Listeria monocytogenes Infection and Activation of Human Brain Microvascular Endothelial Cells

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Listeria monocytogenes **invasion of human brain microvascular endothelial cells (BMEC) and its role as a stimulus for endothelial cell activation were studied. Binding and invasion of intact BMEC monolayers were independent of the *L. monocytogenes inlAB* invasion locus. Cytochalasin D abrogated invasion of BMEC, whereas genistein effected only a 53% decrease in invasion, indicating a requirement for rearrangement of actin microfilaments but less dependence on tyrosine kinase activity. *L. monocytogenes* stimulated surface expression of E-selectin, ICAM-1, and to a lesser extent, VCAM-1, whereas *L. monocytogenes prfA*− and Δ*hly* mutants were severely compromised in this respect. Other experiments showed that BMEC infection stimulated monocyte and neutrophil adhesion and that CD18-mediated binding was the predominant mechanism for neutrophil adhesion to infected BMEC under static conditions. These data suggest that invasion of BMEC is a mechanism for triggering inflammation and leukocyte recruitment into the central nervous system during bacterial meningitis.**

Invasion of the central nervous system (CNS) is a critical event in the pathophysiology of bacterial meningitis. One means by which pathogenic bacteria cross the blood-brain barrier and enter the subarachnoid space is by direct penetration through endothelial cells [1]. This mechanism has been demonstrated in vitro using brain microvascular endothelial cells (BMEC) and pathogens such as *Escherichia coli, Haemophilus influenzae,* and group B streptococci [2–5]. An increasing body of evidence indicates that bacterial invasion of endothelial cells can initiate profound alterations in endothelial cell functions. For example, several microbes known to invade the CNS, including *Borrelia burgdorferi, Leptospira icterohemorrhagiae*, *Listeria monocytogenes,* and *Treponema pallidum,* up-regulate surface adhesion molecule expression and stimulate leukocyte adhesion to human umbilical vein endothelial cells (HUVEC) [6–10]. These data suggest that endothelial cell invasion may play a critical role in triggering immigration of leukocytes into the CNS during bacterial meningitis.

L. monocytogenes is a facultative intracellular bacterium that causes bacteremia and CNS infections, including meningitis, meningoencephalitis, and brain abscess, in humans and domesticated animals [11, 12]. The proclivity of *L. monocytogenes* for infecting the CNS suggests that invasion of vascular endothelial cells may be an important means for circulating *L. monocytogenes* to cross the blood-brain barrier. This hypothesis is supported by histologic findings in experimental *L. monocytogenes* meningitis in mice and by electron microscopy of the brainstem in a fatal case of human rhombencephalitis showing *L. monocytogenes* within endothelial cells [13, 14]. Additionally, in vitro data show that *L. monocytogenes* is able to invade and replicate within endothelial cells, consistent with its biology as a facultative intracellular parasite of eukaryotic cells [15].

Recent data from this laboratory and from others showed that *L. monocytogenes* infection of HUVEC elicits a vigorous cellular response. This includes generation of a lipid mediator response, up-regulation of surface adhesion molecules such as P-selectin (CD62P), E-selectin (CD62E), ICAM-1 (CD54), and VCAM-1 (CD106), increased neutrophil (PMNL) adhesion to infected monolayers, and increased expression of interleukin (IL)-6 and IL-8 [9, 10, 16, 17]. These data suggested that infection of vascular endothelial cells could be an important event in the pathophysiology of neurolisteriosis, particularly as a means for recruiting leukocytes to the CNS. Nevertheless, there is phenotypic and functional heterogeneity between large-vessel endothelial cells such as HUVEC and specialized microvascular endothelial cells [18, 19]. Thus, it is possible that the HUVEC response to *L. monocytogenes* might not be predictive of the BMEC response to *L. monocytogenes.* To test this, we studied interactions between *L. monocytogenes* and cultured primary human BMEC from the standpoints of bacterial and cellular factors associated with invasion and subsequent cellular activation in response to infection.

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Materials and Methods

Antibodies. Mouse monoclonal antibodies (MAbs) directed against human ICAM-1 (CD54, IgG1), E-selectin (CD62E, IgG1), P-selectin (CD62P, IgG1), and VCAM-1 (CD 106, IgG1) were obtained from Serotec (Washington, DC). Horseradish peroxidase–conjugated goat anti-mouse IgG was purchased from Bio-Rad (Hercules, CA). Mouse anti-human CD18 (IgG1) was purchased from Collaborative Biomedical Products (Bedford, MA). Mouse anti-human α_4 chain of CD49d (IgG1) was purchased from PharMingen (San Diego).

Bacteria. *L. monocytogenes* strain EGD, originally obtained from G. B. Mackaness, was a gift from P. Campbell (National Jewish Medical and Research Center, Denver, CO). *L. monocytogenes* containing in-frame deletion mutants of *hly* (DP-L2161) and *plcA* + *plcB* (DP-L1936) and the wild type parent strain, 10403s, were gifts from D. Portnoy (University of California, Berkeley) [20]. Mutants of *L. monocytogenes* containing in-frame deletions of *inlA* (BUG 947), *inlB* (BUG1047), and *inlAB* (BUG 949) and the wild type parent strain, EGD (designated EGD-Pasteur), were gifts from P. Cossart (Institut Pasteur, Paris) [21]. The nonhemolytic, avirulent *L. monocytogenes* strain 43250, which harbors a mutation in the *prfA* gene, was purchased from the American Type Culture Collection (Rockville, MD) [22, 23].

Cultures containing 10^9 bacteria/mL were stored at −70°C in tryptose phosphate broth (Difco, Detroit) or brain-heart infusion broth (Difco) containing 15% glycerol. For each experiment, a fresh culture of bacteria was prepared by inoculating 10 μL of stock culture into 4 mL of broth. Cultures were incubated overnight at 37°C with shaking or, in the case of DP-L2161 and DP-L1936, at 30°C without shaking. Bacteria were washed by centrifugation at 12,000 *g* for 3 min, followed by resuspension and vortex mixing in either BMEC medium or Hanks' balanced salt solution (HBSS).

Endothelial cell culture. Human BMEC were purchased from Cell Systems (Kirkland, WA). These are primary cells isolated from normal human brain cortical tissue at autopsy by enzymatic digestion. They are >95% positive by immunofluorescence for cytoplasmic uptake of Di-I-Ac-LDL and for cytoplasmic von Willebrand's factor/factor VIII, confirming their identity as endothelial cells. The cells were cultured in CS-C 4Z0-500 medium without antibiotics. Cells were maintained in tissue culture flasks, fed daily to every other day, and split 1:2–1:3 into 96-well plates coated with attachment factor (Cell Systems). BMEC were used between passages 4 and 10; early-passage cells were cryopreserved and then thawed as needed for experiments. As cells aged, their doubling time slowed, whereas their general morphology did not change.

Leukocyte separation. Human blood was obtained from healthy donors by venipuncture and was drawn into EDTA-containing tubes (Vacutainer; Becton Dickinson, Lincoln Park, NJ). PMNL were separated by layering whole blood over neutrophil isolation medium (Cardinal Associates, Santa Fe, NM) as described previously [9]. Monocytes were separated by mixing EDTA-anticoagulated whole blood 10:1 with 6% dextran 500 (Sigma, St. Louis) in 0.9% NaCl and allowing the erythrocytes to settle. The resulting leukocyte-rich plasma was layered over Nycoprep 1.068 (Gibco BRL, Gaithersburg, MD) and centrifuged for 15 min at 600 *g* at room temperature. The monocyte-containing band was collected and washed with 1% bovine serum albumin in PBS containing 3.5 m*M* EDTA. Platelets were removed by centrifugation through 30% sucrose in PBS. Monocyte preparations routinely were >85% pure as determined by light microscopy of stained (Diff-Quik; Baxter Healthcare, McGaw Park, IL) cytosmears. Contaminating cells were mainly lymphocytes with rare PMNL.

Endothelial cell infection. BMEC cultured in 96-well plates were infected with serial dilutions of wild type *L. monocytogenes* or *L. monocytogenes* mutants by centrifugation of bacteria onto cells at 1000 *g* for 8–10 min as previously described [9, 15]. For experiments measuring binding and invasion of *L. monocytogenes,* triplicate wells from duplicate 96-well plates were infected with 10^4 wild type or mutant *L. monocytogenes.* After 60 min, cells from one plate were washed three times with 100 μL/well of a solution containing 3 parts PBS and 1 part F12K medium plus 2.5% fetal calf serum. Cells were collected by pipetting with 0.3 mL of sterile distilled H_2O containing 0.5% saponin plus 3 m*M* EDTA. Colony-forming units (cfu) of bacteria in cell lysates and in the inoculum were quantified by serial dilution and plating on trypticase soy agar (Difco). The percentage of the bacterial inoculum bound was calculated as follows: (cfu cell-associated bacteria/cfu bacterial inoculum) × 100. The remaining plate was incubated for an additional 60 min in the presence of gentamicin (Gibco BRL), final concentration 50 μg/mL, to kill extracellular bacteria; then cfu of intracellular *Listeria* organisms were quantified as before [9, 15]. The percentage of invasion was calculated as follows: (cfu intracellular bacteria/cfu bacterial inoculum) × 100.

Other experiments tested the effect of cytochalasin D and genistein (Sigma) on bacterial binding and invasion. For this, endothelial cells in duplicate 96-well plates were incubated with 2-fold dilutions of cytochalasin D or genistein for 60 min and then infected with 10^4 bacteria. Cells and bacteria were cultured for 60 min and washed, and bacterial binding and invasion were measured as described above.

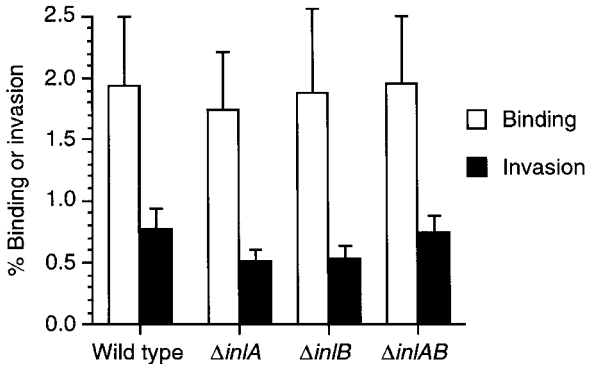

Figure 1. Binding and invasion of confluent brain microvascular endothelial cells by *L. monocytogenes* is independent of *inlAB* locus. Endothelial cells were infected with 10^4 wild type *L. monocytogenes* or Δ*inlA,* Δ*inlB,* and Δ*inlAB* mutants, and bacterial binding and invasion were measured. Binding was calculated as (cfu cell-associated bacteria/cfu bacterial inoculum) × 100. Invasion was calculated as (cfu intracellular bacteria/cfu bacterial inoculum) × 100. Results from 3 similar experiments are shown (mean + SE).

In experiments that measured BMEC adhesion molecule surface expression or leukocyte adhesion, BMEC were infected with the indicated bacteria or, in some cases, stimulated with recombinant human tumor necrosis factor-α (TNF-α) (R&D Systems, Minneapolis) or lipopolysaccharide (LPS) from *Salmonella typhimurium* (Sigma). Cells were cultured for 60 min before adding gentamicin and then were cultured for a total of 5–24 h. At the indicated time, surface adhesion molecule expression was measured by whole cell ELISA as previously described, and the mean absorbance at 490 nm ± SD from quadruplicate wells was calculated [9]. Relative E-selectin and ICAM-1 expression on BMEC following infection with *L. monocytogenes* mutants were calculated as a percentage of the ELISA signal obtained after infection with wild type bacteria according to the following formula: absorbance after infection with mutant bacteria/absorbance after infection with 5×10^4 *L. monocytogenes* strain 10403s [24].

Leukocyte adhesion assay. Immediately before incubation with *Listeria*-infected or TNF-α–stimulated BMEC, PMNL or monocytes (1.0×10^7/mL) were loaded with 5 m*M* calcein AM (Molecular Probes, Eugene, OR) in HBSS at room temperature for 30 min [25]. The cells were washed twice with HBSS without Mg^{2+} or Ca^{2+} to remove excess label. Leukocytes were resuspended at 1×10^6/mL in CS-C complete medium, 100 μL was added to the appropriate wells, and the cells were cocultured for 30 min at 37°C under static conditions. Nonadherent leukocytes were removed by washing the plate five times with HBSS containing Mg^{2+}/Ca^{2+} plus 1% fetal calf serum. Fluorescence intensity of adherent leukocytes was measured in a fluorescence microplate reader (CytoFluor 4000; PerSeptives Biosystems, Framingham, MA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The percentage of leukocyte adhesion was calculated with CytoCalc software (PerSeptives Biosystems) from standards containing serial 2-fold dilutions of labeled leukocytes.

Other experiments tested the effects of anti–adhesion molecule MAbs on PMNL binding to infected BMEC. For this, BMEC were infected with 10^5 *L. monocytogenes* and incubated for 4–5 h. PMNL or BMEC were incubated with 10 μg/mL of the indicated MAb prior to coculture, as described previously [9]. The percentage of PMNL adhesion was quantified as described above. The percentage of PMNL adhesion to infected cells in the absence of any antibody (control) was standardized to 100 and the percentage of control PMNL adhesion for each group was calculated as follows: (% PMNL adhesion with MAb/% PMNL adhesion control) × 100.

Statistical analysis. *P* values between means were calculated by the Student's *t* test by use of Excel software (Microsoft, Redmond, WA) with a level of significance set at <.05.

Results

L. monocytogenes binding and invasion of BMEC. First, we compared binding and invasion of BMEC by wild type *L. monocytogenes* and gene deletion mutants of *L. monocytogenes* that lack the invasion proteins internalin (Δ*inlA*), internalin B (Δ*inlB*), or both (Δ*inlAB*). Figure 1 shows that mutant *L. monocytogenes* bound to and invaded BMEC as well as wild type did. The Δ*inlA* and Δ*inlB* mutants showed slightly decreased invasion; however, these differences were not statistically significant ($P > .05$) compared with wild type bacteria. By comparison, invasion by wild type bacteria was inhibited in a dose-dependent manner by cytochalasin D, culminating in near complete absence of intracellular bacteria (figure 2). The tyrosine kinase inhibitor, genistein, has also been shown to inhibit *L. monocytogenes* invasion of nonphagocytic cells, primarily epithelial cells [26, 27]. Figure 2 shows that pretreatment of BMEC with genistein increased bacterial binding by 619% ± 80% (mean ± SE, $n = 3$) over control; however, this did not achieve statistical significance because of variability between experiments ($P = .14$). Genistein did inhibit invasion, to 52.7% ± 1.5% ($P < .01$ compared with untreated cells), at the highest concentration tested. The same concentration of genistein also inhibited *L. monocytogenes* invasion of HUVEC, to 58.7% ± 11.6% of control ($P < .01$ compared with untreated cells), but did not significantly affect binding (data not shown). Control experiments showed that the diluent, DMSO, did not affect binding or invasion at the concentrations used (data not shown).

L. monocytogenes infection of BMEC up-regulates surface adhesion molecules. BMEC were infected with increasing numbers of *L. monocytogenes* and incubated for a total of 5–24 h after infection, and then surface adhesion molecule expression was measured by whole cell ELISA. We found that BMEC tolerated infection with up to 3×10^5 bacteria/well in a 96-well plate for at least 6 h without detaching, ~10-fold more bacteria than HUVEC could tolerate under similar conditions [9]. However, after infection with this inoculum, the monolayer was completely infiltrated with bacteria and quite deteriorated following overnight growth in medium containing gentamicin. Thus, 3×10^4 cfu of wild type *L. monocytogenes* per well was

,,,,,,(v52),(v52),(v54),C,C

Figure 2. Differential effects of cytochalasin D and genistein on *L. monocytogenes* binding and invasion of brain endothelial cells. Brain microvascular endothelial cells were incubated with indicated concentrations of cytochalasin D (**A**) or genistein (**B**) for 60 min and then infected with *L. monocytogenes,* and bacterial binding and invasion were measured. Results are mean + SE % of control binding or invasion from 1 of 2 identical experiments with similar results (**A**) or mean + SE % of control binding or invasion from 3 separate experiments (**B**).

used for experiments lasting >6 h. Figure 3 shows that expression of surface E-selectin, ICAM-1, and, to a lesser extent, VCAM-1 were stimulated by *L. monocytogenes* infection and that the magnitude increased with increasing cfu of infecting bacteria. Further studies showed that increased surface E-selectin and ICAM-1 were noted as early as 2 h after infection and that E-selectin expression peaked at 8 h and then declined, while ICAM-1 remained elevated (not shown).

Next, we tested the roles of bacterial virulence factors for stimulating BMEC E-selectin and ICAM-1. Previous studies with HUVEC showed that the only single mutations in the *L. monocytogenes* virulence genome that significantly altered its ability to induce adhesion molecule expression were those that rendered a nonhemolytic phenotype [24]. Similarly, the nonhemolytic Δ*hly* and *prfA*− mutants were quite crippled in their ability to up-regulate surface E-selectin or ICAM-1 on BMEC (figure 4). About 150-fold more *prfA*− mutants were required to elicit a cellular response of a magnitude similar to that of wild type, whereas Δ*hly* mutants at the same inoculum still did not elicit a comparable response. In contrast, Δ*plcA* + Δ*plcB* mutants were only somewhat less active than the parent strain in stimulating BMEC and were clearly more active than the nonhemolytic bacteria in this respect.

Other experiments tested the roles of bacterial binding and invasion as stimuli for E-selectin and ICAM-1 up-regulation. For this, BMEC incubated with increasing concentrations of cytochalasin D were infected with *L. monocytogenes* or stimulated with LPS, and surface E-selectin and ICAM-1 expression were measured 6 and 24 h later, respectively. Cytochalasin D caused a dose-dependent decrease in surface E-selectin and ICAM-1 expression on infected cells (figure 5), with expression reduced to preinfection levels at a concentration shown in figure 2 to cause nearly complete inhibition of invasion despite preserved binding. By comparison, surface E-selectin and ICAM-1 remained elevated above control levels on cytochalasin D–treated cells stimulated with LPS. These data suggest that binding of *L. monocytogenes* to BMEC is not sufficient to trigger adhesion molecule expression and that internalization is required for cytostimulation.

L. monocytogenes infection stimulates leukocyte adhesion to BMEC. Figure 6 shows that 6 h after *L. monocytogenes* infection, there was increased PMNL adhesion to BMEC in an inoculum-dependent fashion. In these experiments, there was 3.72% ± 0.72% (mean ± SE, $n = 6$) PMNL adhesion to uninfected monolayers and 17.22% ± 3.18% adhesion to monolayers infected with 3×10^5 cfu of bacteria/well, representing a 4.85-fold increase over baseline ($P < .01$). Monocyte adhesion to BMEC was increased after 6 and 24 h of infection with 10^4 *L. monocytogenes* and was similar to the effect of incubation with 0.2 ng/mL TNF-α (figure 7).

Previous studies showed that PMNL adhesion to *L. monocytogenes*–infected HUVEC could be blocked by MAb to E-selectin as well as anti-CD18 MAb to a greater [10] or lesser [9] degree. To determine the relative roles of E-selectin and ICAM-1 in PMNL adhesion to *L. monocytogenes*–infected BMEC, MAbs directed against endothelial cell and PMNL adhesion molecules were added, and PMNL adhesion was quantified. In contrast to the situation with HUVEC, we found that PMNL adhesion was nearly completely abrogated by anti-CD18 MAb (figure 8). However, anti–E-selectin MAb as a single agent had little inhibitory effect that was not different from the anti–VLA-4 (CD49d) MAb that functioned as control, because this molecule is not involved in PMNL adhesion [28]. Additional control MAbs included anti–VCAM-1 (CD106) and the anti–P-selectin (CD62p) MAb, which is directed against a nonblocking epitope. These data suggest that *L. monocytogenes* stimulates differential binding mechanisms on BMEC and HUVEC despite similarities in expression of these adhesion molecules.

Discussion

Endothelial cells interact with a diverse array of pathogenic bacteria, fungi, and viruses in the course of many infectious diseases [29]. These interactions then can initiate an inflammatory response and contribute to host defense as well as to tissue damage. HUVEC have been used as a model for in vitro study of interactions between endothelial cells and neuroinvasive bacteria. One important finding from these studies is that bacterial invasion or infection of endothelial cells can alter endothelial cell functions, including adhesion molecule expression and leukocyte adhesion [2–10]. Previous work in this laboratory and in others used HUVEC to study these aspects of endothelial cell function in response to *L. monocytogenes* infection [9, 10, 15–17]. Nevertheless, *L. monocytogenes* infection of specialized brain microvascular endothelium had not yet been studied, and it was unknown whether BMEC and HUVEC would respond differentially to this stimulus.

The *L. monocytogenes* surface invasion proteins, internalin and InlB, are important for mediating binding and invasion of nonprofessional eukaryotic cells [30]. In a prior study, we showed that *L. monocytogenes* binding to HUVEC was internalin-independent in the presence of serum, and that a transposon-induced *inlAB*− mutant invaded endothelial cells ~5-fold less efficiently than did wild type [15]. Similarly, the data reported here found that gene deletion mutants lacking *inlA, inlB,* and *inlAB* bound to BMEC as well as did the parent strain. However, these mutants also invaded BMEC as efficiently as did wild type. This discrepancy may be due to differences in the mutants, transposon-induced versus gene deletion, as suggested by similar findings that the *inlAB* locus does not contribute to HUVEC invasion [17]. However, biologic differences could exist between macrovascular and specialized microvascular endothelium that affect expression of surface molecules that participate in binding and invasion of bacteria. Such a finding has been demonstrated for invasion of endo-

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Figure 3. *L. monocytogenes* infection up-regulates surface E-selectin, ICAM-1, and VCAM-1 on brain microvascular endothelial cells. Endothelial cells were infected for 60 min with increasing amounts of *L. monocytogenes* and cultured for total of 5 h (**A**) or 24 h (**B**). Surface E-selectin, ICAM-1, and VCAM-1 were quantified by whole cell ELISA. Mean + SD absorbance of quadruplicate wells from representative experiment is shown.

thelial cells by *E. coli* [4]. Additionally, other newly described members of the *L. monocytogenes inl* gene family, or non–*inl*-related invasion proteins, may have a role in binding and invasion of endothelial cells [31–34].

Experiments with cytochalasin D showed that bacterial invasion of BMEC required rearrangement of actin microfilaments, as previously shown for HUVEC [24]. In contrast, invasion of endothelial cells was less dependent on tyrosine kinase activity, in that genistein decreased invasion in HUVEC and BMEC to ~59% and 53% of control values, respectively. A possible difference between HUVEC and BMEC in this regard was that genistein had no significant effect on *L. monocytogenes* binding to HUVEC, whereas there was a trend toward increased binding to BMEC. An explanation for this is that tyrosine phosphorylation is important for regulation of tight junction permeability in cultured BMEC and for organization of cell-cell adhesions in macrovascular endothelial cells [35]. Thus, it is possible that genistein alters BMEC tight junctions in such a way as to expose more bacterial binding sites.

These data illustrate similarities and differences between endothelial cells and other nonprofessional phagocytes with respect to binding and internalization of *L. monocytogenes.* The main similarity is that invasion of BMEC, HUVEC, and epithelial cells is prevented by cytochalasin D, indicating an absolute dependence on actin polymerization and rearrangement of actin microfilaments [24, 36] (this work). However, differences appear to exist among nonprofessional phagocytes with regard to the molecules used by *L. monocytogenes* for binding and invasion. Recent studies show that molecules encoded by the *inl* family of invasion genes, particularly internalin and InlB, promote invasion of epithelial cell–like lines and hepatocytes in vitro [30, 37–40]. In contrast, binding and internalization of endothelial cells and fibroblasts in vitro seem independent of these invasion proteins [15, 17, 38] (this work). Another difference is that the tyrosine kinase inhibitor, genistein, inhibits 90%–99% of *L. monocytogenes* invasion of epithelial cell lines [26, 27] but seems much less effective on endothelial cells (this work). This could be due to the presence of growth factors in endothelial cell cultures that alter basal states of phosphorylation of kinases important to internalization [39]. However, it also raises the possibility that *inlAB*-mediated invasion is dependent on tyrosine kinase activity, whereas *inlAB*-independent binding triggers internalization without it.

The response of BMEC to *L. monocytogenes* invasion was also studied. *L. monocytogenes* infection up-regulated surface expression of E-selectin, ICAM-1, and to a lesser extent, VCAM-1. We also found that production of listeriolysin O and bacterial invasion are critical factors for stimulating BMEC as well as HUVEC [24]. Nevertheless, there was a significant difference between HUVEC and BMEC with regard to which adhesion molecules were important for mediating PMNL adhesion to infected monolayers. Anti-CD18 MAb completely blocked the increased PMNL adhesion to infected BMEC, whereas anti–E-selectin MAb by itself had little effect. By contrast, PMNL adhesion to *L. monocytogenes*–infected HUVEC is blocked by anti–E-selectin MAb [9, 10]. This difference may be a result of assay conditions in that PMNL binding experiments with HUVEC were conducted in RPMI medium [9]. BMEC did not tolerate RPMI, so these assays were conducted in the endothelial cell growth medium, which may have interfered with E-selectin–mediated binding or dramatically increased CD18-mediated binding. It is also possible that infected HUVEC and BMEC differentially produce other factors, such as IL-8 or cell-bound IL-1, important for PMNL binding that would bring about this difference.

The importance of CD18-mediated binding of PMNL to BMEC activated by *L. monocytogenes* infection complements in vivo studies showing that anti-CD18 MAb blocks PMNL migration into the cerebrospinal fluid in experimental meningitis [41]. However, the role of E-selectin as a mediator of CNS inflammation is less clear. A recent study found that serum levels of soluble isoforms of E-selectin and ICAM-1 were sig-

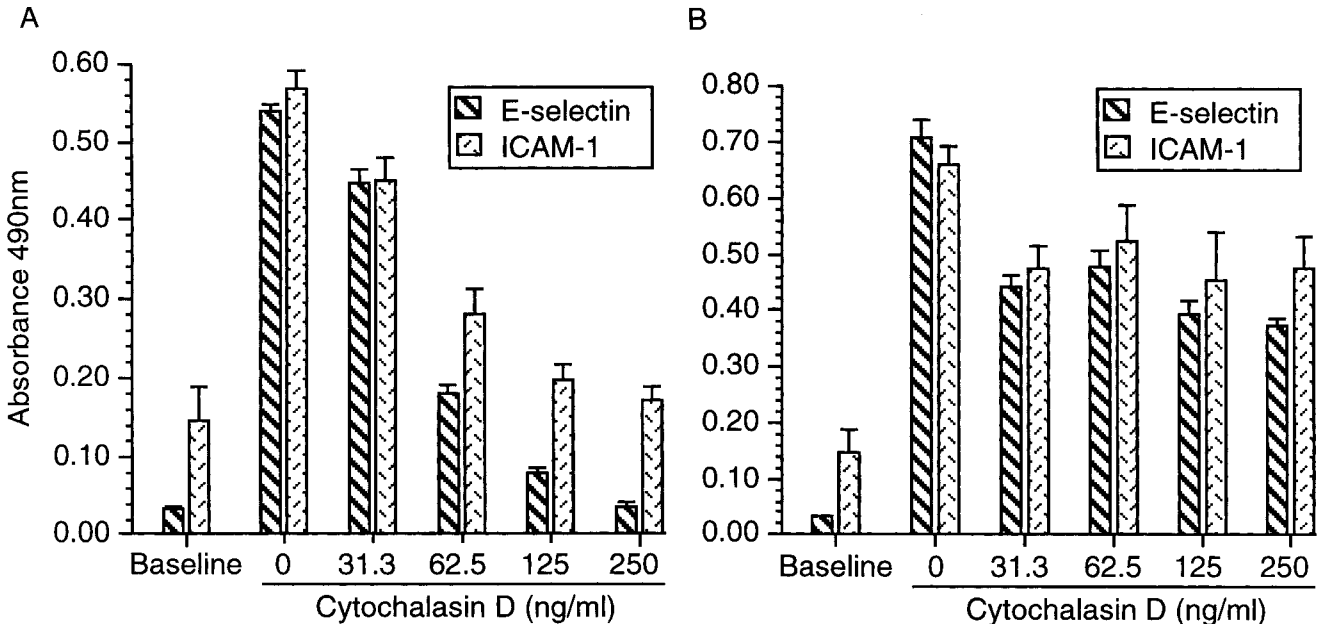

Figure 5. Cytochalasin D inhibits up-regulation of surface E-selectin and ICAM-1 in response to *L. monocytogenes* infection but not in response to lipopolysaccharide (LPS) stimulation. Brain microvascular endothelial cells were incubated with cytochalasin D and then were infected with *L. monocytogenes* (**A**) or stimulated with LPS, 100 ng/mL (**B**). E-selectin and ICAM-1 surface expression was measured after 5 h and 24 h, respectively, by whole cell ELISA. Results are mean + SD absorbance from quadruplicate wells from 1 of 2 similar experiments with comparable results.

nificantly increased in patients with bacterial meningitis and that soluble E-selectin levels correlated positively with cerebrospinal fluid pleocytosis, IL-1β, and TNF-α [42]. Additionally, E-selectin has been shown to function in a complementary fashion to P-selectin for mediating cerebrospinal fluid pleocytosis in mice genetically deficient in P-selectin or both E- and P-selectin [43]. In vitro studies have shown that TNF, LPS, and IL-1β can up-regulate surface E-selectin on cultured BMEC; however, one report suggests that its surface expression may be suppressed by the brain microenvironment [44, 45]. Taken together, these data suggest that E-selectin may contribute to leukocyte recruitment into the CNS during bacterial meningitis, but functional redundancy in the selectins or microenvironmental factors may limit its importance as a single molecule.

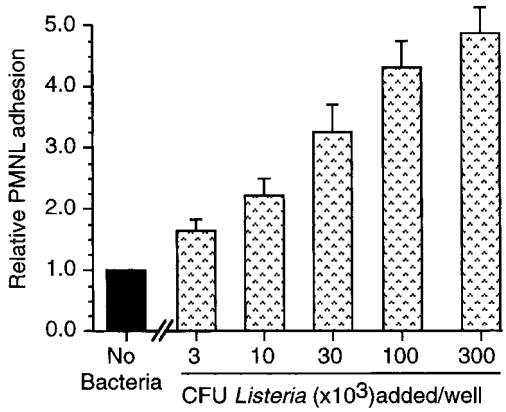

Figure 6. *L. monocytogenes* infection of endothelial cells stimulates neutrophil (PMNL) adhesion. Brain microvascular endothelial cells were infected with *L. monocytogenes* and cultured for 5–6 h before addition of calcein AM–loaded PMNL. % of PMNL adhesion was quantified from quadruplicate wells using fluorescence plate reader. Results from 6 separate experiments are shown as mean + SE relative increase in neutrophil adhesion, calculated as % of adherent PMNL (infected cells)/% adherent PMNL (control).

The data presented here support the idea that infection of the brain microvascular endothelium can play an important role in facilitating leukocyte recruitment into the CNS during *L. monocytogenes* meningitis. This notion is supported by the relationship between bacteremia, bacterial CNS invasion, and leukocyte accumulation in the CNS during experimental *L. monocytogenes* meningitis in mice [13]. This is not, however, the only mechanism for *L. monocytogenes* to initiate CNS inflammation. For example, intracerebral inoculation of *L. monocytogenes* into mice induces production of cytokines, including IL-6 and TNF-α, in the cerebrospinal fluid and C5a receptor expression on neurons [46, 47]. Cytokine stimulation can then contribute to leukocyte recruitment through up-regulation of BMEC surface adhesion molecules, including E-selectin, ICAM-1, and VCAM-1 [44, 45, 48].

In summary, BMEC and HUVEC share certain responses to infection and activation by *L. monocytogenes.* The most significant similarities include cellular requirements for bacterial

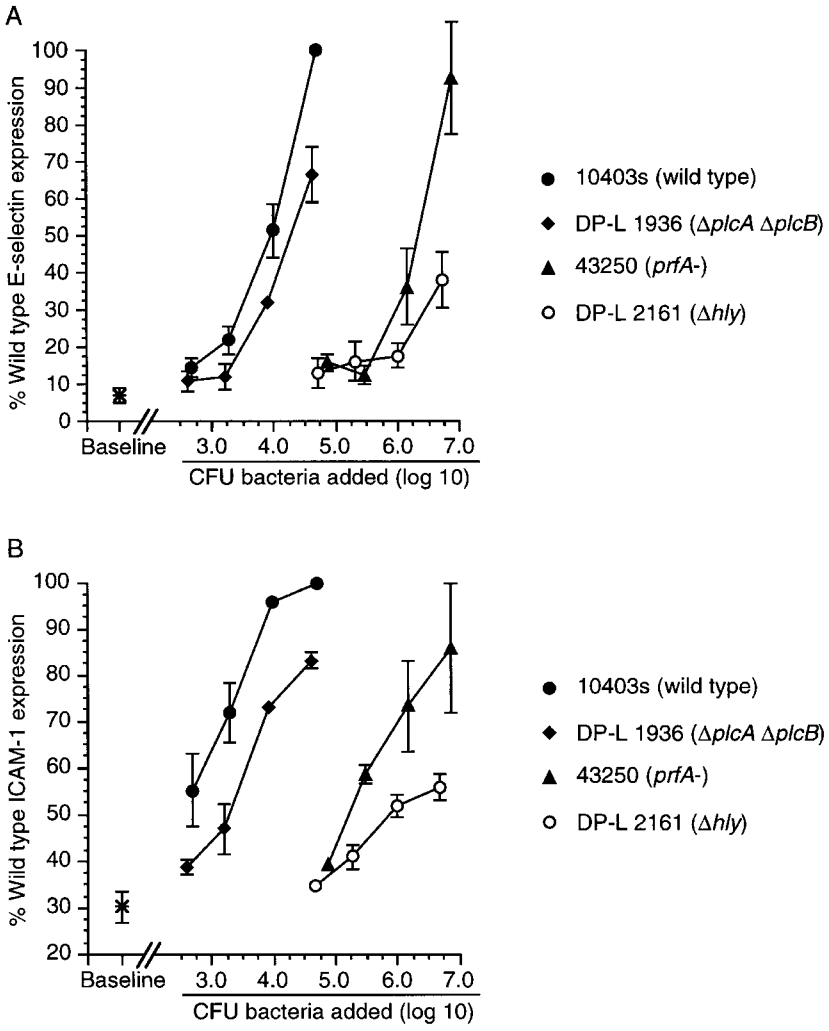

Figure 4. Nonhemolytic *L. monocytogenes* mutants are crippled in their ability to increase surface expression of E-selectin and ICAM-1 on brain microvascular endothelial cells. Endothelial cells were infected with increasing amounts of wild type *L. monocytogenes* 10403s, Δ*plcA* + Δ*plcB*, *prfA*−, and Δ*hly* mutants. Relative surface E-selectin expression (**A**) and ICAM-1 (**B**) were quantified by whole cell ELISA after 5- and 18-h incubations, respectively. Relative % of adhesion molecule expression for each bacterium was calculated as (absorbance at 490 nm bacteria/absorbance at 490 nm after infection with 5×10^4 *L. monocytogenes* 10403s) × 100. Mean + SE from 3–4 experiments is shown. Points without error bars have SE < 1.5.

invasion, bacterial factors that contribute to endothelial cell stimulation, an endothelial cell phenotype following infection marked by increased adhesion molecule expression, and subsequent leukocyte adhesion to infected monolayers. However, there were also notable differences between the two cell types, including the endothelial cell adhesion molecules that mediate PMNL binding to infected cells and possibly the bacterial molecules that mediate binding and invasion. These results show that HUVEC are a reasonable model for studying certain interactions between bacterial pathogens and endothelial cells, including aspects of the cellular response to infection. Nevertheless, endothelial cell heterogeneity makes it important that in vitro data derived from a model endothelial cell system be validated by use of the appropriate specialized endothelium.

Acknowledgment

This article is dedicated to the memory and scientific legacy of Priscilla (Pixie) A. Campbell, who was a friend and mentor of D.A.D.

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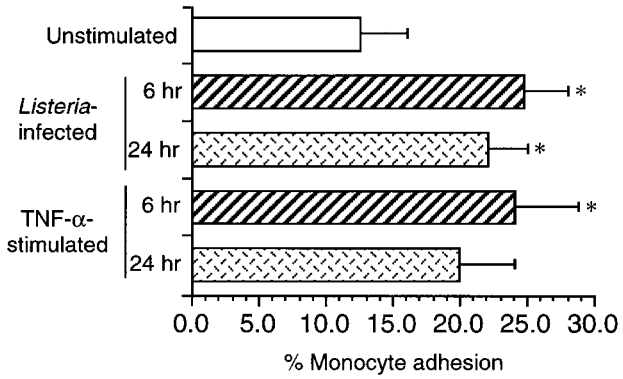

Figure 7. *L. monocytogenes* infection and tumor necrosis factor (TNF)-α stimulate monocyte adhesion to brain microvascular endothelial cells. Endothelial cells were infected with *L. monocytogenes* or stimulated with TNF-α (0.2 ng/mL) and cultured for total of 6 or 24 h before addition of calcein AM–loaded monocytes. % of monocyte adhesion in quadruplicate wells was quantified using fluorescence plate reader. Results are mean + SE from 6 experiments. * $P < .05$ compared with unstimulated cells.

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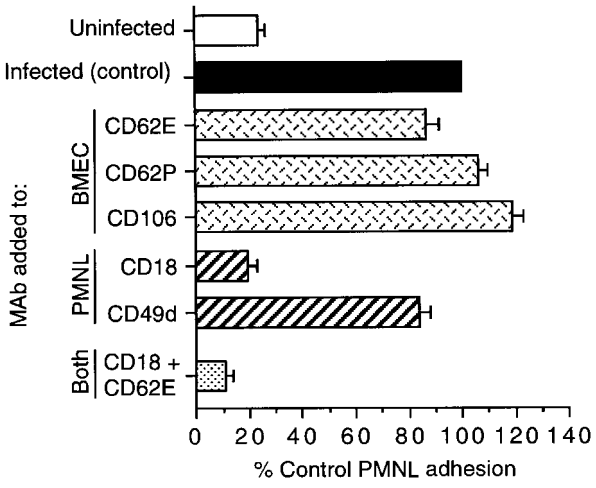

Figure 8. Anti-CD18 monoclonal antibody (MAb) inhibits neutrophil (PMNL) adhesion to *L. monocytogenes*–infected brain microvascular endothelial cells. Endothelial cells were infected with *L. monocytogenes* and then cultured for total of 5–6 h, and PMNL adhesion was measured in presence or absence of indicated MAb (10 μg/mL). % of control neutrophil adhesion was calculated as (% neutrophil adhesion + MAb/% control neutrophil adhesion) × 100. Results are mean + SE from 6 experiments.

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