. The actin-based motility of the facultative intracellular pathogen *Listeria monocytogenes*. Mol. Microbiol. **13**:395-402.

22. **Cossart, P., M. F. Vicente, J. Mengaud, F. Baquero, J. C. Perez-Diaz, and P. Berche.** 1989. Listeriolysin O is essential for virulence of *Listeria monocytogenes*: direct evidence obtained by gene complementation. Infect. Immun. **57**:3629-3636.

23. **Cowart, R. E., J. Lashmet, M. E. McIntosh, and T. J. Adams.** 1990. Adherence of a virulent strain of *Listeria monocytogenes* to the surface of a hepatocarcinoma cell line via lectin-substrate interaction. Arch. Microbiol. **153**:282-286.

24. **Croize, J., J. Arvieux, P. Berche, and M. G. Colomb.** 1993. Activation of the human complement alternative pathway by *Listeria monocytogenes*: evidence for direct binding and proteolysis of the C3 component on bacteria. Infect. Immun. **61**:5134-5139.

25. **Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. *Listeria monocytogenes* moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87**:6068-6072.

26. **De Chastellier, C. and P. Berche.** 1994. Fate of *Listeria monocytogenes* in murine macrophages: evidence for simultaneous killing and survival of intracellular bacteria. Infect. Immun. **62**:543-553.

27. **Decker, C. F., G. L. Simon, R. A. DiGioia, and C. U. Tuazon.** 1991. *Listeria monocytogenes* infections in patients with AIDS: report of five cases and review. Rev. Inf. Dis. **13**:413-417.

28. **Domann, E., M. Leimeister-Wachter, W. Goebel, and T. Chakraborty.** 1991. Moleular cloning,sequencing, and identification of a metalloprotease gene from *Listeria monocytogenes* that is species specific and physically linked to the listeriolysin gene. Infect. Immun. **59**:65-72.

29. **Domann, E., J. Wehland, K. Niebuhr, C. Haffner, M. Leimeister-Wachter, and T. Chakraborty.** 1993. Detection of a *prfA*-independent promoter responsible for listeriolysin gene expression in mutant *Listeria monocytogenes* strains lacking the PrfA regulator. Infect. Immun. **61**:3073-3075.

30. **Dramsi, S., C. Kocks, C. Forestier, and P. Cossart.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature, and the pleiotropic activator *prfA*. Mol. Microbiol. **9**:931-941.

31. **Drevets, D. A. and P. A. Campbell.** 1991. Roles of complement and complement receptor type 3 in phagocytosis of *Listeria monocytogenes* by inflammatory mouse peritoneal macrophages. Infect. Immun. **59**:2645-2652.

32. **Drevets, D. A., B. P. Canono, and P. A. Campbell.** 1992. Listericidal and nonlistericidal mouse macrophages differ in complement receptor type 3-mediated phagocytosis of *L. monocytogenes* and in preventing escape of the bacteria into the cytoplasm. J. Leuk. Biol. **52**:70-79.

33. **Drevets, D. A., B. P. Canono, P. J. M. Leenen, and P. A. Campbell.** 1994. Gentamicin kills intracellular *Listeria monocytogenes*. Infect. Immun. **62**:2222-2228.

34. **Drevets, D. A., P. J. M. Leenen, and P. A. Campbell.** 1993. Complement receptor type 3 (CD11b/CD18) involvement is essential for killing of *Listeria monocytogenes* by mouse macrophages. J. Immunol. **151**:5431-5439.

35. **Freitag, N. E. and D. A. Portnoy.** 1994. Dual promoters of the *Listeria monocytogenes prfA* transcriptional activator appear essential*in vitro* but are redundant *in vivo*. Mol. Microbiol. **12**:845-853.

36. **Freitag, N. E., L. Rong, and D. A. Portnoy.** 1993. Regulation of the *prfA* transcriptional activator of *Listeria monocytogenes*: multiple promoter elements contribute to intracellular growth and cell-to-cell spread. Infect. Immun. **61**:2537-2544.

37. **Gaillard, J. -L., P. Berche, C. Frehel, E. Gouin, and P. Cossart.** 1991. Entry of *L. monocytogenes* into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci. Cell **65**:1127-1141.

38. **Gaillard, J. -L., P. Berche, J. Mounier, S. Richard, and P. Sansonetti.** 1987. *In vitro* model of penetration and intracellular growth of *Listeria monocytogenes* in the human enterocyte-like cell line Caco-2. Infect. Immun. **55**:2822-2829.

39. **Gaillard, J. -L., P. Berche, and P. Sansonetti.** 1986. Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. Infect. Immun. **52**:50-55.

40. **Geoffroy, C., J. -L. Gaillard, J. E. Alouf, and P. Berche.** 1987. Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes*. Infect. Immun. **55**:1641-1646.

41. **Gibson, F. C., A. O. Tzianabos, and F. G. Rodgers.** 1994. Adherence of *Legionella pneumophila* to U-937 cells, guinea-pig alveolar macrophages, and MRC-5 cells by a novel, complement-independent binding mechanism. Can. J. Microbiol. **40**:865-872.

42. **Goebel, W., T. Chakraborty, E. Domann, S. Kohler, M. Kuhn, M. Leimeister-Wachter, Z. Sokolovic, and M. Wuenscher.** 1991. Studies on the pathogenicity of *Listeria monocytogenes*. Infection **19 Suppl. 4**:S195-S197.

43. **Goldfine, H. and C. Knob.** 1992. Purification and characterization of *Listeria monocytogenes* phosphatidylinositol-specific phospholipase C. Infect. Immun. **60**:4059-4067.

44. **Gray, M. L. and A. H. Killinger.** 1966. *Listeria monocytogenes* and listeric infections. Bact. Rev. **30**:309-382.

45. **Guhathakurta, B., D. Sasmal, and A. Datta.** 1992. Adhesion of *Shigella dysenteriae* type 1 and *Shigella flexneri* to guinea-pig colonic epithelial cells *in vitro*. J. Med. Microbiol. **36**:403-405.

46. **Gutekunst, K. A. and L. Pine.** 1992. A filamentous-like mutant of *Listeria monocytogenes* with reduced expression of a 60-kilodalton extracellular protein invades and grows in 3T6 and Caco-2 cells. Can. J. Microbiol. **38**:843-851.

47. **Hasty, D. L., I. Ofek, H. S. Courtney, and R. J. Doyle.** 1992. Multiple adhesins of streptococci. Infect. Immun. **60**:2147-2152.

48. **Hayes, P. S., L. M. Graves, G. W. Ajello, B. Swaminathan, R. E. Weaver, J. D. Wenger, A. Schuchat, and C. V. Broome.** 1991. Comparison of cold enrichment and U.S. Department of Agriculture methods for isolating *Listeria monocytogenes* from naturally contaminated foods. Appl. Environ. Microbiol. **57**:2109-2113.

49. **Hirsch, C. S., J. J. Ellner, D. G. Russell, and E. A. Rich.** 1994. Complement receptor-mediated uptake and tumor necrosis factor-n -mediated growth inhibition of *Mycobacterium tuberculosis* by human alveolar macrophages. J. Immunol. **152**:743-753.

50. **Hof, H.** 1991. Therapeutic activities of antibiotics in Listeriosis. Infection **19 Suppl. 4**:S 229-S233.

51. **Horwitz, M. A.** 1984. Phagocytosis of the Legionnaires' disease bacterium (*Legionella pneumophila*) occurs by a novel mechanism: engulfment within a pseudopod coil. Cell **36**:27-33.

52. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St.Geme, and S. Normark.** 1993. Pilus and nonpilus bacterial adhesins: assembly and function in cell recognition. Cell **73**:887-901.

53. **Jones, D.** 1990. Foodborne illness: Foodborne listeriosis. Lancet **336**:1171-1174.

54. **Jurado, R. L., M. M. Farley, E. Pereira, R. C. Harvey, A. Schuchat, J. D. Wenger, and D. S. Stephens.** 1993. Increased risk of meningitis and bacteremia due to *Listeria monocytogenes* in patients with human immunodeficiency virus infection. Clin. Infect. Dis. **17**:224-227.

55. **Kampfer, P., S. Bottcher, W. Dott, and H. Ruden.** 1991. Physiological characterization and identification of *Listeria* species. Zbl. Bakt. **275**:423-435.

56. **Karunasagar, I., B. Senghaas, G. Krohne, and W. Goebel.** 1994. Ultrastructural study of *Listeria monocytogenes* entry into cultured human colonic epithelial cells. Infect. Immun. **62**:3554-3558.

57. **Keller, R., W. Fischer, R. Keist, and S. Bassetti.** 1992. Macrophage response to bacteria: induction of marked secretory and cellular activities by lipoteichoic acids. Infect. Immun. **60**:3664-3672.

58. **Kocks, C., R. Hellio, P. Gounon, H. Ohayon, and P. Cossart.** 1993. Polarized distribution of *Listeria monocytogenes* surface protein ActA at the site of directional actin assembly. J. Cell Science **105**:699-710.

59. **Kohler, S., A. Bubert, M. Vogel, and W. Goebel.** 1991. Expression of the *iap* gene coding for protein p60 of *Listeria monocytogenes* is controlled on the posttranscriptional level. J. Bact. **173**:4668-4674.

60. **Kuhn, M., M. -C. Prevost, J. Mounier, and P. J. Sansonetti.** 1990. A nonvirulent mutant of *Listeria monocytogenes* does not move intracellularly but still induces polymerization of actin. Infect. Immun. **58**:3477-3486.

61. **Lachica, R. V.** 1990. Simplified Henry technique for initial recognition of *Listeria* colonies. Appl. Environ. Microbiol. **56**:1164-1165.

62. **Langermans, J. A. M. and R. Van Furth.** 1994. Cytokines and the host defense against *Listeria monocytogenes* and *Salmonella typhimurium*. Biotherapy 7:169-178.

63. **Leimeister-Wachter, M., E. Domann, and T. Chakraborty.** 1992. The expression of virulence genes in *Listeria monocytogenes* is thermoregulated. J. Bact. **174**:947-952.

64. **Leimeister-Wachter, M., C. Haffner, E. Domann, W. Goebel, and T. Chakraborty.** 1990. Identification of a gene that positively regulates expression of listeriolysin, the major virulence factor of *Listeria monocytogenes*. Proc. Natl. Acad. Sci. USA **87**:8336-8340.

65. **Linnan, M. J., L. Mascola, X. D. Lou, V. Goulet, S. May, and C. Salminen.** 1988. Epidemic listeriosis associated with Mexican-style cheese. !!!BADKEY -- Journal Name Lost **28257**:29301-26980.

66. **MacGowan, A. P., H. A. Holt, and D. S. Reeves.** 1990. *In-vitro* synergy testing of nine antimicrobial combinations against *Listeria monocytogenes*. J. Antimicrob. Chemother. **25**:561-566.

67. **MacKenness, G. B.** 1962. Cellular resistance to infection. J. Exp. Med. **116**:381-417.

68. **Mainou-Fowler, T., A. P. MacGowan, and R. Postlethwaite.** 1988. Virulence of *Listeria* spp.: course of infection in resistant and susceptible mice. J. Med. Microbiol. **27**:131-140.

69. **Mengaud, J., C. Geoffroy, and P. Cossart.** 1991. Identification of a new operon involved in *Listeria monocytogenes* virulence: its first gene encodes a protein homologous to bacterial metalloproteases. Infect. Immun. **59**:1043-1049.

70. **Mengaud, J., M. F. Vicente, and P. Cossart.** 1989. Transcriptional mapping and nucleotide sequence of the *Listeria monocytogenes hlyA* region reveal structural features that may be involved in regulation. Infect. Immun. **57**:3695-3701.

71. **Meyer, R. D. and S. Liu.** 1987. Determination of the effect of antimicrobics in combination against *Listeria monocytogenes*. Diagn. Microbiol. Infect. Dis. **6**:199-206.

72. **Michelet, C., J. L. Avril, F. Cartier, and P. Berche.** 1994. Inhibition of intracellular growth of *Listeria monocytogenes* by antibiotics. Antimicrob. Agents Chemother. **38**:438-446.

73. **Mielke, M. E. A., S. Ehlers, and H. Hahn.** 1993. The role of cytokines in experimental listeriosis. Immunobiol. **189**:285-315.

74. **Mork, T. and R. E. W. Hancock.** 1993. Mechanisms of nonopsonic phagocytosis of *Pseudomonas aeruginosa*. Infect. Immun. **61**:3287-3293.

75. **Mounier, J., A. Ryter, M. Coquis-Rondon, and P. J. Sansonetti.** 1990. Intracellular and cell-to-cell spread of *Listeria monocytogenes* involves interaction with F-actin in the enterocytelike cell line Caco-2. Infect. Immun. **58**:1048-1058.

76. **Neidhardt, F. C., J. L. Ingraham, and M. Schaechter.** 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Inc., Sunderland,MA.

77. **Newman, S. L. and L. K. Mikus.** 1985. Deposition of C3b and iC3b onto particulate activators of the human complement system: quantitation with monoclonal antibodies to human C3. J. Exp. Med. **161**:1414-1431.

78. **Niebuhr, K., T. Chakraborty, M. Rohde, T. Gazlig, B. Jansen, P. Kollner, and J. Wehland.** 1993. Localization of the ActA polypeptide of *Listeria monocytogenes* in infected tissue culture cell lines: ActA is not associated with actin "comets". Infect. Immun. **61**:2793-2802.

79. **Notermans, S. H. W., J. Dufrenne, M. Leimeister-Wachter, E. Domann, and T. Chakraborty.** 1991. Phosphatidylinositol-specific phospholipase C activity as a marker to distinguish between pathogenic and nonpathogenic *Listeria* species. Appl. Environ. Microbiol. **57**:2666-2670.

80. **Parrisius, J., S. Bhakdi, M. Roth, J. Tranum-Jensen, W. Goebel, and H. P. R. Seeliger.** 1986. Production of listeriolysin by beta-hemolytic strains of *Listeria monocytogenes*. Infect. Immun. **51**:314-319.

81. **Peel, M., W. Donachie, and A. Shaw.** 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE, and western blotting. J. Gen. Microb. **134**:2171-2178.

82. **Peterkin, P. I., M. -A. Gardiner, N. Malik, and E. S. Idziak.** 1992. Plasmids in *Listeria monocytogenes* and other *Listeria* species. Can. J. Microbiol. **38**:161-164.

83. **Platt, M. W., N. Correa, and C. Mold.** 1994. Growth of group B streptococci in human serum leads to increased cell surface sialic acid and decreased activation of the alternative complement pathway. Can. J. Microbiol. **40**:99-105.

84. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60**:1263-1267.

85. **Poyart, C., E. Abachin, I. Razafimanantsoa, and P. Berche.** 1993. The zinc metalloprotease of *Listeria monocytogenes* is required for maturation of phophatidylcholine phospholipase C: direct evidence obtained by gene complementation. Infect. Immun. **61**:1576-1580.

86. **Rawn, J. D.** 1989. Biochemistry. Neil Patterson Publishers, Burlington,N.C..

87. **Rocourt, J. and R. Brosch.** 1993. Human listeriosis in 1990. Bulletin of the World Health Organization **71**:259-263.

88. **Rodgers, F. G. and F. C. Gibson.** 1993. Opsonin-independent adherence and intracellular development of *Legionella pneumophila* within U-937 cells. Can. J. Microbiol. **39**:718-722.

89. **Roll, J. T. and C. J. Czuprynski.** 1990. Hemolysin is required for extraintestinal dissemination of *Listeria monocytogenes* in intragastrically inoculated mice. Infect. Immun. **58**:3147-3150.

90. **Ross, G. D., S. L. Newman, J. D. Lambris, J. E. Devery-Pocius, J. A. Cain, and P. J. Lachmann.** 1983. Generation of three different fragments of bound C3 with purified factor I or serum: location of binding sites in the C3 fragments for factors B and H, complement receptors, and bovine conglutinin. J. Exp. Med. **158**:334-352.

91. **Ruhland, G. J. and F. Fiedler.** 1987. Occurrence and biochemistry of lipoteichoic acids in the genus *Listeria* . System. Appl. Microbiol. **9**:40-46.

92. **Ruhland, G. J., M. Hellwig, G. Wanner, and F. Fiedler.** 1993. Cell-surface localization of *Listeria*-specific protein p60- detection of *Listeria* cells by indirect immunofluorescence. J. Gen. Microbiol. **139**:609-616.

93. **Sanger, J. M., J. W. Sanger, and F. S. Southwick.** 1992. Host cell actin assembly is necessary and likely to provide the propulsive force for intracellular movement of *Listeria monocytogenes*. Infect. Immun. **60**:3609-3619.

94. **Schlesinger, L. S. and M. A. Horwitz.** 1994. A role for natural antibody in the pathogenesis of leprosy: antibody in nonimmune serum mediates C3 fixation to the *Mycobacterium leprae* surface and hence phagocytosis by human mononuclear phagocytes. Infect. Immun. **62**:280-289.

95. **Schuchat, A., B. Swaminathan, and C. V. Broome.** 1991. Epidemiology of human listeriosis. Clin. Microbiol. Rev. **4**:169-183.

96. **Schwan, W. R., A. Demuth, M. Kuhn, and W. Goebel.** 1994. Phosphatidylinositol-specific phospholipase C from *Listeria monocytogenes* contributes to intracellular survival and growth of *Listeria innocua*. Infect. Immun. **62**:4795-4803.

97. **Schwartz, B., D. Hexter, C. V. Broome, A. W. Hightower, R. B. Hirschhorn, J. D. Porter, P. S. Hayes, W. F. Bibb, B. Lorber, and D. G. Faris.** 1989. Investigation of an outbreak of listeriosis: new hypotheses for the etiology of epidemic *Listeria monocytogenes* infections. J. Infect. Dis. **159**:680-685.

98. **Siragusa, G. R., L. A. Elphingstone, P. L. Wiese, S. M. Haefner, and M. G. Johnson.** 1990. Petite colony formation by *Listeria monocytogenes* and *Listeria* species grown on esculin-containing agar. Can. J. Microbiol. **36**:697-703.

99. **Skogberg, K. Syrjanen,J., J. Syrjanen, M. Jahkola, O. -V. Renkonen, J. Paavonen, J. Ahonen, S. Kontiainen, P. Ruutu, and V. Valtonen.** 1992. Clinical presentation and outcome of listeriosis in patients with and without immunosuppressive therapy. Clin. Infect. Dis. **14**:815-821.

100. **Small, P. L. C., L. Ramakrishnan, and S. Falkow.** 1994. Remodeling schemes of intracellular pathogens. Science **263**:637-639.

101. **Sokolovic, Z., J. Riedel, M. Wuenscher, and W. Goebel.** 1993. Surface-associated, PrfA regulated proteins of *Listeria monocytogenes* synthesized under stress conditions. Mol. Microbiol. **8**:219-227.

102. **Speert, D. P. and S. Gordon.** 1992. Phagocytosis of unopsonized *Pseudomonas aeruginosa* by murine macrophages is a two-step process requiring glucose. J. Clin. Invest. **90**:1085-1092.

103. **Stryer, L.** 1988. Biochemistry. W.H. Freeman and Co., New York.

104. **Sun, A. N., A. Camilli, and D. A. Portnoy.** 1990. Isolation of *Listeria monocytogenes* small-plaque mutants defective for intracellular growth and cell-to-cell spread. Infect. Immun. **58**:3770-3778.

105. **Tilney, L. G., P. S. Connelly, and D. A. Portnoy.** 1990. Actin filament nucleation by the bacterial pathogen, *Listeria monocytogenes*. J. Cell Biol. **111**:2979-2988.

106. **Tilney, L. G. and D. A. Portnoy.** 1989. Actin filaments and the growth, movement, and spread of the intracellular bacterial parasite, *Listeria monocytogenes*. J. Cell. Biol. **109**:1597-1608.

107. **Tzianabos, A. O. and F. G. Rodgers.** 1989. Pathogenesis and chemotherapy of experimental *Legionella pneumophila* infection in the chick embryo. Zbl. Bakt. **271**:293-303.

108. **Vasquez-Boland, J. -A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60**:219-230.

109. **Wood, S., N. Maroushek, and C. J. Czuprynski.** 1993. Multiplication of *Listeria monocytogenes* in a murine hepatocyte cell line. Infect. Immun. **61**:3068-3072.

110. **Wright, S. D. and S. C. Silverstein.** 1982. Tumor-promoting phorbol esters stimulate C3b and C3b' receptor-mediated phagocytosis in cultured human monocytes. J. Exp. Med. **156**:1149-1164.

111. **Wuenscher, M. D., S. Kohler, A. Bubert, U. Gerike, and W. Goebel.** 1993. The *iap* gene of *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic activity. J. Bact. **175**:3491-3501.

APPENDICES

7.1 Biological Studies

A. Bacteria

1.1 Storage of Bacterial Cultures

A fully virulent clinical isolate of *L. monocytogenes* serotype 1/2b was isolated on blood agar, subcultured once on T-soy agar, and stored as stock cultures frozen at -70°C in 1% serum-sorbitol.

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 megohm resistivity was reached.

1.3 Bacterial Media

Trypticase soy agar and broth (Difco) was used for cultivation of the organism during all assays. This media was purchased in dehydrated form from VWR Scientific and is resuspended in Milli-Q water as per directions on the bottle. Agar and broth tube preparations were sterilized by autoclave using 121°C at 12 psi for 20 min. Plates were poured after allowing the agar to cool to 50°C. After the plates cooled, they were placed in an incubator at 37°C overnight to check for sterility and were stored at 4°C until needed.

1.4 Phosphate Buffered Saline

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:

Component	Amount (g/L)
NaCl	8.0
KCl	0.2
KH_2PO_4	0.2
Na_2HPO_4	0.15

PBS was adjusted to a pH of 7.2 by addition of 10N NaOH and was sterilized by autoclaving. Solutions were stored at room temperature in 800mL batches until needed.

1.5 1% Peptone

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

B. Cell Culture

1.6 De-ionized Water

See Appendix Section 1.1 above.

1.7 Thioglycollate

Thioglycollate (Difco) was purchased in dehydrated form from VWR Scientific and is resuspended in 100mL Milli-Q water as per directions on the

bottle. The solution was sterilized by autoclaving and stored at room temperature in the dark for at least 1 month prior to use.

1.8 Glutamine

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

1.9 Fetal Bovine Serum

Sterile fetal bovine serum (Hybrimax) was purchased from Sigma in 500mL batches. The contents of each bottle was thawed and aliquoted into 25mL batches in sterile 50mL conical tubes and stored at -20°C until needed. Aliquots were heat-inactivated by incubation at 55°C for 30 min prior to use in preparation of RPMI-1640.

1.10 Sodium Bicarbonate

A 7.5% sodium bicarbonate solution was prepared by added 7.5g Na_2HCO_3 to 100mL fresh Milli-Q water. This solution was filter sterilized by positive pressure filtration through a 0.22µm membrane filter into a sterile 100mL dilution bottle and was stored at 4°C until needed.

1.11 Sodium Hydroxide

A 1N NaOH solution was prepared by added 4g NaOH to 100mL Milli-Q water and this solution was sterilized by autoclaving.

1.12 Hank's Balanced Salt Solution (HBSS)

Sterile batches of 500mL 10X concentrated HBSS with supplemented

with phenol red was purchased from Sigma and stored at -20°C. To 448mL of sterile Milli-Q water, 50mL of sterile 10X HBSS was added. To this solution 2.4mL of 7.5% Na_2HCO_3 was added as a buffer and the pH adjusted to 7.2 with sterile 1N NaOH. These batches were tested for sterility by placing the prepared bottles at 37°C overnight and sterile media stored at 4°C.

1.13 RPMI-1640

Murine peritoneal macrophages were cultivated in RPMI-1640 without antibiotics. Basal RPMI-1640 cell culture media containing phenol red was purchased as 10X stock solutions in 500mL bottles from Sigma and stored at -20°C. For culture, the basal media was prepared in 500mL batches as follows:

Component	Amount (mL)
Milli-Q water	387
RPMI-1640	45
Heat-inactivated FBS	50
L-glutamine	5.1
7.5% sodium bicarbonate	13.4

The RPMI-1640 medium was adjusted to pH 7.2 with NaOH. Media was tested for sterility by incubation at 37°C overnight and sterile media was stored at 4°C.

7.2 Lethal Dose $_{50}$ Calculations

Calculations were carried out using the method of Reed and Muench (1938) as follows:

$$\frac{\text{(\% mortality at dilution next above 50\%) - 50\%}}{\text{(\% mortality at dilution next above 50\%) - (\%mortality at dilution next below)}} = \text{Proportionate distance}$$

Negative logarithm of LD_{50} endpoint titer = negative log of the dilution above the 50% mortality plus the proportionate distance factor

Or

Negative logarithm of the lower dilution (next above 50% mortality) = -X
Proportionate distance x dilution factor = -Y
LD50 titer = -X +-Y
log LD_{50} titer = 10^{-X+-Y}

Fertile Hens' Egg Assays

Assay 1: LD_{50} = 8.8 CFU

				Accumulated Values			
						Mortality	
Bacterial Dilution	Mortality Ratio	Died	Survived	Died	Survived	Ratio	Percent
7×10^6	10/10	10	0	74	0	74/74	100
7×10^5	10/10	10	0	64	0	64/64	100
7×10^4	10/10	10	0	54	0	54/54	100
7×10^3	10/10	10	0	44	0	44/44	100
7×10^2	10/10	10	0	34	0	34/34	100
7×10^1	10/10	10	0	24	0	24/24	100
7×10^0	10/10	9	1	14	1	14/15	93
7×10^{-1}	10/10	5	5	5	6	5/11	45

Assay 2: LD_{50} = 6.3 CFU

Bacterial Dilution	Mortality Ratio	Died	Survived	Accumulated Values: Died	Accumulated Values: Survived	Mortality: Ratio	Mortality: Percent
2×10^5	10/10	10	0	60	0	60/60	100
2×10^4	10/10	10	0	50	0	50/50	100
2×10^3	10/10	10	0	40	0	40/40	100
2×10^2	10/10	10	0	30	0	30/30	100
2×10^1	10/10	10	0	20	0	20/20	100
2×10^0	8/10	8	2	10	2	10/12	83
2×10^{-1}	2/10	2	8	2	10	2/12	17

Assay 3: LD_{50} = 35.2 CFU

Bacterial Dilution	Mortality Ratio	Died	Survived	Accumulated Values: Died	Accumulated Values: Survived	Mortality: Ratio	Mortality: Percent
1.5×10^5	10/10	10	0	52	0	52/52	100
1.5×10^4	10/10	10	0	42	0	42/42	100
1.5×10^3	10/10	10	0	32	0	32/32	100
1.5×10^2	10/10	10	0	22	0	22/22	100
1.5×10^1	10/10	10	0	12	0	12/12	100
1.5×10^0	2/10	2	8	2	8	2/10	20

Assay 4: LD_{50} = 37.9 CFU

				Accumulated Values			
Bacterial Dilution	Mortality Ratio	Died	Survived	Died	Survived	Mortality Ratio	Percent
1.2×10^3	10/10	10	0	30	0	30/30	100
1.2×10^2	10/10	10	0	20	0	20/20	100
1.2×10^1	8/10	8	2	10	2	10/	83
1.2×10^0	2/10	2	8	2	10	2/12	17

Assay 5: LD_{50} = 2.2 CFU

				Accumulated Values			
Bacterial Dilution	Mortality Ratio	Died	Survived	Died	Survived	Mortality Ratio	Percent
1.5×10^3	10/10	10	0	41	0	41/41	100
1.5×10^2	10/10	10	0	31	0	31/31	100
1.5×10^1	10/10	10	0	21	0	21/21	100
1.5×10^0	7/10	7	3	11	3	11/14	79
1.5×10^{-1}	4/10	4	6	4	9	4/9	44

Assay 6: LD_{50} = 33 CFU

				Accumulated Values			
						Mortality	
Bacterial Dilution	Mortality Ratio	Died	Survived	Died	Survived	Ratio	Percent
1.6×10^3	10/10	10	0	32	0	32/32	100
1.6×10^2	10/10	10	0	22	0	22/22	100
1.6×10^1	8/10	8	2	12	2	12/14	86
1.6×10^0	4/10	4	6	4	8	4/12	33

7.3 Mouse Care and Collection of Peritoneal Macrophages

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 license 930803 and its predecessors issued to Dr. Frank G. Rodgers.

3.1 Animal Specifications

The animals used for production of peritoneal macrophages were Balb/c inbred mice raised in the Department of Microbiology colony.

3.2 Animal Living Conditions

All cages conformed to Animal Care and Use Committee guidelines for size. In addition, each cage was maintained with a generous amount of wood chips which were changed on a twice-weekly basis.

3.3 Food and Water

Mouse chow (Rawlston Purina) and water were supplied to animals *ad libitum*.

3.4 Euthanization and Collection of Peritoneal Macrophages

Each animal was injected with 1.5mL thioglycollate 2.5-3.5 d prior to sacrifice and harvest of macrophages. Euthanization occurred by CO_2 asphyxiation using 5% CO_2 injected into a small plastic autoclave bag. The peritoneal cavity was exposed without penetrating the membrane, and a series of 3-10mL lavages of HBSS were injected into cavity and the

macrophages collected by aspiration. Collected samples were placed on ice until they were processed.

7.4 Electron Microscopy

Methodology outlined here is in standard use in our laboratory and was originally developed by Rodgers (1976)

A. Transmission Electron Microscopy

4.1 Grids

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

4.2 Collodion

A stock solution of collodion was purchased as 10% nitrocellulose dissolved in EM grade amyl acetate. This solution was diluted to 1% with EM grade amyl acetate immediately prior to use for coating of grids. All procedures involving collodion were carried out in a fume hood.

4.3 Collodion Coating of Grids

3 drops of 1% collodion solution were dropped onto the surface of a standard petri dish containing 20mL 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 overnight at 37°C.

4.4 Cacodylate Buffer

A 0.1M cacodylate buffer with 10mM $MgSO_4$ (EMS) was made by adding 21.4g sodium cacodylate-trihydrate and 1.2g $MgSO_4$ to 1L Milli-Q water. The buffer was adjusted to pH 7.4 and stored in the dark at 4°C until needed.

4.5 Glutaraldehyde

EM grade glutaraldehyde was purchased from EMS as a 50% solution and stored at 4°C in the dark. Prior to TEM and SEM studies, glutaraldehyde was diluted to 5% in cacodylate buffer and used for specimen fixation. Glutaraldehyde was used in a fume hood.

4.6 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 1g vial in 50mL cacodylate buffer to make a 2% (w/v) stock solution. Equal volumes of 2% osmium tetroxide solution and cacodylate buffer were placed over each sample and samples were fixed overnight at room temperature. OsO_4 was used in a fume hood and waste was disposed of in proper fashion according to University policy.

4.7 Graded Ethanol Series

Absolute ethanol was diluted with water to create a series of 50%, 70%,

75%, 90%, and 95% ethanol. Each dilution was stored in sealed bottles and stored at room temperature.

4.8 Propylene Oxide

Propylene oxide was purchased from EMS ready for use and was stored tightly stoppered at room temperature in the dark.

4.9 Resins

An epon-araldite 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 these studies were as follows:

Resin	Proportion
Epon 812	5 parts
Araldite	11 parts
Dodecyl succinic anhydride	3 parts
Dibutyl phthalate (plasticizer)	0.8 parts
DMP-30 (accelerator)	0.4 parts

All resins were measured using plastic tri-pour beakers or plastic syringes. The resins were added in sequence to a 75cm^3 cell culture flask (except for the DMP-30) and were shaken vigorously for 10-15 min. The resin mixture was divided into 4 equal parts and was completed by the addition of DMP-30 at the appropriate concentration (see TEM procedure Appendix Section 4.10). The complete resin was then stirred vigorously for an

additional 3 min to facilitate even distribution of DMP-30. All manipulations were performed in a fume hood.

4.10 TEM Procedure

Experimental design for TEM is outlined in Section 3.3.3. At no point in the embedding process were samples allowed to dry. Samples were fixed overnight with 5% glutaraldehyde in cacodylate buffer. Following fixation, cells were washed 10X by centrifugation with cacodylate buffer. After the final wash, pelleted cells were embedded in 2% molten Noble 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%, and 95% ethanol at 10 min in each. This was followed by 2 changes of absolute ethanol for 15 min each. The samples were further dehydrated in propylene oxide with 2 changes of 15 min each. Following the preparation of the resin mixture, a gradient resin series was made to facilitate impregnation of specimens. The samples were processed using a gradient as shown below:

%Resin/%Propylene Oxide	Time
33/66	1 h
66/33	1 h
100/0	1 h
100/0 (degassed)	1 h

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 100% 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 60-90nm sections cut with a LKB Ultratome III ultramicrotome using a Diatom diamond knife. Sections were collected using collodion-coated copper grids and were stained using uranyl acetate for 1 min, washed with 20mL of boiled, cooled Milli-Q water. Sections were subsequently stained with lead citrate for 30 seconds followed by 50mL rinsing with boiled, cooled Milli-Q water. After drying, stained grids were observed and photographed in the transmission mode of on a Hitachi scanning-transmission electron microscope at an accelerating voltage of 75kV.

4.11 Uranyl Acetate Staining

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

4.12 Lead Citrate Staining

A sodium hydroxide pellet was dissolved in 15mL boiled, cooled Milli-Q water. A 0.5% lead citrate solution was made by dissolving 0.1g lead citrate

into this solution and subsequent addition of 10mL Milli-Q water. This solution was allowed to dissolve overnight and 1mL aliquots were removed and centrifuged at 12,400 Xg for 15 min followed by filtration through a 0.45μm filter immediately before use.

B. Scanning Electron Microscopy

4.13 Hexamethyldisalizane (HMDS)

HMDS was purchased from EMS and stored tightly stoppered at room temperature in the dark. All manipulations with HMDS were performed in a fume hood.

4.14 SEM Procedure

Samples in 6-well plates on coverslips were fixed with 5% glut-araldehyde in cacodylate buffer overnight. Following fixation, samples were washed extensively with cacodylate buffer and dried in a graded ethanol series *in situ* (50%, 70%, 75%, 90%, 95%, and 100%) in similar fashion to TEM studies (Appendix Section 4.10). Specimens on coverslips were dried from HMDS, fixed on the top of a carbon coated stub, and stored at room temperature in a desiccated environment. The coverslips were placed in a sputter coater and coated with 20nm gold-palladium using a target to specimen distance of 5cm and 15mA for 4 min. Specimens were placed in an AMR-1000 scanning electron microscope with an accelerating voltage of 60kV.

7.5 ELISA Data: ANOVA (One-Way Analysis of Variance) Statistics

All data were analyzed by ANOVA using Statview SE + Graphics v. 1.02 (Abacus Concepts, Berkeley, CA) on a Macintosh LC III.

5.1 Prokaryotic treatment

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	33	.059	.002	3.613	.0001
Within subjects	102	.05	4.948E-4		
treatments	3	.013	.004	11.192	.0001
residual	99	.038	3.807E-4		
Total	135	.109			

Reliability Estimates for- All treatments: .723 Single Treatment: .395

Note: 9 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Beta-Gal	-.018	.009*	4.76*	3.779
Control vs. Lipase	-.026	.009*	10.07*	5.496
Control vs. Neuraminidase	-.009	.009	1.27	1.952
Beta-Gal vs. Lipase	-.008	.009	.983	1.717
Beta-Gal vs. Neuraminidase	.009	.009	1.113	1.827

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_3

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	35	.041	.001	.682	.893
Within subjects	72	.124	.002		
treatments	2	.061	.03	33.249	.0001
residual	70	.064	.001		
Total	107	.166			

Reliability Estimates for- All treatments: -.467 Single Treatment: -.119

Note: 12 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_3

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Na Meta	-.004	.014	.153	.554
Control vs. Glutaraldehyde	.048	.014*	22.909*	6.769
Na Meta vs. Glutaraldehyde	.052	.014*	26.811*	7.323

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	35	.182	.005	1.04	.4205
Within subjects	144	.721	.005		
treatments	4	.066	.017	3.546	.0087
residual	140	.655	.005		
Total	179	.903			

Reliability Estimates for- All treatments: .038 Single Treatment: .008

Note: 52 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Chymotrypsin	.052	.032*	2.564*	3.202
Control vs. Protease	.047	.032*	2.136	2.923
Control vs. Trypsin	.031	.032	.913	1.911
Control vs. NAcNeu Aldol...	.048	.032*	2.251	3.001
Chymotryp... vs. Protease	-.005	.032	.019	.279

* Significant at 95%

5.2 Eukaryotic treatment

One Factor ANOVA-Repeated Measures for X_1 ... X_7

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	32	.09	.003	8.195	.0001
Within subjects	198	.068	3.445E-4		
treatments	6	.013	.002	7.332	.0001
residual	192	.055	2.890E-4		
Total	230	.159			

Reliability Estimates for- All treatments: .878 Single Treatment: .507

Note: 3 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_7

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Chymotrypsin	3.848E-4	.008	.001	.092
Control vs. Lipase	-.007	.008	.495	1.723
Control vs. Neuraminidase	-.006	.008	.343	1.434
Control vs. Pepsin	.004	.008	.143	.927
Control vs. Protease	.01	.008*	1.016	2.469

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_7

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Trypsin	.015	.008*	2.013	3.475
Chymotryp... vs. Lipase	-.008	.008	.549	1.815
Chymotryp... vs. Neurami...	-.006	.008	.388	1.526
Chymotryp... vs. Pepsin	.003	.008	.116	.835
Chymotryp... vs. Protease	.01	.008*	.942	2.377

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	32	.044	.001	2.758	.0001
Within subjects	132	.065	4.951E-4		
treatments	4	.011	.003	6.324	.0001
residual	128	.055	4.264E-4		
Total	164	.109			

Reliability Estimates for- All treatments: .637 Single Treatment: .26

Note: 3 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Nonidet P40	.011	.01*	1.197	2.188
Control vs. Glutaraldehyde	.014	.01*	1.775	2.665
Control vs. Formaldehyde	.019	.01*	3.47*	3.726
Control vs. Na Meta	-.002	.01	.045	.423
Nonidet P40 vs. Glutarald...	.002	.01	.057	.477

* Significant at 95%

5.3 Competitive binding assays

A. Sugars

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	51	.086	.002	2.912	.0001
Within subjects	208	.121	.001		
treatments	4	.012	.003	5.83	.0002
residual	204	.109	.001		
Total	259	.207			

Reliability Estimates for- All treatments: .657 Single Treatment: .277

Note: 6 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Fucose	.006	.009	.412	1.284
Control vs. Galactose	.013	.009*	2.213	2.975
Control vs. Glucose	.017	.009*	3.674*	3.834
Control vs. Mannose	.018	.009*	3.814*	3.906
Fucose vs. Galactose	.008	.009	.715	1.692

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	31	.049	.002	1.744	.0212
Within subjects	96	.087	.001		
treatments	3	.043	.014	30.756	.0001
residual	93	.044	4.711E-4		
Total	127	.136			

Reliability Estimates for- All treatments: .427 Single Treatment: .157

Note: 3 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. NAcGal	.029	.011*	9.277*	5.276
Control vs. NAcGlu	.019	.011*	3.954*	3.444
Control vs. NAcNeu	.051	.011*	29.557*	9.416
NAcGal vs. NAcGlu	-.01	.011	1.118	1.831
NAcGal vs. NAcNeu	.022	.011*	5.716*	4.141

* Significant at 95%

B. Lectins

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	35	.045	.001	1.08	.365
Within subjects	144	.172	.001		
treatments	4	.058	.014	17.529	.0001
residual	140	.115	.001		
Total	179	.218			

Reliability Estimates for- All treatments: .074 Single Treatment: .016

Note: 25 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. Maackia	.015	.013*	1.243	2.229
Control vs. Limulus	-.001	.013	.002	.095
Control vs. WGA	-.025	.013*	3.549*	3.768
Control vs. Concanavalin A	-.033	.013*	6.142*	4.956
Maackia vs. Limulus	-.016	.013*	1.35	2.324

* Significant at 95%

5.5 NAcNeu at varying concentrations

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	34	.011	3.330E-4	.641	.9304
Within subjects	105	.055	.001		
treatments	3	.029	.01	39.93	.0001
residual	102	.025	2.460E-4		
Total	139	.066			

Reliability Estimates for- All treatments: -.56 Single Treatment: -.099

Note: 1 case deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_4

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. 50 mM NAcNeu	.039	.007*	35.17*	10.272
Control vs. 25 mM NAcNeu	.031	.007*	23.292*	8.359
Control vs. 10 mM NAcNeu	.021	.007*	10.742*	5.677
50 mM NAc... vs. 25 mM ...	-.007	.007	1.219	1.913
50 mM NAc... vs. 10 mM ...	-.017	.007*	7.038*	4.595

* Significant at 95%

5.6 Opsonin-dependent adherence: NAcNeu

One Factor ANOVA-Repeated Measures for X_1 ... X_3

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	32	.043	.001	2.426	.0012
Within subjects	66	.037	.001		
treatments	2	.009	.005	10.731	.0001
residual	64	.028	4.324E-4		
Total	98	.08			

Reliability Estimates for- All treatments: .588 Single Treatment: .322

Note: 2 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_3

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. NMS	-.022	.01*	9.109*	4.268
Control vs. NAcNeu/NMS	-.003	.01	.165	.574
NMS vs. NAcNeu/NMS	.019	.01*	6.823*	3.694

* Significant at 95%

5.7 M1/70 Adherence studies

A. Opsonized *L. monocytogenes*

One Factor ANOVA-Repeated Measures for X_1 ... X_8

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	11	.006	.001	.479	.9111
Within subjects	84	.091	.001		
treatments	7	.074	.011	49.91	.0001
residual	77	.016	2.125E-4		
Total	95	.096			

Reliability Estimates for- All treatments: -1.086 Single Treatment: -.07

Note: 37 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_8

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. NMS	-.014	.012*	.829	2.408
Control vs. 25 ug/mL	-.021	.012*	1.695	3.444
Control vs. 10 ug/mL	-.019	.012*	1.534	3.276
Control vs. 5 ug/mL	-.006	.012	.166	1.078
Control vs. 1 ug/mL	-.003	.012	.047	.574

* Significant at 95%

One Factor ANOVA-Repeated Measures for X_1 ... X_8

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. 0.5 ug/mL	.057	.012*	13.103*	9.577
Control vs. .1 ug/mL	.045	.012*	8.319*	7.631
NMS vs. 25 ug/mL	-.006	.012	.153	1.036
NMS vs. 10 ug/mL	-.005	.012	.108	.868
NMS vs. 5 ug/mL	.008	.012	.253	1.33

* Significant at 95%

B. Nonopsonized *L. monocytogenes*

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Source:	df:	Sum of Squares:	Mean Square:	F-test:	P value:
Between subjects	29	.029	.001	3.656	.0001
Within subjects	120	.032	2.689E-4		
treatments	4	.004	.001	3.838	.0058
residual	116	.028	2.456E-4		
Total	149	.061			

Reliability Estimates for- All treatments: .726 Single Treatment: .347

Note: 8 cases deleted with missing values.

One Factor ANOVA-Repeated Measures for X_1 ... X_5

Comparison:	Mean Diff.:	Fisher PLSD:	Scheffe F-test:	Dunnett t:
Control vs. 25 ug/mL	.002	.008	.084	.581
Control vs. 10 ug/mL	.006	.008	.587	1.532
Control vs. 1 ug/mL	.011	.008*	1.996	2.825
Control vs. 0.1 ug/mL	.013	.008*	2.567*	3.204
25 ug/mL vs. 10 ug/mL	.004	.008	.226	.951

* Significant at 95%