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Microbes and Infection xx (2006) 1–9

Microbes and Infection

www.elsevier.com/locate/micinf

Original article

Invasion of human neuronal and glial cells by an infectious strain of *Borrelia burgdorferi*

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Received 13 June 2006; accepted 30 August 2006

Abstract

Human infection by *Borrelia burgdorferi*, the etiological agent for Lyme disease, can result in serious acute and late-term disorders including neuroborreliosis, a degenerative condition of the peripheral and central nervous systems. To examine the mechanisms involved in the cellular pathogenesis of neuroborreliosis, we investigated the ability of *B. burgdorferi* to attach to and/or invade a panel of human neuroglial and cortical neuronal cells. In all neural cells tested, we observed *B. burgdorferi* in association with the cell by confocal microscopy. Further analysis by differential immunofluorescent staining of external and internal organisms, and a gentamicin protection assay demonstrated an intracellular localization of *B. burgdorferi*. A non-infectious strain of *B. burgdorferi* was attenuated in its ability to associate with these neural cells, suggesting that a specific borrelial factor related to cellular infectivity was responsible for the association. Cytopathic effects were not observed following infection of these cell lines with *B. burgdorferi*, and internalized spirochetes were found to be viable. Invasion of neural cells by *B. burgdorferi* provides a putative mechanism for the organism to avoid the host's immune response while potentially causing functional damage to neural cells during infection of the CNS.

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Keywords: *Borrelia burgdorferi*; Cell invasion; Neuroborreliosis

1. Introduction

Human infection by *Borrelia burgdorferi*, a spirochetal bacterium transmitted through the bite of *Ixodes* ticks, results in Lyme borreliosis, an illness that affects several organ systems of the body following bacterial dissemination within the host. Despite the elicitation of a strong antibody response against several borrelial antigens, the organism is able to evade the host's immune defenses and mobilize to various host tissues eventually resulting in arthritis, carditis, and neurological manifestations. Several mechanisms have been postulated for the spirochete to sidestep the immune response, e.g. sequestration into immune privileged sites,

antigenic variation, regulation of surface antigen expression, and suppression of host immune responses (reviewed by Embers et al. [1]).

Lyme neuroborreliosis is associated with an inflammation of the central nervous system (CNS) which can lead to debilitating effects such as neuronal cell damage and loss. Neurologic syndromes occur roughly in 15% of untreated patients with Lyme disease, and the clinical manifestations of neuroborreliosis include encephalitis, cranial neuropathy, and meningitis [2]. Mechanisms by which *B. burgdorferi* affect the nervous system are not known, but damage caused by association of these bacteria to cells of the CNS could be the basis for some of the neurological manifestations seen in neuroborreliosis. Furthermore, *B. burgdorferi* may cross the blood–brain barrier and enter the CNS as a means to circumvent the adaptive immune response of the host.

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The goal of this study was to observe and investigate the interaction between *B. burgdorferi* and human neural cells in vitro. As adherence and entry of a bacterium into host cells is one of the essential elements of microbial pathogenicity, we tested the hypothesis that *B. burgdorferi* have the ability to interact with and infect the cells of CNS thereby disrupting cellular function leading to the pathogenesis described for human neuroborreliosis. Additionally, association and/or invasion of CNS cells may provide seclusion for the organisms to escape immune clearance and survive in the host. In this study we demonstrate the association of *B. burgdorferi* with a panel of human neuronal and glial cell lines including the capability for cellular invasion.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. burgdorferi strains used are derivatives of strain B31. B31 clone A (B31-A) is a high-passage non-infectious derivative of type strain B31 (ATCC 35210), and B31 A3 is a clonal, low-passage infectious strain [3]. Frozen stocks of all *B. burgdorferi* strains were maintained in 60% glycerol at -70°C . All bacterial strains were grown in liquid Barbour–Stoenner–Kelly (BSK-II) complete medium at 35°C with 5% CO_2 .

2.2. Mammalian cell culture

All human cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, Virginia, USA). Human umbilical vein endothelial cells (HUVECs) were grown in F12 K medium with 2 mM L-glutamine, adjusted to contain 1.5 g/l sodium bicarbonate, and supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, and 10% fetal bovine serum. The human cortical neuronal cell line (HCN-2), and the two human neuroglial cell lines (HS-683, H4) were grown in Dulbecco's modified Eagle's medium (DMEM) with 4 mM L-glutamine containing 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, and supplemented with 10% fetal bovine serum. All cells were grown at 37°C with 5% CO_2 in a humidified cell incubator. Cells were harvested from confluent monolayers using 0.05% trypsin with 10 mM EDTA and enumerated with a hemocytometer.

2.3. Cell-association assays

Mammalian cells were enumerated and seeded the morning of the experiment on Lab-tek II CC chamber slide system (Nalge Nunc International, Rochester, New York, USA) to a density of 5×10^4 (HCN-2 cells were plated at 3×10^4) and allowed to attach to the slides for 4 h at 37°C . *B. burgdorferi* were grown for 3 days and enumerated using a Petroff-Hausser counting chamber. Bacteria were centrifuged at $500 \times g$ for 20 min and resuspended in pre-warmed DMEM. To formalin-kill the bacteria, pelleted spirochetes were suspended in PBS with 1 mM MgCl_2 , and neutral buffered formalin was added to 10% final concentration. Killed

spirochetes were seeded into BSK-II and allowed to incubate at 35°C with 5% CO_2 . No bacterial growth was detected following formalin treatment. *B. burgdorferi* were added to the mammalian cells at a multiplicity of infection (MOI) of 40, and were incubated at 35°C with 5% CO_2 for approximately 20 h.

2.4. Immunofluorescence labeling

For all staining protocols, the *B. burgdorferi*-infected human cells were first washed 3 times with phosphate buffered saline (PBS) to clear unassociated spirochetes from the monolayers, followed by fixation in 4% paraformaldehyde solubilized in TE (10 mM Tris, 1 mM EDTA, pH 7.0) for 30 min at room temperature, followed by another 3 PBS washes. Fixed cells were incubated in PBS with 2% BSA for 30 min to block non-specific binding. For dual staining of bacteria and eukaryotic cell plasma membrane, the cells were first stained with Alexa fluor 594 wheat germ agglutinin (Image-iT LIVE Plasma Membrane and Nuclear Labeling Kit, Molecular Probes, Invitrogen, Carlsbad, California). Cells were then washed 3 times in PBS and permeabilized by incubating in PBS/2% BSA with 0.5% Triton X-100 for 1 h at room temperature. Bacteria were stained with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-*B. burgdorferi* polyclonal antiserum (1:75) for 1 h at 37°C in a humidified chamber. The cells were washed 3 times in PBS and coverslips were mounted with Molecular Probes ProLong Gold Antifade.

For differential staining of intracellular and extracellular bacteria, fixed monolayers were first stained with a mouse polyclonal anti-*B. burgdorferi* antibody (1:100) for 1 h at 37°C in a humidified chamber. Following 3 PBS washes, a rhodamine-conjugated goat anti-mouse antibody (1:100) was added for 1 h at 37°C in a humidified chamber. Following this step, the slide was washed 3 times in PBS and the host cells were permeabilized by incubating in PBS/2% BSA with 0.5% Triton X-100 for 1 h at room temperature. After a PBS wash, the intracellular bacteria were stained by adding an FITC-conjugated rabbit anti-*B. burgdorferi* polyclonal antiserum (1:75) for 1 h at 37°C in a humidified chamber. The cells were washed with PBS and coverslips were mounted with ProLong Gold Antifade mounting reagent. The samples were viewed under a Zeiss LSM 5 Pascal confocal laser scanning microscope. Images were analyzed using the LSM 5 image browser (Carl Zeiss Inc.).

2.5. Gentamicin protection assay

To enumerate the bacteria that invaded the human cells, infections were performed as stated above. After incubation with *B. burgdorferi* for approximately 20 h, the monolayers were washed with PBS, gentamicin (150 g/ml) was added to the growth medium, and the cells were incubated for 4 h at 35°C to kill the extracellular bacteria. After incubation with gentamicin, the supernatant was collected, and the cell monolayers were washed 3 times in PBS, scraped from the wells, and placed in 500 μL of prewarmed BSK-II complete media

for less than 30 min. Dilutions of this mixture containing human cells and spirochetes were made in BSK-II, and plated on solid BSK medium. Internalized spirochetes grew on the solid BSK-II agar in 7–10 days. Numbers of internalized *B. burgdorferi* were estimated by counting the individual colonies formed in the solid medium. Platings of each infected cell line were performed at least 3 times. Calculations were based on using a monolayer of 5×10^4 cells with an input of 2×10^6 *B. burgdorferi*, an MOI of 40 (except for HCN-2 neuronal cells which grew to a lower density, whereby 3×10^4 cells were infected with the same MOI of 40 (1.2×10^6 *B. burgdorferi*)).

2.6. Cell viability assays

Trypan blue staining was performed to assess the viability of the mammalian cells following incubation with *B. burgdorferi*. Mammalian cells were enumerated and seeded on 24-well plates the morning of the experiment at a density of 1×10^5 (HCN-2 cells were plated at 1×10^4). *B. burgdorferi* were added to the monolayers at an MOI of 40 as described above. Following approximately 20 h incubation, the cells were washed 3 times in PBS, and trypsinized to remove them from their growth chamber. Trypan blue dye (10%) was added and the viable cells were enumerated using a hemocytometer. Each infection was performed in triplicate and counted 4 times each.

3. Results

3.1. Microscopic observation of bacterial association with human cells in vitro

The ability of *B. burgdorferi* to associate with 4 different human cell lines in vitro was examined. The panel included two different human neuroglioma cell lines (HS-683 and H4), a human cortical neuronal cell line (HCN-2), and human endothelial cells as a positive control (HUVEC). *B. burgdorferi* have previously been shown to attach and invade HUVECs [4]. Because the attachment assays were carried out in DMEM, *Borrelia* were tested for their ability to remain viable in this culture medium. The spirochetes retained viability for at least 24 h following incubation in this tissue culture medium (data not shown). After testing different multiplicities of infection, we found that an MOI of 40 allowed 1–3 spirochetes to associate per cell. Incubation times ranging from 1 to 24 h were tested, and no quantitative differences regarding the number of cell-associated spirochetes were observed by confocal microscopy. Other investigators have found that the number of radiolabeled spirochetes associated with host cells in vitro increased up to 20 h [4,5]. For this study, we chose an incubation time of approximately 20 h to allow for optimum spirochete/cell interaction prior to performing further analysis.

Following incubation for approximately 20 h, the cells were extensively washed of the unassociated spirochetes, fixed, immunostained using antibodies directed against the mammalian cells and spirochetes, and imaged by confocal microscopy.

The mammalian cell plasma membranes were stained red with Alexa Fluor 594, and the spirochetes were stained green with a polyclonal FITC-conjugated anti-*B. burgdorferi*. Using this assay, *B. burgdorferi* were observed to be associated with all cell lines tested. Fig. 1 displays a representative confocal image of each of the infected cell lines with both fluorescent stains merged into one image. *B. burgdorferi* were co-localized with the cell membrane as evidenced by the spirochetes appearing yellow (red plus green) in each merged panel. *B. burgdorferi* were not observed in spaces between cells, further evidence that *Borrelia* were associated with cells and not simply lying freely across cells. Gentamicin treatment of infected cells prior to fixation produced similar results (data not shown) demonstrating borrelial interaction with the cells thereby shielding the organisms from the antibiotic. These data suggested that *B. burgdorferi* became associated with the human neural cells in vitro.

3.2. Mammalian cell viability after incubation with *B. burgdorferi*

We examined the effects of *B. burgdorferi* association on the neuronal and glial cell lines, specifically being interested in any cytopathic effects caused by the organism. Trypan blue staining was performed to assay for cell viability after incubation with *B. burgdorferi*. In all cell lines tested, there were no observable adverse effects under microscopic observation of the infected mammalian cells after incubation with *B. burgdorferi* when compared to the uninfected controls. Measurement of cell viability by trypan blue staining showed no significant difference between infected and non-infected cells (Table 1). An incubation of 7 days was also performed to observe extended association effects on each of the cell lines. No cytopathic effects were apparent following 7 day incubations with *B. burgdorferi* on any of the cell lines tested compared to the uninfected controls (data not shown). These observations demonstrated the ability of *B. burgdorferi* to interact with neural cells for several days without causing observable deleterious effects.

3.3. Specificity of *B. burgdorferi* association with human cells in vitro

The specificity of *B. burgdorferi* association with these cells was tested by comparing attachment abilities of infectious *B. burgdorferi* against a non-infectious strain. Following fixation and immunostaining of the cells, the infectious *B. burgdorferi* were observed to have associated with the mammalian cells in greater numbers than the non-infectious strain. Fig. 2 shows immunofluorescence images comparing a $25 \times$ field view of human cells infected with the two strains of *B. burgdorferi*. On average, the non-infectious strain showed a 10-fold reduction in association as compared to the infectious *B. burgdorferi*, after counting spirochetes in 5 fields. Additionally, experiments comparing the cell-association properties of formalin-killed *Borrelia* with infectious *B. burgdorferi* were performed. Formalin-killed *Borrelia* had an 11-fold reduced ability to associate with all cell lines

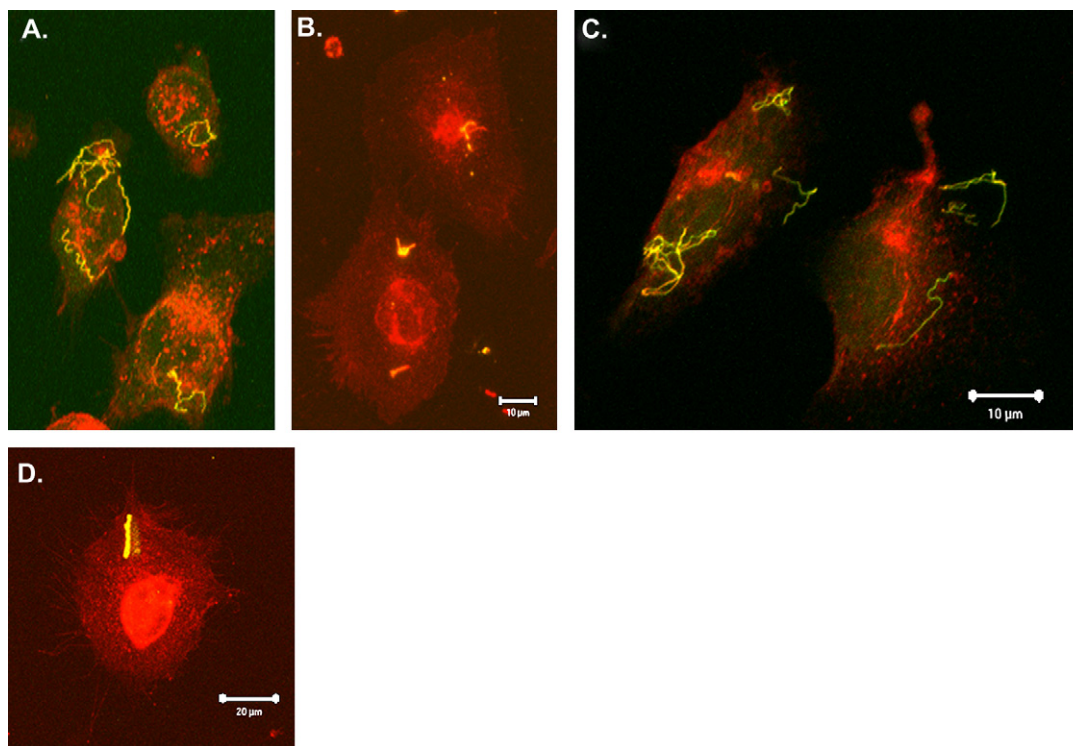


Fig 1. Association of *Borrelia burgdorferi* with human cells in vitro. Confocal microscopy images of human cells incubated with *B. burgdorferi*. Human cell plasma membranes were immunostained red, and *B. burgdorferi* were labeled green according to Section 2, with the 2 images being merged whereby the yellow indicates co-localization of *B. burgdorferi* with the cellular membrane. Images represent the entire Z-stack of the sections. Panels: (A) human umbilical vein endothelial cells; (B) H4 human neuroglial cells; (C) HS-683 human neuroglial cells; and (D) human cortical neurons.

tested, results that were nearly identical to those of the non-infectious strain (data not shown). These results suggest that the association of infectious *B. burgdorferi* with these cell lines is a specific and targeted binding mechanism, rather than a non-specific endocytic event.

3.4. Differential fluorescent antibody staining of intracellular and extracellular spirochetes

To determine whether spirochetes were truly internalized as opposed to being associated externally with the human cell plasma membrane, differential immunofluorescent staining of intracellular and extracellular bacteria was performed. Following fixation, *B. burgdorferi*-infected cells were subjected to a rhodamine-conjugated secondary antibody after incubation with a polyclonal *B. burgdorferi* antibody which stained the extracellular bacteria red. The fixed monolayer was then permeabilized with Triton X-100, and subjected to immunostaining with a polyclonal FITC-conjugated anti-*B. burgdorferi* antibody, thereby resulting in both extracellular and intracellular *B. burgdorferi* to fluoresce green. The resultant images show extracellular bacteria stained both red and green, while intracellular bacteria stained only green (Fig. 3). Therefore, when the images were merged, the extracellular spirochetes appeared yellow, while the internalized spirochetes remained green (Fig. 3, panels C, F, I, L). Each cell type demonstrated extracellular adherent organisms and also internalized

B. burgdorferi, thereby demonstrating the ability of *B. burgdorferi* to attach to and invade human neuronal and glial cell lines.

3.5. Gentamicin protection assay

To investigate further whether the bacteria were internalized and viable, gentamicin was added to the host cell culture medium following infection with *B. burgdorferi*, thereby killing extracellular bacteria while leaving any internalized spirochetes unharmed. Gentamicin does not readily penetrate mammalian host cells, therefore internally localized bacteria are protected from the activity. Preliminary experiments were performed testing different concentrations of gentamicin with *B. burgdorferi* to assure 100% killing. Following the addition of *B. burgdorferi* to the cell monolayer and incubation,

Table 1
Cell viability assay

Host cells ^a	Strains used for infection		
	B31-A3 (infectious)	B31-A (non-infectious)	No <i>Borrelia</i>
Number of viable host cells following <i>B. burgdorferi</i> infection			
HUVEC	1.4×10^5	1.5×10^5	1.5×10^5
HS-683	2.1×10^5	2.2×10^5	2.3×10^5
H4	1.8×10^5	1.5×10^5	1.8×10^5
HCN-2 ^b	2.8×10^3	N.D.	2.4×10^3

N.D. not determined.

^a 1×10^5 Cells infected.

^b 1×10^4 Cells infected.

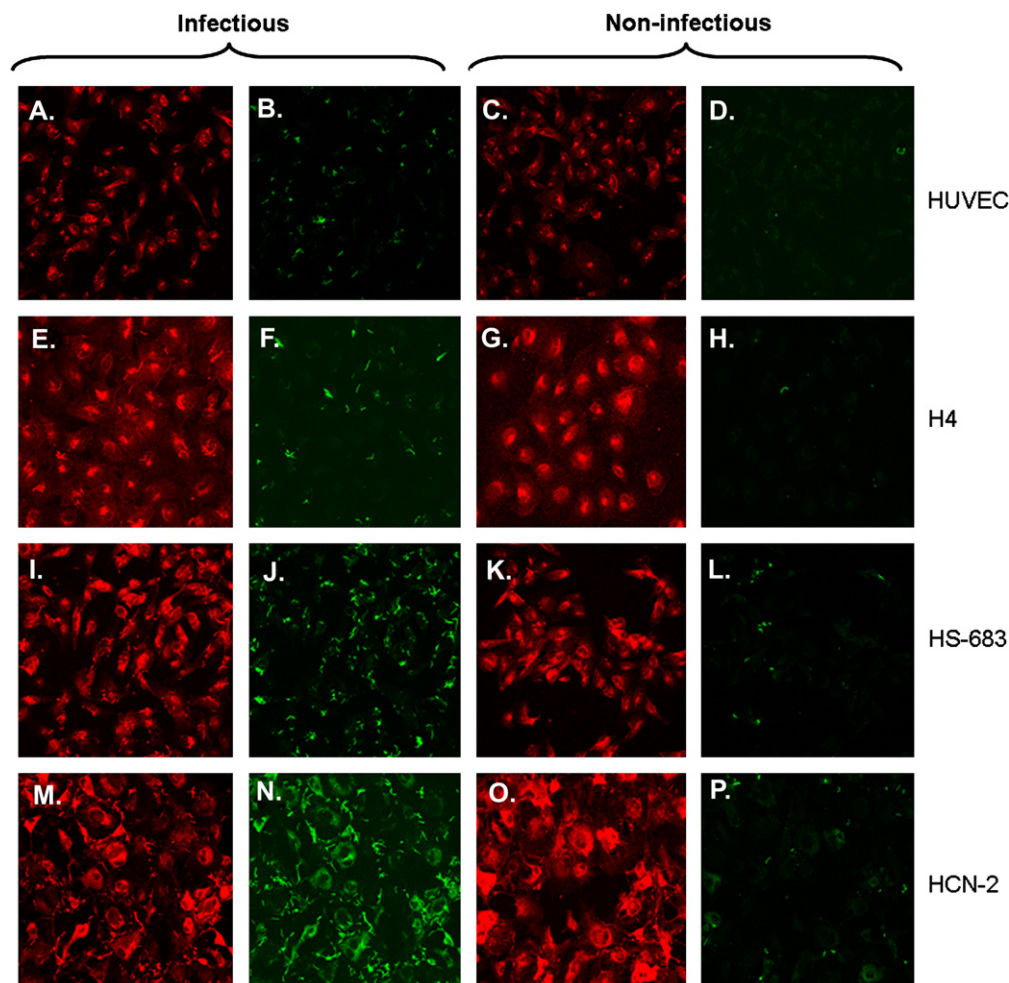


Fig 2. Infectious and non-infectious *B. burgdorferi* strain association with human cells. *B. burgdorferi*-infected cells with plasma membranes stained red and spirochetes stained green as described for Fig. 1. Left and right panels of each infected cell line are the same microscopic field. Panels: (A–D) human umbilical vein endothelial cells; (E–H) H4 human neuroglial cells; (I–L) HS-683 human neuroglial cells; and (M–P) human cortical neuronal cells. Panels: (A, B, E, F, I, J, M, N) cells infected with the infectious strain; (C, D, G, H, K, L, O, P) cells infected with the non-infectious strain.

analysis of the cell culture supernatant following the gentamicin treatment demonstrated no borrelial growth, indicating that extracellular *B. burgdorferi* were killed (data not shown).

To release internalized spirochetes, lysis of the human cells was tested using water, Triton X, or RLT buffer (Qiagen, Valencia, California, USA) with the intent of leaving the spirochetes intact for downstream culturing. However, these treatments also proved to lyse and kill the spirochetes, therefore an alternate strategy was employed. Following a 4-h gentamicin treatment, the *Borrelia*-infected human cells were extensively washed with PBS, scraped from their growth chambers, and suspended in pre-warmed BSK-II media for less than 30 min. The placement of the *B. burgdorferi*-infected cell suspension into BSK-II media successfully allowed the organisms to be released from the host cells undamaged once plated onto solid BSK-II agar. Dilutions of the cell suspensions were plated onto solid BSK-II media and incubated for 7–10 days at 35 °C. Viable spirochetes that were protected from gentamicin grew on the solid media, and the number of spirochetes that were internalized in each of the neural cell lines was enumerated (Table 2). All the cell lines tested

resulted in borrelial growth on solid media indicative of viable internalized spirochetes. The two glial cell lines (H4 and HS-683) had the lowest percentages of internalized *B. burgdorferi* when factored against the input bacterial numbers, whereby the cortical neuronal cells supported the highest percentage. Additionally, while the fluorescent images in Fig. 3 showed intracellular *B. burgdorferi*, viable vs. dead spirochetes could not be differentiated, although there were no apparent morphological aberrations suggesting borrelial death. This experiment demonstrated the viability of the intracellular organisms following gentamicin treatment, as well as the percentage of the input spirochetes that were being internalized.

4. Discussion

Several studies have demonstrated that *B. burgdorferi* can attach to many different cell types from various animal species including human endothelial cells [4,6,7], human skin fibroblasts [8], human synovial cells [9], tick cells [10], murine macrophages [11], human peripheral blood fibrocytes [12], and human and murine neural cells [5,13]. The borrelial

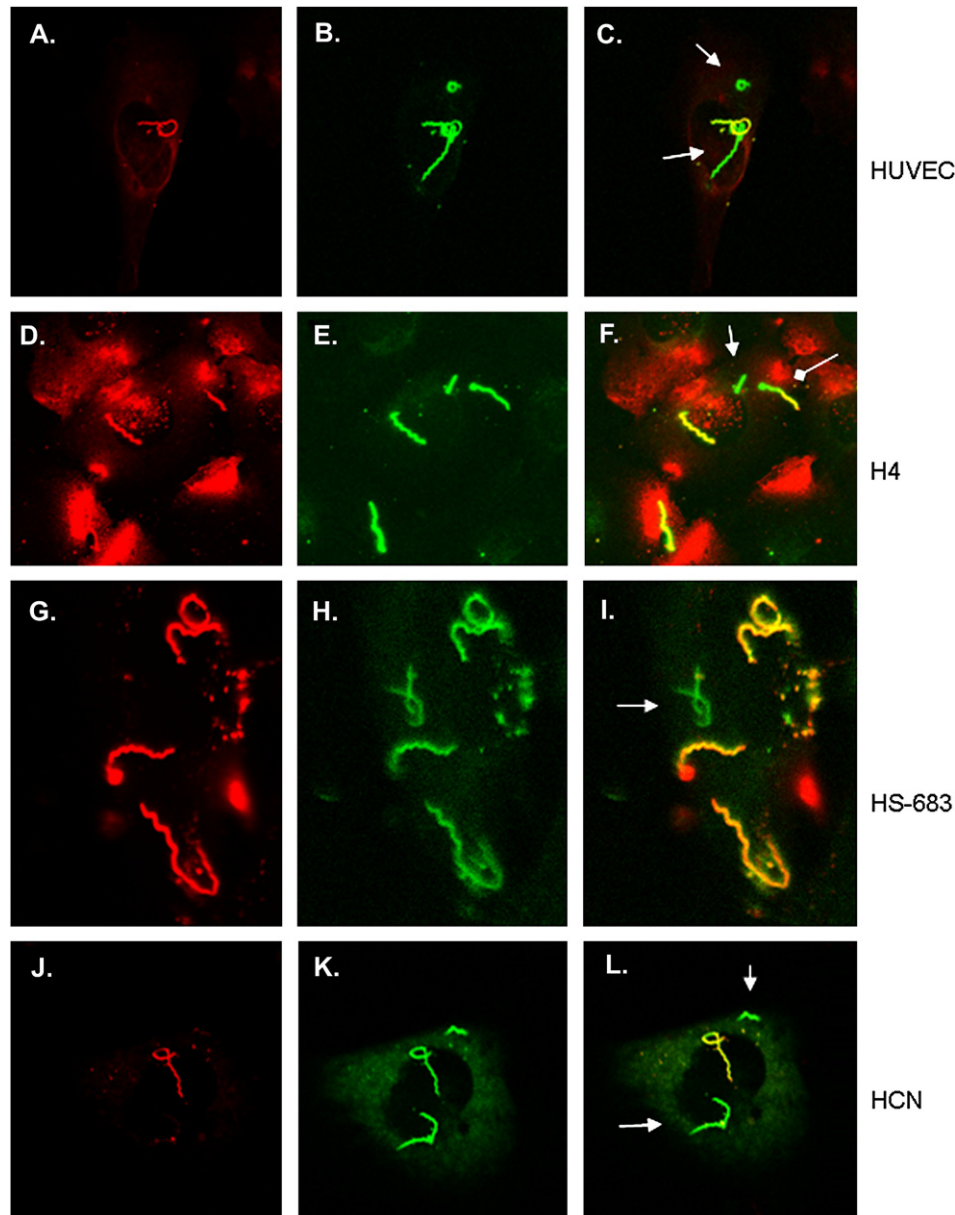


Fig. 3. Double immunostaining of *B. burgdorferi*-infected human cells. Left panels represent extracellular spirochetes stained with a rhodamine-conjugated anti-*B. burgdorferi* antibody. Middle panels show the same microscopic field of spirochetes stained with an FITC-conjugated anti-*B. burgdorferi* antibody following permeabilization of fixed cells. Right panels show a merged image of the two stains. Panels: (A–C) human umbilical vein endothelial cells; (D–F) H4 human neuroglial cells; (G–I) HS-683 human neuroglial cells; and (J–L) Human cortical neuronal cells. Panels: (C, F, I) yellow-appearing *Borrelia* indicate extracellular organisms that have been stained with both rhodamine and FITC. Arrows point to the green-staining *Borrelia* indicating intracellular organisms. The diamond arrow (F) points to a spirochete staining part green and part yellow indicative of an organism that has partially entered the cell.

associations in these studies were based on radioactive quantification of membrane-associated spirochetes, and by confocal microscopy. Interestingly, in addition to binding to the outer membranes of the host cells, *B. burgdorferi* have been shown by independent studies to localize intracellularly in human endothelial cells [4], skin fibroblasts [8], and synovial cells [9].

Direct interactions of *B. burgdorferi* with human neural cells have not extensively been examined. Investigations have shown that *B. burgdorferi* DNA can be detected by PCR in the cerebrospinal fluid of patients diagnosed with Lyme disease [14], but there is little information on the

localization of the organism in CNS tissues. The non-human primate is currently the best model to study neuroborreliosis, and researchers have described spirochetal localization in CNS tissues of infected monkeys by PCR and histopathological staining, however, intracellular *Borrelia* have not been observed [15,16]. Additional research dealing with the pathogenesis of Lyme neuroborreliosis has shown that *B. burgdorferi* infection of rhesus monkeys resulted in the proliferation and apoptosis of astrocytes, a type of glial cell, leading to a dysregulated production of inflammatory cytokines that are believed to contribute to CNS pathogenesis [17,18].

Table 2
Gentamicin protection assay to enumerate internalized *B. burgdorferi*

Host cells	Avg. colonies counted (dilution)	<i>B. burgdorferi</i> internalized (average % of input) ^a	<i>B. burgdorferi</i> cell (average) ^b
HUVEC	45.0 ± 10.8 (1:1000)	2.3 ± 0.54	0.9 ± 0.22
HS-683	53.5 ± 36.5 (1:100)	0.27 ± 0.18	0.11 ± 0.07
H4	6.0 ± 7.0 (1:100)	0.03 ± 0.035	0.012 ± 0.014
HCN-2	91.3 ± 36.7 (1:1000)	7.6 ± 3.1	3.0 ± 1.2

^a Number of colonies per plate × dilution correction/number input *Borrelia* × 100.

^b Number of colonies × dilution correction/number of human cells plated.

Borrelial lipoproteins were shown to stimulate this proliferation and apoptosis activity in cultured astrocytes. In humans, an increase in glial fibrillary acidic protein (GFAP) [19] and tumor necrosis factor- α [20] in the cerebrospinal fluid is sign of inflammation that appears to be associated with Lyme neuroborreliosis, further evidence of borrelial interactions with the cells of the CNS to upset the balance of cytokine production resulting in pathogenesis. Fundamental questions regarding *B. burgdorferi* biology and host cell interactions in humans can begin to be answered using a human in vitro cell culture model to study neuroborreliosis. We have initiated the study by demonstrating that *B. burgdorferi* can invade and remain viable inside human neuronal and glial cells.

In the present work, incubation of these cell lines up to 7 days with *B. burgdorferi* had no adverse effect on host cell viability when compared to the uninfected control cells. Our result is consistent with the study by Thomas et al. [13] who also reported no cellular cytotoxicity when *B. burgdorferi* were incubated with human neural cells. The lack of cytopathic effects suggests that these cells could serve as a site for *B. burgdorferi* to be sequestered from the host's immune defenses, and/or act as a locale for prolonged infection without causing immediate harm or cell death to their host. Although morphological cell damage was not apparent in *B. burgdorferi*-infected cells after 7 days incubation in vitro, one could reasonably expect that infected cells would nevertheless be compromised physiologically, and would eventually break down. Explanations regarding the putative *B. burgdorferi* interactions with CNS cells in vivo as mechanisms either to evade host defenses or to cause aberrant cellular function await further experimentation.

Confocal microscopy of cells incubated with *B. burgdorferi* demonstrated a co-localization of the spirochetes with the mammalian cells, results consistent with previous researchers' findings. Significantly, non-infectious, and formalin-killed *B. burgdorferi* had roughly a 10-fold reduction in their ability to associate with human cell lines when compared to infectious *B. burgdorferi*, a result similar to the findings by Peters and Benach studying borrelial adherence to rat C6 glioma and PC-12 cells [21], and by Szczepanski et al. using HUVECs [7], suggesting that the interaction was mediated by borrelial factors encoded by *B. burgdorferi* strains that have not undergone loss of genomic material. It has been well documented

that in vitro passage of *B. burgdorferi* results in the loss of plasmids, rendering the strain non-infectious [22]. These results suggest that there is a genetic component(s) that augments the ability for *B. burgdorferi* to invade human cells.

Although the confocal microscopy data displaying borrelial adherence to the host cells depicted in Fig. 1 were intriguing, they did not conclusively determine whether the spirochetes seen associated with each of the cell types tested were internalized, surrounded by deep invaginations of the outer cell membrane, or integrated externally to the outer cellular surface. To further explore these possibilities, two additional experiments were performed: differential immunostaining of internalized spirochetes and the gentamicin protection assay. The former experiment clearly showed that some *B. burgdorferi* were immunostained only after the host cells were permeabilized. Although some of the spirochetes were attached to the outer plasma membranes of the cells, each infected human cell showed the invasion of approximately 1–2 spirochetes. Additionally, viable spirochetes were recovered from infected human cells that were gentamicin treated, indicating that *B. burgdorferi* were sheltered from the antibiotic, presumably by being localized inside the cell which is impermeable to gentamicin activity. Interestingly, the gentamicin protection assays showed differing abilities of *B. burgdorferi* to invade each of the four cell types tested. The two cell types that showed the least amount of internalized *B. burgdorferi* were the two neuroglial cell lines. However, the human endothelial cell line and the cortical neurons showed a greater ability to harbor viable spirochetes, with the human cortical neuronal cells displaying the largest number of gentamicin-protected spirochetes. This result indicates that human neuronal cells could be a more preferable site for active infection in humans. These experiments demonstrated that spirochetes incubated with the human cells became internalized, providing further evidence supporting the hypothesis that CNS is a site where *B. burgdorferi* could disseminate and cause pathological damage.

Further supporting the hypothesis that *B. burgdorferi* invade cells during in vivo infection has been the findings that *B. burgdorferi* can bind a number of components associated with mammalian cells such as integrins, fibronectin, decorin, and glycosaminoglycans (reviewed by Coburn et al. [23]). Furthermore, the identification of several borrelial adhesin molecules, P66 [24], fibronectin-binding protein BBK32 [25], decorin-binding proteins DbpA and DbpB [26], and Bgp (*Borrelia* GAG-binding protein) [27] have been recognized to bind the above host cell receptors. Also, a recent study has implicated the outer surface protein A (OspA) of *Borrelia garinii* as an adhesin to proteoglycans on neural cell lines [28]. Adherence to host cells is critical for infection and subsequent invasion, therefore the activity of these borrelial adhesins to functionally mediate cellular adherence lends considerable support for an eventual *B. burgdorferi* intracellular localization when the environmental conditions warrant such a state during the course of infection. Our study focused on the extra-cellular and intracellular interactions of *B. burgdorferi* with human neural cells, but the mechanisms involved in cell

penetration and the determinants for borrelial survival within cells are yet to be determined. Leong et al. demonstrated that *B. burgdorferi* attachment to endothelial and neural cells was mediated by different classes of proteoglycans, but may be recognized by a single borrelial molecule [29]. Other studies have observed a polar, or end-on adhesion property of the spirochetes as they attach to the plasma membrane of cells [6,21,30], with a non-membrane bound cytosolic localization seen within human synovial cells [9]. Unquestionably, there is much to be done to elucidate the pathways and mechanisms involved in the intracellular lifestyle of *B. burgdorferi*. Clearly, the major challenge will be to describe and document *B. burgdorferi* intracellular interactions as a function of in vivo infections, and not only as an observation of in vitro cell culture systems.

The current direction of our laboratory research involves the examination of specific proteins for their ability to mediate adherence and invasion. Also, we have begun to utilize knock out gene mutations of *B. burgdorferi* to investigate the roles of individual genes in human neuronal and glial cell attachment and invasion. Finally, using the model developed here, we are interested in examining differential gene expression of *B. burgdorferi* following adherence and invasion of neural cells. Conversely, it will be important to study differential expression of the neural cellular genes to understand the regulation and molecular genetics of the host cell response. Understanding the mechanisms of *B. burgdorferi* infection of the human CNS is an important step to learn more about the pathogenesis of Lyme neuroborreliosis, and will be a key advancement to develop better diagnostics and treatment for this disease.

Acknowledgments

We thank Becky Byram for critical reading of the manuscript, and Phil Stewart for providing strain B31 A3. We would also like to thank Rebekah Howison, Barbara Johnson, Martha Folmsbee, Mark Pilgard, Ginny Schmit, and Steve Sviat for discussion and general laboratory support. JAL was supported by a post-doctoral fellowship from the American Society for Microbiology and Centers for Disease Control and Prevention.

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