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Human brain microvascular endothelial cell traversal by *Borrelia burgdorferi* requires calcium signaling

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Abstract

Neurological manifestations of Lyme disease (or neuroborreliosis) occur variably and while it is clear that *Borrelia burgdorferi* can invade the nervous system, how it does so is not well understood. Pathogen penetration through the blood brain barrier (BBB) is often influenced by calcium signaling in the endothelial cells triggered by extracellular host-pathogen interactions. We examined the traversal of *B. burgdorferi* across the human BBB using *in vitro* model systems constructed of human brain microvascular endothelial cells (HBMEC) grown on Costar Transwell™ inserts. Pretreatment of the cell monolayers with BAPTA-AM (an intracellular calcium chelator) or phospholipase C (PLC) inhibitor U73122 inhibited *B. burgdorferi* transmigration. By 5 h, BAPTA-AM significantly inhibited (82–99%; $p < 0.017$) spirochete traversal of HBMEC compared to DMSO controls. Spirochete traversal was almost totally blocked (≥99%; $p < 0.017$) after pretreatment with the PLC-β inhibitor U73122 as a result of barrier tightening based on electric cell-substrate impedance sensing (ECIS). The data suggest a role for calcium signaling in CNS spirochete invasion through endothelial cell barriers.

Keywords: Blood-brain barrier, *Borrelia burgdorferi,* calcium signaling, endothelial cells, Lyme disease

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Introduction

Lyme disease is the most frequent arthropod-borne infection in North America and Europe [1,2]. The bacterium which is transmitted to humans by the bites of infected *Ixodes persulcatus* complex ticks can disseminate into multiple tissues including the peripheral and central nervous systems [1,2]. Neurological manifestations of Lyme disease (or neuroborreliosis) occur variably [2–4] and include meningitis, cranial neuritis, and radiculoneuritis [4]. While it is clear that *B. burgdorferi* can invade the nervous system, how it does so is not well understood. Using an *in vitro* model of the blood-brain barrier (BBB) consisting of human brain microvascular endothelial cells (HBMEC), we previously showed that host-cell proteases [5] are required for a human neurotropic *B. burgdorferi* strain to efficiently cross the BBB. We now show that host cell calcium signaling also plays an important role in this process.

Materials and Methods

Materials

The cell membrane-permeable intracellular calcium chelator BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis)acetoxymethyl ester) was obtained from Invitrogen, and the phospholipase C (PLC) inhibitor U-73122 (1-(6-[([17β]-3Methoxyestra-1,3,5[10]-trien-17-yl) amino]hexyl)-1H-pyrrole-2,5-dione) was from Sigma.

The spirochetes

Low-passage (<8 *in vitro* passages from original isolate) human *B. burgdorferi* isolates 297 (from CSF), B331, B356,

and BL206 spirochetes [6–9] were provided courtesy Barbara Johnson (CDC, Ft. Collins, CO) and Ira Schwartz (New York Medical College, Valhalla, NY). The spirochetes were cultured at 34°C in BSK II medium containing 6% rabbit serum [10]. Bacteria were examined for motility by dark-field microscopy to verify viability and that organisms were thoroughly dispersed. *Borrelia burgdorferi* were quantified using real-time PCR (qPCR) targeting the single copy chromosomal *flgB* [11]. *Borrelia burgdorferi* strain 297 was tested by PCR and determined to possess both linear plasmids 25 and 28-1 [12].

The human brain microvascular endothelial cells

A HBMEC cell line whose phenotypic expression was stabilized by immortalizing the cells with pSVT, a pBR322-based plasmid containing DNA sequence encoding the SV40-large T antigen [13] was used in these studies. Similar to the primary HBMEC cell line from which they were derived, the transfected HBMEC express FVIII-Rag, carbonic anhydrase IV, *Ulex europus* agglutinin I, take up acetylated low-density lipoprotein, and express gamma glutamyl transpeptidase [13,14]. HBMEC were cultured at 37°C in Medium 199 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen) and 1X Glutamax (GIBCO) in a humidified environment of 95% air/5% CO_2. When incubated with the borrelia, the FBS concentration was increased to 20% to maintain spirochete viability.

Spirochete transmigration across HBMEC

HBMEC were preincubated for 30 min with 15 μM BAPTA-AM or 5 μM U73122 in 0.5% DMSO, conditions known to prevent subsequent seven transmembrane G-protein coupled receptor (GPCR)-induced $[Ca^{2+}]_i$ rise in HBMEC, then washed three times in medium to free the HBMEC of the inhibitors before spirochete addition [15,16]; 0.5% DMSO alone instead of the inhibitors was used in controls. For both BAPTA-AM and U73122-treated and untreated cultures, based on trypan blue dye exclusion (>99% viability), $[Ca^{2+}]_i$ measurements [15,16], and on electric cell substrate impedance sensing (ECIS) as a biosensor of cell toxicity [17], both drugs and DMSO were found to be non-toxic to HBMEC at concentrations used. *Borrelia burgdorferi* transmigration assays were performed as described previously [5]. Endothelial cells were seeded on collagen type I-coated 3 μm pore Transwell™ inserts (Costar) and cultured until the transendothelial electrical resistance (TEER) reached ≥25 Ω x cm^2 as measure in an EndOhm chamber and EVOM (AC voltmeter) meter (World Precision Instruments) [5]. For 6.5 mm diameter inserts, intact HBMEC monolayers have TEER ≥ 25 Ω x cm^2 [5,18], values comparable to those of epithelial cells grown on inserts [19]. Exponential growth phase spirochetes maintained in BSK II medium were centrifuged at 4000 *g* for 10 min at 4°C, and then washed twice in HBMEC medium; 2×10^5 spirochetes were immediately added (final volume = 200 μL) to the HBMEC-containing inserts. The approximate MOI of *Borrelia* to HBMEC was 1:1. Some inserts lacked HBMEC to establish a baseline for *B. burgdorferi* transmigration (typically 5–20% of the initial inoculum at 5 h) and viability. Cultures were incubated for 5 h, spirochete motility and viability were assessed by dark field microscopy, and the number of spirochetes that crossed the HBMEC and migrated into the bottom chamber was determined by qPCR. Statistical analyses were performed using one- or two-sided unequal variance Student's *t*-tests with $p < 0.05$ considered significant.

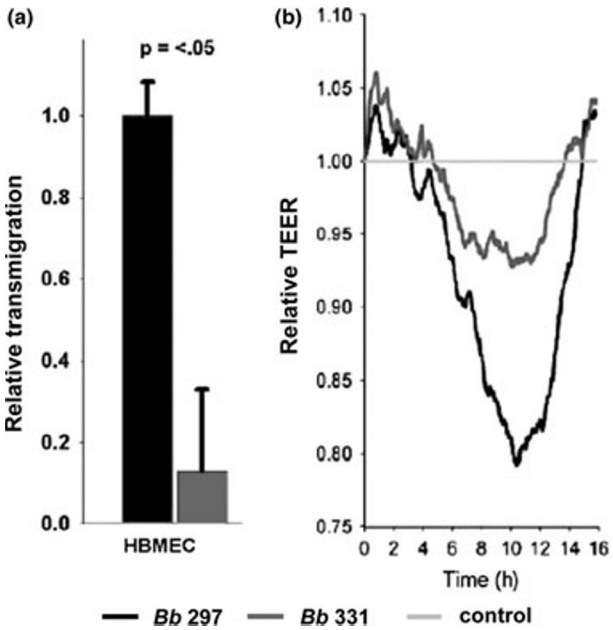

FIG. 1. Comparison of HMBEC transmigration and TEER changes in response to *Borrelia burgdorferi* strains 297 (invasive) and B331 (non-invasive). (a) 2×10^5 *B. burgdorferi* 297 or 331 were incubated for 5 h with HBMEC in Transwell inserts. qPCR was used to enumerate the number of spirochetes in the inoculum and that crossed the monolayers. The data shown are an example of three independent experiments of triplicate determinations calculated as the number of *B. burgdorferi* (±SEM) that crossed relative to *B. burgdorferi* 297 (p value calculated by Student's *t*-test). (b) Real-time TEER changes by ECIS comparing *B. burgdorferi* strains 297 (black line) and B331 (mid-gray line) relative to the HBMEC controls lacking spirochetes (light gray line). The data are plotted as the mean of duplicate runs. Based on the lower transmigration and effect of *B. burgdorferi* non-invasive strain B331 on TEER, subsequent experiments focused solely on invasive strain 297.

Assessment of barrier function by electric cell substrate impedance sensing

The changes in transendothelial electrical resistance (TEER) were monitored in real-time by electric cell-substrate impedance sensing (ECIS) using an ECIS Model 1600R instrument (Applied BioPhysics, Troy, NY, USA). ECIS measures the resistance and impedance on small gold electrodes that serve as substrates for cell attachment and growth [20]. In our study, HBMEC were grown on 8W10E+ ECIS arrays until stable resistances of ≥1000 Ω were reached. Unlike the single electrode 8W1E array in which HBMEC resistances are >10 000 Ω, the ten-fold higher capacitance of the 8W10E+ arrays due to 20 small active electrode groups arranged in series leads to reduced resistances (<1/10th that of the single electrode array) while providing a more statistically relevant result (ECIS 1600R instruction manual). Resistances were recorded every 80 s for 5 h post infection, and triplicate samples were averaged. Statistical analyses were performed using two-sided paired Student's *t*-tests with significance levels of $p < 0.05$.

Results

Selecting *B. burgdorferi* strains for analysis

We initially screened several strains of *B. burgdorferi* previously documented by other investigators to cause disseminated (CSF strain 297 and BL206 from blood) or localized (strains B331 and B356 from individual erythema migrans skin lesions) human infections (Fig. 1, Table 1). Of these

TABLE 1. Transmigration of HBMEC by different *Borrelia burgdorferi* strains

B.burgdorferi strain (passage #)	Infection type	Transmigration relative to 297	*t*-test*	References
B356 (P7)	Localized	0.147 ± 0.005	p 0.043	[9]
BL206 (P5)	Disseminated	1.21 ± 0.30	p 0.667	[9]
297 (P6)	Disseminated	1.00 ± 0.25		[6,7]

*p Value compared to 297 determined by Student's *t*-test.

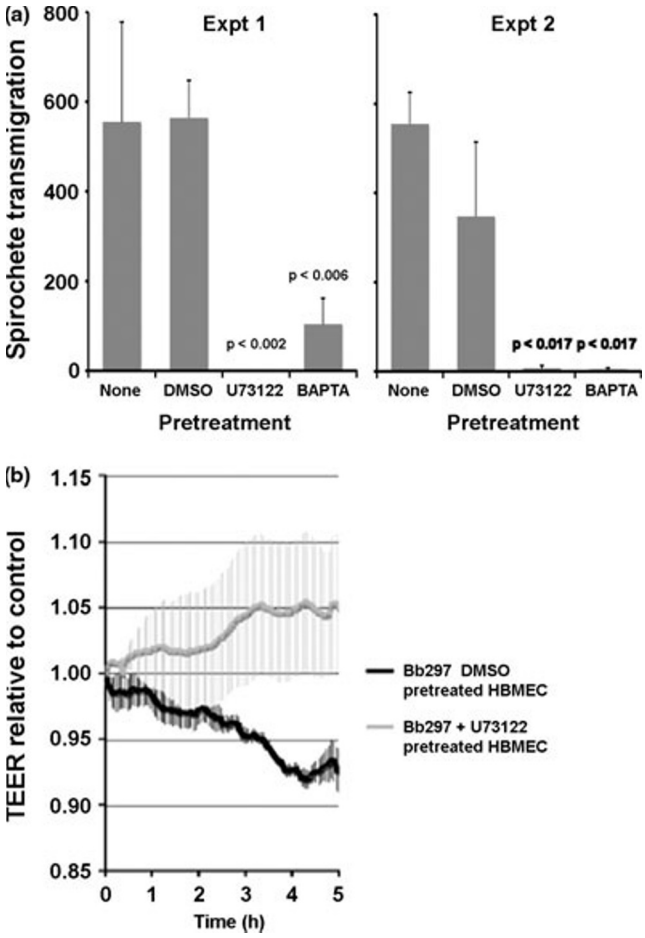

FIG. 2. Changes in HBMEC TEER in response to *Borrelia burgdorferi* are mediated by phospholipase C. (a) 2×10^5 *B. burgdorferi* 297 were incubated for 5 h with HBMEC in Transwell inserts. qPCR was used to enumerate the number of spirochetes in the inoculum and that crossed the monolayers. The data shown from two independent experiments of triplicate determinations are calculated as the number of *B. burgdorferi* 297 (±SEM) that crossed the HBMEC monolayers. * $p \leq 0.017$. DMSO was used as solvent for both U73122 and BAPTA-AM. (b) Real-time TEER changes by ECIS also confirmed that pretreatment of HBMEC with U73122 tightens the barrier to *B. burgdorferi* (gray line) relative to the DMSO pretreated controls (back line). The data are plotted as the Mean ± SEM ($n = 3$); $p < 0.001$.

strains, HBMEC barrier transmigration varied by strain, with 297 reproducibly achieving the highest levels, and B331 and B356, the lowest (Table 1, Fig. 1). The transmigration of HBMEC by 297 was three- to four-fold higher than for strain B331 (Fig. 1a), and this correlated with the ability of strain 297 to induce a three to four-fold greater reduction in TEER by ECIS than observed for B331 (Fig. 1b). Based on the higher degree of transmigration and greater ECIS TEER alteration compared with strain B331, we selected strain 297 for definitive investigation of the role that calcium plays in HBMEC invasion and permeability changes.

Transmigration of *B. burgdorferi* across HBMEC requires calcium signaling

We examined the hypothesis that changes in intracellular calcium ($[Ca^{2+}]_i$) in HBMEC would alter *B. burgdorferi* 297 traversal of this endothelial barrier. As shown in Fig. 2a, by 5 h BAPTA-AM significantly inhibited (82–99%; $p < 0.017$) spirochete traversal of HBMEC compared to DMSO control; the difference in proportions of spirochetes transmigrating between experiments 1 and 2 was not statistically different ($p > 0.12$). Spirochete traversal was almost totally blocked (≥99%; $p < 0.017$) after pretreatment with the PLC-β inhibitor U73122, although there was no statistically significant difference in spirochete traversal when these treatments were compared. Pretreatment of HBMEC with DMSO alone did not significantly alter *B. burgdorferi* transmigration through endothelial cell barriers. Furthermore, spirochetes maintained viability/motility when assayed by darkfield microscopy whether in inserts containing either control or pretreated HBMEC, making it unlikely that the lack of the spirochetes crossing endothelial cell barriers was due to a direct effect of residual chelator or antagonist. Real-time TEER changes by ECIS also confirmed that pretreatment of HBMEC with U73122 tightened the barrier to *B. burgdorferi* (Fig. 2b; $p < 1.51 \times 10^{-28}$). These data suggest that the changes in HBMEC $[Ca^{2+}]_i$ induced by *B. burgdorferi* may be in part mediated through extracellular signaling.

Discussion

Through direct spirochete induction of plasminogen activators and MMP expression, proteases (i.e. MMP-1, plasmin) could compromise endothelial cell barrier integrity [5]. Calcium flux via PLC-β-linked GPCR activation leads to cytoskeletal rearrangements involved in the ability of other pathogens to transmigrate/invade the BBB comprised of the HMBEC we used [15,16]. Thus, we tested the ability of *B. burgdorferi* to transmigrate through HBMECs by modifying intracellular calcium levels using BAPTA and U73122 [15,16,21–23]. Both manipulations can block or reverse decreases in TEER and/or increases in permeability in BMEC-based models of the BBB induced by various biological stimuli.

We now demonstrate that *B. burgdorferi* transmigration across the BBB also depends on the bacterium's ability to influence Ca^{2+}responses in HBMEC. Spirochete traversal of HBMEC requires host-derived MMP-1 and plasmin [5], which are known to stimulate the GPCR *p*rotease *a*ctivated *r*eceptor (PAR)-1 [24,25]. PAR-1 activation can trigger the release of tissue-type plasminogen activator, a process known to be inhibited by U73122 [26]. The finding that U73122 interferes with *B. burgdorferi* transmigration and TEER is consistent with a role for PARs in these processes. Other Ca^{2+}-mediated processes could also play a role in spirochete invasion of the CNS where U73122 exerts its effect through inhibiting Ca^{2+}-entry pathways or pumps (SERCA) [27]. Our data showing abrogation of *B. burgdorferi* transmigration of the BBB in the presence of intracellular Ca^{2+}chelators and U73122 does not allow differentiation of these potential concepts and prompt testing of these hypotheses.

Complete or near complete inhibition of *B. burgdorferi* migration through endothelial cell barriers occurs when calcium signaling pathways are interrupted. Careful examination of this process could identify host cell mechanisms used by the bacterium that promote dissemination as murine models of Lyme neuroborreliosis are developed [28] and applied to investigate other neurotropic and non-neurotropic strains. Moreover, such studies will identify pathways amenable to further investigation, even pharmacological prevention of disseminated disease in humans using advanced agonists or antagonists currently in clinical trials for other purposes.

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Transparency Declaration

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