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The Role of Sialic Acid in Opsonin-Dependent and Opsonin-Independent Adhesion of *Listeria monocytogenes* to Murine Peritoneal Macrophages†

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The adhesion of listeriae to host cells employs mechanisms which are complex and not well understood. *Listeria monocytogenes* is a facultative intracellular pathogen responsible for meningoencephalitis, septicemia, and abortion in susceptible and immunocompromised individuals. Subsequent to colonization and penetration of the gut epithelium, the organism attaches to resident macrophages and replicates intracellularly, thus evading the humoral immune system of the infected host. The focus of these studies was to investigate the attachment of the organism to murine peritoneal macrophages in an opsonin-dependent and opsonin-independent fashion. Assessment of competitive binding experiments by immunofluorescence and enzyme-linked immunosorbent assays showed that adhesion of the organism to macrophages in the presence or absence of opsonins was inhibited (90%) by N-acetyleneuraminic acid (NAcNeu). In addition, the lectin from *Maackia amurensis*, with affinity for NAcNeu-(2,3)galactose, blocked binding of *L. monocytogenes* to host cells. Oxidation of the surface carbohydrates on the organism by using sodium metaperiodate resulted in a dose-dependent reduction (up to 98%) in adherence to macrophages. Monoclonal antibody to complement receptor 3 did not prevent listeriae from binding to mouse macrophages or from replicating within the infected cells whether or not normal mouse serum was present. Based on our results, we propose the involvement of NAcNeu, a member of the sialic acid group, in the attachment of *L. monocytogenes* to permissive murine macrophages.

Adherence is the necessary first step in the infectious process of many intracellular pathogens (17, 28). The molecular mechanisms by which adherence and subsequent phagocytosis of the pathogen occur either through an opsonin-mediated process, involving complement or antibody and their appropriate receptors on the host cell, or through an opsonin-independent process in which bacterial adhesins recognize and attach to specific host cell receptors are not well understood. However, the receptors involved in opsonin-dependent adherence may also be involved in opsonin-independent adherence, as in the case of group B streptococci (GBS), which recognize and bind 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* (18, 26) as well as for *Listeria monocytogenes* (5, 8, 24).

*L. monocytogenes* is the causative agent of food-borne listeriosis, a disease which primarily affects immunocompromised individuals, pregnant women, and neonates. Clinical manifestations range from mild, flu-like symptoms to meningoencephalitis and septic abortion. The organism has long been recognized as a facultative intracellular pathogen capable of infecting and replicating within a wide variety of cells, including fibroblasts, epithelial cells, hepatocytes, and cells from the mononuclear phagocyte system (1, 4, 12, 33). It has been shown that mononuclear phagocytes constitute the major effector cells of immunity in experimental infections (1, 20). *L. monocytogenes* has been shown to induce the deposition of C3b and its cleavage products iC3b and C3d through ester and amide linkages, resulting in the activation of the alternative pathway of human complement (6, 10). Recent studies indicate that CR3 mediates phagocytosis of *L. monocytogenes* in the presence of opsonins by a population of listericidal macrophages (8). However, the same studies demonstrated that nonlistericidal macrophages used CR3 as a minor binding molecule for listeriae (8). Use of a monoclonal antibody (MAb) directed against CR3 (CD11b/CD18) inhibited killing of *L. monocytogenes* in a dose-dependent manner for listericidal macrophages; indeed, when the MAb was used at high doses, these treated cells became permissive hosts (9). In contrast, the use of anti-CR3 antibody to block adherence and phagocytosis by nonlistericidal, permissive macrophages was largely ineffective. This finding 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 (27).

Although phagocytosis of *L. monocytogenes* by macrophages in the presence of opsonins has been investigated (7, 21), the role of opsonin-independent phagocytosis in the initiation of infection is not clear. The aim of this study was to investigate the interaction of *L. monocytogenes* with permissive murine peritoneal macrophages prior to phagocytosis and to partially characterize the bacterial adhesive molecules responsible for binding *L. monocytogenes* to these cells both in the presence and in the absence of opsonic components of serum.
The full virulent strain, UNHNY99, of *L. monocytogenes* serotype 1/2b was isolated on blood agar from the cerebrospinal fluid of an infant who died of neonatal meningoencephalitis. This isolate was subcultured once only on Trypticase soy agar and stored as stock cultures frozen at −70°C in 1% serum-sorbitol. Organism virulence was periodically assessed by the fertile hen egg method (3), and the 50% lethal dose in this system remained at approximately 21 CFU throughout the study period. Aliquots thawed from −70°C were plated on Trypticase soy agar and incubated overnight. Colonies were harvested, resuspended in Trypticase soy broth, and cultured for 8.5 h in a shaking incubator at 37°C. Organisms were collected by centrifugation at 10,000 × g, washed with serum-free Hanks’ balanced salt solution (HBSS; Sigma, St. Louis, Mo.), and resuspended in HBSS to give 10^7 CFU/ml prior to the inoculation of macrophages.

### MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The fully virulent strain, UNHNY99, of *L. monocytogenes* serotype 1/2b was isolated on blood agar from the cerebrospinal fluid of an infant who died of neonatal meningoencephalitis. This isolate was subcultured once only on Trypticase soy agar and stored as stock cultures frozen at −70°C in 1% serum-sorbitol. Organism virulence was periodically assessed by the fertile hen egg method (3), and the 50% lethal dose in this system remained at approximately 21 CFU throughout the study period. Aliquots thawed from −70°C were plated on Trypticase soy agar and incubated overnight. Colonies were harvested, resuspended in Trypticase soy broth, and cultured for 8.5 h in a shaking incubator at 37°C. Organisms were collected by centrifugation at 10,000 × g, washed with serum-free Hanks’ balanced salt solution (HBSS; Sigma, St. Louis, Mo.), and resuspended in HBSS to give 10^7 CFU/ml prior to the inoculation of macrophages.

### Collection and cultivation of murine peritoneal macrophages. BALB/c mice of both sexes were used at 3 to 6 months of age. Animals were housed at the University of New Hampshire animal maintenance facility according to Animal Care and Use Committee guidelines (National Institutes of Health-approved protocol 1995). Murine peritoneal macrophages were elicited by intraperitoneal injection of each mouse with 2 ml of 4% thioglycolate broth aged for a minimum of 3 months. Mice were sacrificed by carbon dioxide asphyxiation 2.5 to 3.5 days poststimulation, and peritoneal exudate cells were extracted in HBSS by using three 10-ml peritoneal cavity lavages. These cell extracts were pooled, centrifuged at 220 × g for 10 min, and resuspended in RPMI 1640 cell culture medium containing 10% heat-inactivated fetal bovine serum (HI-FBS; Sigma). Cells were enumerated by hemocytometer. For immunofluorescence assay (IFA), macrophages were seeded into six-well cell culture plates at 10^6 macrophages/well in 3 ml of RPMI 1640 supplemented with 10% HI-FBS. Each well contained a 22-mm-diameter glass coverslip. For enzyme-linked immunosorbent assay (ELISA), macrophages were seeded into 96-well plates at 10^5 macrophages/well in 200 μl of RPMI 1640 with 10% HI-FBS. All plates containing macrophages were incubated at 37°C for 6 to 8 h in the presence of 5% CO2 and washed three times with HBSS to remove unbound cells, and fresh serum-free RPMI 1640 medium containing 0.5 μg of cytochalasin D per ml was added to the cells to uncouple adherence from bacterial uptake in subsequent studies.

### Host cell-organism interaction assays. Cells were inoculated with 10-fold multiplicities of infection (MOI) ranging from 1 to 10,000. Following 1 h of incubation at 37°C, unbound bacteria were removed by washing and *L. monocytogenes* adherent to macrophages were assayed by IFA to determine the degree of organism binding at each MOI and hence define the kinetics of *L. monocytogenes* attachment to these cells. In this fashion, the optimum inoculation ratio of organisms to macrophage was determined to be an MOI of 100 (data not shown). This inoculum was used for all subsequent adherence assays.

### Opsonin-independent adherence assays. For organism treatment studies, bacteria were exposed to the various modifying agents listed in Table 1. After treatment, organisms were washed with serum-free HBSS and added to untreated host cells.

### Role of CR3 in opsonin-independent and opsonin-dependent adherence. The rat B-cell hybridoma line MI/70, expressing the MAb immunoglobulin G2b (IgG2b) anti-CR3, with specificity for mouse and human CR3 epitopes, was used to investigate the role of CR3 in opsonin-independent and opsonin-dependent adherence of *L. monocytogenes* to murine macrophages. This antibody blocks the binding of iC3b-coated targets to CR3 (9). The MI/70 cell line was cultured in RPMI 1640 containing 10% fetal bovine serum, and the supernatant was collected and concentrated in a Centriprep-10 (Amicon, Beverly, Mass.). The concentrated MAB was purified by protein G-Sepharose 4 Fast Flow column chromatography (Pharmacia, Piscataway, N.J.) and eluted with 0.2 M glycine at pH 2.0. Fractions were further concentrated after adjusting pH to neutrality, and the

### TABLE 1. Treatment protocol for the surface expressed adhesins of *L. monocytogenes*

<table>
<thead>
<tr>
<th>Agent*</th>
<th>Activity</th>
<th>Treatment time (min)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAc-neuraminic aldolase</td>
<td>10 U/ml</td>
<td>60</td>
<td>Degradation of protein-, sugar-, and lipid-containing moieties on the surface of <em>L. monocytogenes</em></td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>100 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>500 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Lipase</td>
<td>100 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Neuraminidase</td>
<td>20 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>10 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>250 U/ml</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.1%</td>
<td>10</td>
<td>Immobilization of protein moieties</td>
</tr>
<tr>
<td>Sodium metaperiodate</td>
<td>500 mM</td>
<td>60</td>
<td>Oxidation of carbohydrate moieties</td>
</tr>
</tbody>
</table>

* Prepared to pH 7.2 in HBSS (except for sodium metaperiodate, which was used at pH 5.0 in acetic buffer, and neuraminidase, used at pH 5.0 in HBSS) and added to bacterial cells for the specified time then washed to remove excess.

<table>
<thead>
<tr>
<th>Agent*</th>
<th>Activity</th>
<th>Treatment time (min)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin</td>
<td>0.1 U/ml</td>
<td>30</td>
<td>Degradation of sensitive</td>
</tr>
<tr>
<td>Lipase</td>
<td>100 U/ml</td>
<td>30</td>
<td>host cell receptors</td>
</tr>
<tr>
<td>Pepsin</td>
<td>100 U/ml</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Protease</td>
<td>0.005 U/ml</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
<td>50 U</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>1.0%</td>
<td>10</td>
<td>Immobilization of protein</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>0.1%</td>
<td>10</td>
<td>moieties</td>
</tr>
<tr>
<td>Nonidet P-40</td>
<td>0.005%</td>
<td>60</td>
<td>Oxidation of lipids</td>
</tr>
<tr>
<td>Sodium metaperiodate</td>
<td>5 mM</td>
<td>10</td>
<td>Oxidation of carbohydrate moieties</td>
</tr>
</tbody>
</table>

* Prepared to pH 7.2 in HBSS (except for sodium metaperiodate, which was used at pH 5.0) and added to macrophage host cells for the specified time then washed to remove excess.
protein content was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Macrophage monolayers were incubated for 1 h with RPMI 1640 medium containing cytochalasin D (0.5 μg/ml) and rat IgG (10 μg/ml) to prevent organism uptake through phagocytosis and to block Fc receptor-mediated binding. Following cytochalasin treatment, macrophage monolayers were incubated at 37°C for 1 h with NAcNeu or for 30 min with increasing concentrations of the purified anti-CR3 MAb ranging from 0.1 to 25 μg/ml prior to the addition of listeriae.

Adherence assays. (i) Indirect IFA. Macrophages, washed to remove nonadherent bacteria, were fixed in 10% (vol/vol) formalin in HBSS (pH 7.2) for 30 min at 25°C. After fixing, the cells were wash with phosphate-buffered saline (PBS) and treated with rabbit polyclonal anti-L. monocytogenes antiserum for 1 h at 37°C. Unbound globulin was removed, and goat anti-rabbit fluorescein isothiocyanate-conjugated antibody (Boehringer Mannheim, Indianapolis, Ind.) was added for 1 h at 37°C. Cells were stained with 0.01% propidium iodide in PBS for 20 min, washed, air dried, and mounted in glycerol containing 1% 1,4-diazobicyclo(2,2,2)octane. For the purposes of this study, a 50% decrease of listeriae to host cells. In addition, a 40% reduction in listeria binding (60% adhesion) to macrophages was observed (Fig. 1). For L. monocytogenes, this destruction of binding by sodium metaperiodate was seen to be concentration dependent (Fig. 2). When GBS were treated with various concentrations of sodium metaperiodate, a similar dose-dependent decrease in binding of the organisms to macrophages was observed (Fig. 3). However, unlike Listeria attachment, which was unaffected by pH treatment alone, GBS binding was decreased by 45%. These data demonstrated that while sodium metaperiodate abolished binding, the use of pH 5.0 in the treatment buffers in the absence of the oxidizing agent had no effect on the attachment of listeriae to host cells. In addition, a 40% reduction in listeria binding (60% adhesion) to macrophages was effected following treatment with neuraminidase. With the exception of glutaraldehyde treatment, which showed an increase in binding of greater than 60% compared with control cells, no treatment listed in Table 1 was shown to decrease the viability of the bacterial population as determined by viable bacterial cell colony counts. However, modification of adhesin molecules on the bacterial surfaces showed that treatment with the carbohydrate-oxidizing agent sodium metaperiodate abolished binding of the organism to macrophages (Fig. 1). For L. monocytogenes, this destruction of binding by sodium metaperiodate was seen to be concentration dependent (Fig. 2). When GBS were treated with various concentrations of sodium metaperiodate, a similar dose-dependent decrease in binding of the organisms to macrophages was observed (Fig. 3). However, unlike Listeria attachment, which was unaffected by pH treatment alone, GBS binding was decreased by 45%. These data demonstrated that while sodium metaperiodate abolished binding, the use of pH 5.0 in the treatment buffers in the absence of the oxidizing agent had no effect on the attachment of listeriae to host cells. In addition, a 40% reduction in listeria binding (60% adhesion) to macrophages was effected following treatment with neuraminidase. 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protocol influenced adherence of listeriae to macrophages. These data indicated that the presence of neuraminidase-sensitive carbohydrate residues on the organism surface were involved in wash-resistant attachment to the receptors on the host cell and that bacterial viability was not essential for this binding to occur.

**Host cell surface modification.** In similar fashion, host cell surface-modifying agents, with the exception of the aldehyde fixatives and Nonidet P-40, did not adversely affect macrophage viability as assayed by cell counts and trypan blue vital staining. Macrophages exposed to those enzymes or oxidizing agents listed in Table 2 showed no significant change in ability to bind the pathogen in an opsonin-independent manner. However, treatment with either glutaraldehyde or formalde-
binding of the organism when the anti-CR3 MAb was used in a blocking fashion but in the absence of organism uptake was seen to be independent of the concentration of MAb used.

**DISCUSSION**

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. It is possible that these two processes result in different reactions by the host cell to the bacterium. For *L. monocytogenes*, uptake by lysterical macrophages via the CR3 receptor resulted in death of the organism, whereas uptake by nolisterical macrophages occurred through some other recognition factor and resulted in intracellular replication of the organism (8). 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 macrophages in a wash-resistant manner (24), the rate at which host cell receptors were saturated with listeriae was investigated. In all cases, an MOI of 1.000 resulted in high levels of organism attachment to the receptors on macrophage surface membranes; however, at this inoculum, the level of nonspecific binding of *L. monocytogenes* to culture plates as seen by IFA proved problematic. An MOI of 100 resulted in a reduced background binding while affording the advantage of greater significance to a 50% reduction in binding as a result of a particular treatment. In essence, this resulted in an inhibition of adherence which was defined as the concentration and nature of a treatment agent that reduced the binding of the organism to the host cells by 50% or greater. In this study, the presence of opsonin-independent attachment of *L. monocytogenes* to murine peritoneal macrophages has been established.

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 adhesion of the organism to macrophages, while Nonidet P-40 and sodium metaperiodate were also ineffective at influencing the microbe-host cell binding phenomenon. These results indicated that the macrophage receptors involved in the attachment of the organism are protein in nature but that these are either sterically protected from the effects of proteolytic enzymes or present in sufficient quantities on the host cell membranes to allow binding of listeriae to occur at numbers similar to that found in the control macrophage cells.

Attachment of bacteria to mammalian host cells is often mediated by sugar-lectin interactions (5, 17, 23). Mannose (27) and polysialic acid (29) have been shown to facilitate the attachment of *Mycobacterium tuberculosis* and *Neisseria meningitidis*, respectively, to host cells, while *Salmonella typhimurium* competitve binding assays using sugars suggested a role for NAcNeu in the binding of *L. monocytogenes* to macrophages. Indeed, a strong inhibition of attachment (90%) was seen in the presence of 100 mM NAcNeu, while other sugars tested had no effect on the adherence process. Data similar to those reported here showed that the addition of NAcNeu in competitive binding assays impaired the adhesion of strains of *Shigella dysenteriae* and *S. flexneri* to epithelial cells (17). Results from the use of lectins in similar binding assays strongly support the involvement of NAcNeu in adhesion. The lectin from *M. amurensis* showed inhibitory effects similar to those of NAcNeu on binding of *L. monocytogenes*; this lectin has a binding affinity for oligosaccharides which possess the terminal NAcNeuα(2,3)Gal linkage. Wheat germ agglutinin reduced binding of *L. monocytogenes* by almost 50%. This lectin has binding specificity for both NAcNeu and NAcGlu. *L. polyphagus* lectin is known to bind to NAcNeu as well as N-acetylglycolylneuraminic acid. However, this lectin is extremely large, with an aggregate molecular mass of between 35 and 50 kDa, with four major protein bands seen on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25), whereas the lectin from *M. amurensis* is a single polypeptide of 8 to 9 kDa on SDS-PAGE (32). Steric hindrance may therefore be an important factor in preventing the
competition of *L. polyphemus* lectin with the adhesins of this organism.

Bacterial surface treatments indicated a 40% decrease in the binding of the organism following treatment with neuraminidase when used at pH 5.0, the optimal pH for the functioning of this enzyme. Oxidation of the bacterial surface with sodium metaperiodate resulted in a dose-dependent reduction in the binding of *L. monocytogenes* to mouse macrophages. Following treatment with 10, 100, and 500 mM sodium metaperiodate, this microbial adhesion was reduced by 30, 70, and 98%, respectively. Treatment with other enzymes and fixatives did not inhibit adherence of the organism significantly; in contrast, 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.

Recent studies have indicated that CR3 is involved in the phagocytosis of *L. monocytogenes* by listerial macrophages; however, CR3 is not involved in the phagocytosis of the organism by permissive, nonlisterial macrophages (8, 9). Accordingly, we wished to determine the effect of the anti-CR3 MAb on adherence of *L. monocytogenes* as measured by our assay. The presence of this MAb had little or no inhibitory effect on the binding of *L. monocytogenes* to macrophages in the presence or absence of normal mouse serum. In the present study, a reduction of 30% in the binding of *L. monocytogenes* to nonlisterical, thioglycollate-elicited macrophages was effected under non-opsonin-mediated conditions using anti-CR3 antibody. These results were similar to those reported by others (7, 8). However, in contrast to their reported results, the presence or absence of normal mouse serum in our system had no effect on binding in that approximately the same number of listeriae bound to both groups of control macrophage cells. The results reported here indicate that adherence and uptake in permissive macrophages occur by mechanisms other than through CR3 mediation. Significantly, NAcNeu at a concentration of 100 mM was highly inhibitory to the binding of opsonized-*L. monocytogenes*.

In contrast to GBS, *L. monocytogenes* does not utilize CR3 as a means for adhering to nonbactericidal, permissive macrophages (2, 8). However, the adhesin molecule used by listeriae to initiate the cellular infectious process was identical whether permissive or listerialicidal macrophages were used as host cells. Attachment in both the presence and absence of opsonins is strongly inhibited by NAcNeu. In addition, treatment of the bacterial cell surface with the carbohydrate-oxidizing agent sodium metaperiodate also inhibited listerial adhesion to macrophages. Based on our results, we propose the involvement of NAcNeu, a member of the sialic acid group, in the attachment of *L. monocytogenes* to nonlisterical, permissive host cells. The process of attachment occurred through receptors other than CR3 and allowed the intracellular replication of the organism within host cells to proceed.

Entry of facultative intracellular pathogens into cells that are not professionally phagocytic has been described as a multifactorial process and has been studied extensively in *Yersinia, Salmonella*, and *Shigella* as well as *Listeria*. Indeed, it has been shown that entry and subsequent internalization is effected either through a trigger mechanism as is the case with *Salmonella* and *Shigella* or via a zipper mechanism as reported for *Yersinia* and *Listeria* (11, 19, 22, 30). Efforts to elucidate the nature of the bacterial ligand (invasin protein) as well as the host cell receptor (β1 integrin receptor) involved in the binding and entry of some facultative intracellular bacterial pathogens into cells has met with some success. Indeed, the critical role of the internalin molecule mediating bacterial entry and invasion of epithelial host cells has been demonstrated (13). In addition, E-cadherin, a calcium-dependent cell-to-cell adhesion glycoprotein located on the surface of epithelial cells, was shown to be the receptor for internalin (22). Further studies have shown that the bacterial surface protein p60 may be involved in uptake of *L. monocytogenes* by transformed nonproliferating epithelial cells, while the internalin proteins InlA and InlB mediate the invasion of these host cells when in an actively multiplying state (31). From the data presented here, it is possible that internalin proteins, in addition to regulating invasion, are involved in facilitating attachment to permissive peritoneal macrophages. However, the nature of the receptors located on macrophage membranes involved in opsonin-independent binding remains unclear. Additional studies are in progress to determine whether bacterial molecules other than internalin are involved in the attachment of *L. monocytogenes* to permissive macrophages. Understanding the mechanisms by which *L. monocytogenes* binds to host cells both in the presence and in the 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 preventative treatment strategies for listeriosis.

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