Borrelia burgdorferi Adherence and Injury to Undifferentiated and Differentiated Neural Cells In Vitro

David J. Peters and Jorge L. Benach

The role of outer surface proteins (Osp) A and B and length of time in culture on the adhesion and cytotoxicity of Borrelia burgdorferi to C6 glioma and PC-12 pheochromocytoma cells was investigated using 6 different spirochete strains in an ELISA. Statistically significant differences in adhesion between OspB mutants and parental isolates were not seen, yet clear differences in adhesion were noted between low- and high-passage isolates. Polar adhesion and penetration by the tips of spirochetes resulted in the formation of surface cavities and blebs. Adhesion of spirochetes to C6 and to undifferentiated PC-12 cells did not result in significant cytotoxicity, but adhesion of spirochetes to PC-12 cells differentiated with nerve growth factor resulted in a loss of confluence of the monolayer and cytotoxicity at high spirochete-to-cell ratios. These results demonstrate that B. burgdorferi can induce damage to neural cells directly.

Lyme disease is a chronic infection caused by the spirochete Borrelia burgdorferi [1]. The clinical manifestations of Lyme disease may include a wide spectrum of acute and chronic neurologic disorders that begin with alterations of the blood-brain barrier [2]. The acute neurologic manifestations include cranial neuritis, radiculoneuritis, and meningitis [3–6]. The Garin-Bujadoux-Bannwarth syndrome is a lymphocytic meningoradiculitis seen more in Europe than in the United States [3]. Later manifestations include peripheral neuritis [7, 8], an encephalopathy with impaired cognitive functions and memory loss [9, 10], and, rarely, leukoencephalitis [11]. Magnetic resonance imaging of the brain has disclosed damage to the white matter in some patients [10, 12, 13]. In addition, electromyographic studies have also shown axonal damage and provided evidence suggestive of demyelination [7, 14, 15]. Brain and peripheral nerve biopsies have disclosed both axonal and myelin disruption as well as microgliosis [16]. Peripheral nerve biopsies have disclosed mononuclear cell infiltration of the nerve vessels without evidence of necrosis [17, 18].

Meningitis and peripheral nerve disorders have been demonstrated in experimentally infected nonhuman primate models of neuroborreliosis [19, 20]. The clinical presentations of both acute and chronic neuroborreliosis in patients and in nonhuman primates indicate that the central nervous system (CNS) and the peripheral nervous system can be affected. In the CNS, the neurons as well as glial cells could be involved in the pathogenesis of this disease.

Experimental studies have demonstrated that B. burgdorferi can adhere to a variety of cell types, including tick epithelium [21], human endothelium [22, 23], and both human and rodent neural cells [24, 25]. Evidence for B. burgdorferi invasion of the CNS includes the detection of spirochetal antigens by immunologic methods [2, 26] and by polymerase chain reaction (PCR) amplification of spirochetal DNA [3]. Using primary cultures of neonatal rat brains, it was demonstrated that spirochetes could adhere to cells of the CNS [24] and damage oligodendrocytes as measured by a 31Cr release assay and electron microscopy [27]. Spirochetes are able to adhere to extracellular matrix proteins [22, 24], proteoglycans [28, 29], integrin receptors [30], and nonprotein components such as glycosphingolipids [31, 32].

Outer surface proteins, namely OspA and OspB, have been implicated as potential adhesins to eukaryotic cells [33]. Recently, B. burgdorferi mutants have been generated using bactericidal monoclonal antibodies (MAbs) to OspB. These mutants either do not express OspB or express truncated forms of the protein that lack the epitope for these MAbs [34]. A mutant that does not express OspA, B, C, or D has also been described [35]. Previous studies have not been able to demonstrate statistically greater adhesion of these ospB mutants to glycosphingolipids [32] and to endothelium [35]. Other studies have shown different membrane protein variations associated with passage of spirochetes in vitro [36].

To further investigate the role of OspA and OspB and the effect of time in culture on the adhesion of B. burgdorferi spirochetes to neural cells in vitro, these ospB mutants were tested alongside their parental isolates for their ability to adhere to and damage PC-12 and C6 neural cell lines as models for neurons and glia, respectively. With the most adhesive isolate, further experiments were performed to determine the effect of coincubation of live spirochetes with neural cells over an extended period of time (72 h).

Materials and Methods

Spirochete isolates. Low-passage Ixodes tick–derived spirochete isolates WW1 and TI-1, the TI-1 mutant TI-EV, and the B31

Received 8 January 1997; revised 24 March 1997.
Grant support: NIH (AI-27044).
Reprints or correspondence: Dr. Jorge L. Benach, Dept. of Pathology, BHS Tower 9, Room 140, State University of New York, Stony Brook, NY 11794.

The Journal of Infectious Diseases 1997;176:470–7
© 1997 by The University of Chicago. All rights reserved.
0022–1899/97/7602–0021$02.00
mutant B313 (gift of A. Sadziene [35]) were inoculated from frozen stocks stored at −70°C and grown at 34°C in Barbour Stoenner Kelly (BSK) medium with 5% rabbit serum. High-passage strain B31 and its mutant EvB—[34] were grown in BSK medium without serum.

**Cell lines.** The PC-12 rat pheochromocytoma cell line, derived from an adrenal gland tumor that can be differentiated into cells resembling neurons with nerve growth factor (NGF), was obtained from American Type Culture Collection (ATCC CRL 1721; Rockville, MD) [37]. PC-12 cells were grown in 1× Dulbecco’s modified Eagle medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated horse serum (Hyclone, Logan, UT) and 5% heat-inactivated fetal bovine serum (HyClone) with sodium pyruvate (1 mM) and penicillin-streptomycin (100 U/mL). PC-12 cells were differentiated using NGF 7S (Boehringer Mannheim, Indianapolis) at 100 ng/mL. Differentiation was monitored visually by the presence of neurite outgrowths. The C6 glioma cell line (ATCC CCL 107), which was derived from a rat glial cell tumor induced by N-nitosomethylurea, was grown in Ham’s F-12 media (GIBCO) supplemented with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum with penicillin-streptomycin [38]. Cells were grown at 37°C in 5% CO₂ in a humidified cell incubator. PC-12 and C6 cells were harvested from 80% confluent monolayers using 0.05% trypsin with 10 mM EDTA (GIBCO) and incubated at 37°C for ~3 min. The cells were then pelleted by centrifugation in a swinging bucket rotor at 500 g at 5°C for 5 min. Cells were washed once in sterile PBS (GIBCO), resuspended in incubation medium (without antibiotics), and enumerated with a hemacytometer.

**SDS-PAGE and immunoblotting.** Spirochetes were harvested from BSK medium by centrifugation at 7000 g at 20°C for 10 min. Spirochete pellets were washed three times with 1× PBS with 5 mM MgCl₂, pH 7.4. Spirochetes were resuspended in PBS and sonicated with an ultrasonic processor (W380; Heat Systems–Thermotronics, Farmingdale, NY) for 5 min at 1-s intervals on ice. A Bradford assay was performed to determine the total protein concentration of each of the samples prior to electrophoresis. SDS-PAGE was performed using a Laemmli buffer system (Bio-Rad, Hercules, CA) in gels of 12.5% acrylamide (National Diagnostics, Atlanta). Spirochetal protein (10 μg/lane) was loaded under reducing conditions with sample buffer (5% glycerol, 10 mM EDTA, 3% SDS, 31 mM TRIS, 2.5% β-mercaptoethanol, with 0.004% bromophenol blue) on 10-cm stacking gels (Hoefer Scientific Instruments, San Francisco).

Four replicate gels were run simultaneously at 100 V for 1 h at room temperature. One gel was stained with 0.025% Coomassie brilliant blue. The three remaining gels were transferred to 0.45-μm nitrocellulose membranes (Bio-Rad) in transfer buffer at 30 mA overnight for immunoblotting. Blots were blocked with 1× PBS with 2% bovine serum albumin (BSA) for 1 h and probed with the MAbs (in parentheses) for detection of flagellin (CB1) [39], wild type OspA (CB10), and the C-terminal region of wild type OspB (CB2) [34]. All primary MAbs used were of the mouse IgG isotype and were obtained as concentrated hybridoma supernatants. Blots were incubated for 1 h at room temperature with shaking and washed three times with blocking buffer. Secondary goat anti-mouse IgG heavy- and light-chain-specific antibody conjugated to alkaline phosphatase (Kirkegaard & Perry, Gaithersburg, MD) was used at the optimal dilution in blocking buffer. Blots were washed three times with blocking buffer and incubated with substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Kirkegaard & Perry) according to manufacturer’s instructions.

**DNA sequencing of Osp mutants.** Mutant spirochete isolates selected for growth in the presence of CB2 MAb have been described [34]. The gene encoding OspB of the TI-EV mutant was sequenced by PCR amplification of the entire OspA/B operon. Spirochetes were harvested from growth in BSK medium as previously described. Spirochetal template DNA was obtained by resuspending the pellet in buffer containing 10 mM TRIS with 1 mM EDTA, pH 8.0, and boiling for 10 min. PCR was performed using the GeneAmp kit (Perkin-Elmer Cetus, Norwalk, CT) using oligonucleotide primers previously described [40]. PCR products were visualized by 0.8% agarose gel electrophoresis in 1× TRIS-borate-EDTA buffer and stained with ethidium bromide. PCR products were purified using a kit (Magic PCR Preps; Promega, Madison, WI). Sequencing was performed (Taq DyeDeoxy Terminator Cycle sequencing kit; Applied Biosystems, Foster City, CA) and analyzed (373A DNA sequencing system; Applied Biosystems).

**Adherence assay.** Flat-bottomed 96-well plates (Falcon 3872; Becton Dickinson, Lincoln Park, NJ) were coated with 100 μL/well poly-l-lysine (100 μg/mL; Sigma, St. Louis). PC-12 cells were seeded at 5 × 10⁴ cells/mL. C6 cells were seeded at 2.5 × 10⁵ cells/mL. Plates were used at the time when they reached ~80% confluence as determined by visual inspection (usually within 2 days). Spirochetes were harvested by centrifugation at 7000 g at 20°C for 10 min. Spirochetes were resuspended in incubation medium, which consisted of 1× DMEM with 10% heat-inactivated horse serum and 5% heat-inactivated fetal bovine serum without antibiotics. Both the horse and bovine sera were tested for the presence of antibodies to *B. burgdorferi* by an indirect IFA using fluorescein isothiocyanate–labeled goat anti–horse IgG. A 100-μL aliquot of each resuspended spirochete strain was centrifuged and washed three times with PBS with 5 mM MgCl₂ and used in a Bradford assay to determine the total protein concentration for each strain. Spirochete suspensions were adjusted to equal spirochetal protein concentrations, using additional coincubation medium. Cocultures were incubated overnight at 37°C.

The next day, cell monolayers were washed three times with 1× DMEM, fixed with 10% buffered formalin at room temperature for 30 min, and washed three times with 200 μL/well PBS to remove the excess fixative. Nonspecific binding of the primary and secondary antibodies was minimized by adding to each well 200 μL of 2% BSA in PBS, pH 7.4 (blocking buffer), at room temperature for 1 h. The ELISA using CB10 as the primary MAb was used to detect the adherence of all the isolates used, except the B313 mutant, which does not express OspA. For this mutant, CB1, an antiflagellar MAb [37], was used as the primary MAb for the adherence assay, which was performed using essentially the same procedure as for the CB10 MAb. Primary MAb in blocking buffer was reacted with the plate at 37°C for 1 h. The plate was washed three times with PBS, and 100 μL/well of the secondary conjugate was added in PBS with 2% BSA. The secondary conjugate was incubated at 37°C for 1 h, the plate was washed, and 100 μL/well of the substrate p-nitrophenyl phosphate (2 mg/mL; Sigma) was added in alkaline phosphatase buffer (pH 9.8). The plate was incubated at 37°C for 1 h and read in a microplate reader.
NGF was added to a final concentration of 100 ng/mL every 24 h. Differentiated PC-12 cells were plated at 5 x 10^5 cells/mL for the PC-12 cells and 2.5 x 10^5 cells/mL for the C6 cells. Cell medium was harvested from log-phase growth in BSK medium (without serum) by centrifugation and resuspended in coincubation medium (BSK with 10% horse and 5% fetal calf serum). Cell medium was removed from the plate and the resuspended spirochetes were added. A control included coincubation medium alone. The plate was incubated at 34°C with 5% CO₂.

At 24, 48, and 72 h after addition of spirochetes, cells were harvested by disrupting the monolayer with 0.05% trypsin with 5 mM EDTA (GIBCO) and resuspended in 0.5 mL of freshly made assay buffer (5 μL of calcine acetoxy-methylester and 20 μL of ethidium homodimer stock solution in 10 mL of PBS, pH 7.4; Eukolight; Molecular Probes Eugene, OR) and incubated in the dark at room temperature for 30 min. Cells were gently vortexed and counted on a hemacytometer. The total number of cells stained green (live) and red (dead) for each sample were counted using a fluorescent microscope. The means and SDs in the total number of cells for 3 replicate wells were determined for each time point. Mortality of cells was measured by dividing the total number of red-staining cells by the total number of cells and determining the percentage of nonviable cells for each time point. The means and SDs for three separate experiments were analyzed with statistical software (InStat 2.0; Graph Pad, San Diego).

**Results**

**Characterization of spirochete isolates.** Three isolates (B31, TI-1, and WW1) were isolated from *Ixodes scapularis* ticks. Escape variants (EvB– and TI-EV) were generated by growth of parental isolates (B31 and TI-1, respectively) in the presence of the borreliacidal MAb CB2 as described [34] (figure 1). Mutant B313 was selected from a B31 clone [35]. B31 is a high-passage isolate that is not virulent in animal models for Lyme borreliosis [1]. TI-1 is a low-passage tick isolate whose virulence has not been tested. TI-EV is an escape variant of the TI-1 strain that expresses a chimeric OspA protein fused to the C-terminus of OspB. Sequencing of the ospA/B operon of this isolate revealed a deletion from base 841 to 1756 that results in an open reading frame encoding a protein with a deduced molecular mass of 26,135 Da (table 1). WW1 is a primary tick isolate that has not been cloned or tested for virulence.

**Table 1.** Characterization of various spirochete isolates of *B. burgdorferi* used in the adhesion assay.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Passage</th>
<th>OspA/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>B31</td>
<td>Very high</td>
<td>A'B'</td>
</tr>
<tr>
<td>EvB–</td>
<td>Derived from B31</td>
<td>A', C-terminal, truncated OspB</td>
</tr>
<tr>
<td>TI-1</td>
<td>Low passage</td>
<td>A'B'</td>
</tr>
<tr>
<td>TI-EV</td>
<td>Derived from TI-1</td>
<td>Truncated OspA, fused to C-terminal of OspB</td>
</tr>
<tr>
<td>B313</td>
<td>Cloned from B31</td>
<td>A'B'</td>
</tr>
<tr>
<td>WW1</td>
<td>Very low passage tick isolate</td>
<td>A'B'</td>
</tr>
</tbody>
</table>

NOTE. Every isolate expressed flagellin.
B. burgdorferi Injury to Neural Cells

Adherence of B. burgdorferi to neural cells. The tendency of low-passage spirochete isolates to aggregate made direct enumeration of spirochete numbers difficult. To ensure that equal numbers of spirochetes for each isolate were used, the total protein concentration of resuspended whole spirochetes was determined using a Bradford assay. Spirochete suspensions were adjusted to equal protein concentrations by using additional coinubcation medium. ELISAs were done to determine the spirochete-specific signal for a known number of spirochetes/well and establish a standard curve for adhesion using optical density measurements (data not shown). Using the B31 isolate, it was demonstrated that as few as $10^3$ adherent spirochetes could be detected within the linear range of the assay ($OD = 0.1-1.0$) up to $10^9$ spirochetes/well. The results of these experiments indicated that the TI-1 and TI-EV isolates of spirochetes were the most adherent (figure 3; $P = .008$ by one-way analysis of variance). These spirochetes adhered twice as much as B31 and its mutant EvB$^-$ and ~3 times as much as WW1, a tick-derived low-passage isolate. Mutant B313 did not adhere in numbers much above background (figure 3). In addition, it was demonstrated that, on a per cell basis, spirochetes were more adherent to monolayers of C6 cells than of PC-12 cells (data not shown).

Figure 2. Immunoblots of different spirochete isolates (B31, EvB$^-$, TI-1, TI-EV, B313, and WW1). A, Using CB1 monoclonal antibody (MAb) (anti-flagellin). B, CB2 MAb (anti-OspB C-terminal). C, CB10 MAb (anti-OspA). Equal amounts of total protein were loaded for each isolate, and 12.5% acrylamide gels were run in parallel and transferred to nitrocellulose membranes. All isolates expressed p41 flagellin at same level, whereas not all isolates expressed wild type OspA (p31) or OspB (p34).

Figure 3. ELISAs (mean OD ± SD) to determine relative adherence of different isolates of spirochetes to monolayers of C6 glioma cells (A) and undifferentiated PC-12 cells (B) using CB10 (anti-OspA) primary monoclonal antibody (MAb). Controls for background reactivity of secondary conjugate antibody to cell monolayers (no CB10) and for background reactivity of primary MAb to cell monolayers in absence of spirochetes (CB10+2) were used for each assay. * Statistically significant differences in adhesion compared with background reactivity of primary antibody control. All isolates were adjusted to equivalent total protein concentration and coinubcated with neural cells at 37°C with 5% CO$_2$ for 24 h. Results were representative of 3 separate experiments.
by the lack of an increase in the percentage of nonviable cells throughout the time periods tested (figure 4).

*B. burgdorferi* spirochetes were irreversibly cytotoxic for differentiated PC-12 cells as shown by the significantly higher increase in the percentage of nonviable cells coincubated with high spirochete-to-cell ratios ($P = 0.026$, figure 5A). The monolayer was markedly disrupted, with loss of the neurites. A dose-response curve was generated using spirochete-to-cell ratios of 1:1, 10:1, and 100:1 (figure 5B). Significant cytotoxic effects were noted at 72 h of coincubation with 10 spirochetes/cell.

**Figure 4.** Mortality of C6 (A) and undifferentiated PC-12 cells (B) under conditions of cytotoxicity assay. Control cell cultures (□) were incubated without spirochetes under identical conditions as cells coincubated with 100 spirochetes/cell (◆). All cells were coincubated with live TI-1 spirochetes at $5 \times 10^7$ spirochetes/mL for up to 72 h. There was no significant increase in mortality of cells coincubated with spirochetes, nor was there any significant decrease in total cell number. Results are representative of 3 separate experiments.

**Figure 5.** A, Mortality of PC-12 cells differentiated with nerve growth factor for 7 days before coincubation with TI-1 spirochetes under conditions of cytotoxicity assay. There is significant ($P = 0.026$) increase in PC-12 cell mortality after 72 h of coincubation with spirochetes. Control cell cultures (□) and cells coincubated with 100 spirochetes/cell (◆). These results represent means of 3 separate experiments. B, Mortality of differentiated PC-12 cells coincubated with spirochetes at various spirochete-to-cell ratios. Control cells (○) and cells coincubated with spirochetes at 100 (□), 10 (◆), and 1 (△) per cell. Results are derived from representative experiment.
Ultrastructural and confocal microscopy of spirochete adherence and cytotoxicity to neural cells. Although coinoculation of C6 glioma and undifferentiated PC-12 cells with spirochetes did not lead to cell death within a 72-h period, evidence of sublethal damage to these cells was observed by both scanning and transmission electron microscopy. These cells produced membrane blebs and ruffles that were identical in appearance to those shown in figure 6 for NGF-differentiated PC-12 cells. In almost all cases, spirochete adhesion was polar (tip adhesion), with the resulting formation of cell membrane blebs at the point of contact with the organism (figure 6A). Blebs formed in the cell body as well as in the neurites of differentiated PC-12 cells (figure 6D). Penetration of the undifferentiated PC-12 and C6 glioma cell membranes by the tips of spirochetes was observed by both scanning (figure 6A) and transmission (figure 6B) electron microscopy. Additional evidence for this phenomenon was obtained by confocal microscopy (figure 7), with the tip of the spirochete penetrating at a cell depth of 1 μm.

**Discussion**

To investigate the role of OspA and OspB in spirochete adherence to neural cells, 6 different isolates of *B. burgdorferi*

---

**Figure 6.** A, Scanning electron micrograph of a TI-1 spirochete adhering to surface of PC-12 cell differentiated with nerve growth factor. Blebbing of cell surface (arrow) was consistent feature of cells coincubated with 10 spirochetes/cell. Cavity formed at point of attachment of spirochete tip with cell plasma membrane was also visualized using transmission electron microscopy. B, Transmission electron micrograph of PC-12 cell differentiated with nerve growth factor with tip of spirochete (arrow) forming cavity in cell plasma membrane associated with profuse blebbing of plasma membrane that was induced by coincubation with spirochetes. C, Transmission electron micrograph of cross section of spirochete (arrow) demonstrating intimate association with blebs on surface of PC-12 cell differentiated with nerve growth factor. D, Scanning electron micrograph of polar attachment of spirochetes (arrows) to surface of neurite outgrowth of PC-12 cell differentiated with nerve growth factor showing partial tip penetration.
The difference in adhesion may be due to the nature and number of the components expressed on the surface of these two cell lines or to differences in the extracellular matrix produced by these two cell lines [42]. Because spirochetes were cytotoxic for cultures of differentiated PC-12 cells, the ELISA could not be used to determine the adhesion of spirochetes to monolayers of differentiated PC-12 cells, due to loss of integrity of the monolayer.

Scanning and transmission electron microscopy were used to visualize the interface of spirochetes and cell membranes. Scanning electron microscopy showed that spirochetes adhered in a polar orientation (tip adhesion) to the plasma membrane of neural cells (figure 6). Polar adhesion of spirochetes has been previously documented for neural as well as for other types of cells [24, 25, 43, 44] and may well be the preferred mode for cellular contact by this organism.

To determine whether spirochete adhesion to neural cells resulted in reversible (nonlethal) or irreversible (lethal) damage, a cytotoxicity assay was performed using a fluorescein-based viability stain. Essentially no differences were seen in either cell growth or viability between control cells and cells coincubated with spirochetes (figure 4). However, differences in cell viability were noted using PC-12 cells that had been differentiated for 7 days with NGF prior to coincubation with spirochetes (figure 5). Although cell death was noted at ratios of \( \geq 10 \) spirochetes/cell, differentiated PC-12 cells were susceptible to spirochete-induced cytotoxicity. One major difference is that C6 and undifferentiated PC-12 cells can proliferate under the conditions of the cytotoxicity assay, whereas PC-12 cells differentiated with NGF are no longer capable of cell division. This difference in growth may account for the difference in susceptibility to damage by spirochetes. Dividing cells may be able to dilute the effects of spirochete-mediated damage to the integrity of the plasma membrane, but nonproliferating cells may not be able to accomplish this. A similar type of injury was noted with terminally differentiated oligodendrocytes of primary neonatal rat brain cultures [27].

It is possible that such mechanisms may occur in vivo and that the cytotoxic effects of spirochetes may accumulate over long periods of time. Spirochetes may be able to alter the permeability of the plasma membrane by forming pores as a result of tip penetration by spirochetes. This may result in an influx of ions into the cell. Rapid influxes of ions are associated with both cellular stress and cell death [45]. The formation of blebs on the surfaces of C6 and undifferentiated PC-12 cells may be a physiologic reaction to sublethal injury. Post mitotic cells of the CNS may be more susceptible to subtle injury than proliferative cells. Therefore, prolonged exposure to spirochetes in vivo as a result of chronic neuroborreliosis may result in lethal injury. These results may shed light on the mechanisms involved in CNS injury in patients with Lyme disease.

References


44. Montgomery RR, Malawista SE. Entry of *Borrelia burgdorferi* into macrophages is end-on and leads to degradation in lysosomes. Infect Immun 1996; 64:2867–72.