



Analysis of *Borrelia burgdorferi* surface proteins as determinants in establishing host cell interactions

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Borrelia burgdorferi infection causes Lyme borreliosis in humans, a condition which can involve a systemic spread of the organism to colonize various tissues and organs. If the infection is left untreated by antimicrobials, it can lead to manifestations including, arthritis, carditis, and/or neurological problems. Identification and characterization of *B. burgdorferi* outer membrane proteins that facilitate cellular attachment and invasion to establish infection continue to be investigated. In this study, we sought to further define putative cell binding properties of surface-exposed *B. burgdorferi* proteins by observing whether cellular adherence could be blocked by antibodies. *B. burgdorferi* mixed separately with monoclonal antibodies (mAbs) against outer surface protein (Osp) A, OspC, decorin-binding protein (Dbp) A, BBA64, and RevA antigens were incubated with human umbilical vein endothelial cells (HUVEC) and human neuroglial cells (H4). *B. burgdorferi* treated with anti-OspA, -DbpA, and -BBA64 mAbs showed a significant decrease in cellular association compared to controls, whereas *B. burgdorferi* treated with anti-OspC and anti-RevA showed no reduction in cellular attachment. Additionally, temporal transcriptional analyses revealed upregulated expression of *bba64*, *ospA*, and *dbpA* during coincubation with cells. Together, the data provide evidence that OspA, DbpA, and BBA64 function in host cell adherence and infection mechanisms.

Keywords: *Borrelia burgdorferi*, cell attachment, gene expression

INTRODUCTION

Lyme borreliosis, or Lyme disease in humans, is the result of an infection with the spirochete *Borrelia burgdorferi*. This tick-borne illness typically exhibits a rash (termed erythema migrans) at the tick-bite site, and can progress to more severe conditions if left untreated. After introduction into the skin, the organism systemically disseminates and colonizes other tissues and organs ultimately resulting in arthritis, carditis, and/or neurological manifestations (Steere, 2001). The processes underlying the spirochete's ability to spread and localize to host tissues leading to the pathology of Lyme disease are not fully defined.

Borrelia burgdorferi adapts to the varying environments encountered during its enzootic cycle through ticks and mammals by undergoing differential gene expression. For example, the organisms are relatively dormant in an unfed tick; however during tick feeding, *B. burgdorferi* becomes metabolically active by turning on genes encoding proteins essential for survival, replication, and transmission. Accordingly, once inside the mammalian host, *B. burgdorferi* regulates gene expression to facilitate infection. The number of genes expressed and gene products synthesized during mammalian infection is evidenced by the robust antibody response against a large number of borrelial antigens (Dressler et al., 1993). Some *in vivo* differentially expressed genes encode surface lipoproteins that have been identified as adhesins that mediate binding to molecules present in the extracellular matrix or on host cells of tissues (Coburn et al., 2005). Borrelial surface-exposed proteins decorin-binding protein (Dbp) A and

B, BBK32, ErpX, RevA, Bgp, P66, BBB07, BmpA, CRASP-1, and outer surface protein (Osp) A, bind fibronectin, decorin, laminin, integrins, and other glycosaminoglycans and proteoglycans (Guo et al., 1998; Probert and Johnson, 1998; Pal et al., 2000; Parveen and Leong, 2000; Coburn and Cugini, 2003; Zambrano et al., 2004; Fischer et al., 2006; Rupprecht et al., 2006; Behera et al., 2008; Brissette et al., 2009a,b; Verma et al., 2009; Hallstrom et al., 2010).

Numerous studies have demonstrated borrelial adherence to and/or invasion of several cell types *in vitro* (Garcia-Monco et al., 1989; Szczepanski et al., 1990; Comstock and Thomas, 1991; Ma et al., 1991; Klempner et al., 1993; Kurtti et al., 1993; Girschick et al., 1996; Peters and Benach, 1997; Leong et al., 1998; Cinco et al., 2001; Fischer et al., 2003; Livengood and Gilmore, 2006; Wu et al., 2011). However, knowledge of specific processes mediated by known *B. burgdorferi* surface proteins for cell and tissue colonization, as well as the identification of novel proteins involved in host cell infections is lacking. In a previous study, we utilized global transcriptome analysis to examine *B. burgdorferi* gene expression during human host cell interactions as an initial step to identify proteins involved in cellular colonization, including adherence and invasion (Livengood et al., 2008). We hypothesized that genes upregulated in response to host cell signals may function in establishing *B. burgdorferi* infection. In this study, we examined whether *B. burgdorferi* binding to human cells could be blocked by antibodies directed against more well-characterized surface lipoproteins identified by the microarray (i.e., DbpA, BBA64, OspA, OspC, and RevA). Additionally, we measured transcription

of these borrelial genes to observe the level of regulation in response to cellular interaction.

MATERIALS AND METHODS

MONOCLONAL ANTIBODIES, INDIRECT IMMUNOFLUORESCENCE (IFA) AND IMMUNOBLOTTING OF CULTURED *B. BURGDORFERI*

Monoclonal antibodies (mAbs) against BBA64, DbpA, and the anti-*Bartonella* protein were obtained from Barbara J. B. Johnson (CDC, Fort Collins, CO, USA). Anti-BBA64 and -DbpA were generated by recombinant protein immunization, and the anti-*Bartonella* mAb was generated by whole cell lysate immunization utilizing standard procedures for generating hybridomas in mice (Mbow et al., 2002). The anti-*Bartonella* mAb was reactive to a 70-kDa band on immunoblot against a rodent-derived *Bartonella* isolate. Anti-Rev and -OspC (B5) mAbs were generated by tick-bite inoculation of mice and have been described previously (Gilmore and Mbow, 1998; Mbow et al., 1999, 2002). Anti-OspA mAb H5332 was provided by Alan Barbour, UC-Irvine.

Immunofluorescent staining of cultured *B. burgdorferi* was performed as follows. *B. burgdorferi* low passage, infectious, clonal strain B31-A3 (Elias et al., 2002) was grown in complete Barbour-Stoenner-Kelly (BSK-II) medium at 34°C in capped tubes. Cultures were grown to mid-to-late logarithmic stage (approx. 5×10^7 – 1×10^8 organisms/ml), and 2×10^6 bacteria were spun onto Cytospin microscope slides using a Shandon Cytospin 4 (Thermo Electron Corporation, Waltham, MA, USA). After centrifugation, slides were air dried, incubated in blocking solution (2% bovine serum albumin in phosphate buffered saline pH 7.5 [BSA-PBS]) for 1 h at room temperature (rt), and then incubated with the specific mAb (1:75 dilution) for 1 h. Slides were washed (3× with PBS) and stained with goat anti-mouse IgG Alexafluor 594 (Molecular Probes, Eugene, OR, USA) at a 1:75 dilution for 45 m. Slides were washed (3× with PBS) and probed with FITC-conjugated goat anti-*Borrelia burgdorferi* IgG (KPL, Gaithersburg, MD, USA) at a 1:75 dilution for 45 m. Slides were washed (3× with PBS), air dried, and overlaid with Prolong AntiFade (Molecular Probes) and coverslips prior to imaging by epifluorescent microscopy.

Immunoblotting was performed as follows. *B. burgdorferi* were collected from culture and washed in PBS prior to gel fractionation. Cell lysates were denatured by boiling for 10 min in Laemmli buffer containing 5% 2-mercaptoethanol and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10–20% gradient gel followed by transfer to nitrocellulose filters according to standard procedures. Immunoblots were incubated with the appropriate mAb (1:5000 dilution) for 1 h, washed with PBS (3× at 5 min), followed by incubation with alkaline phosphatase conjugated goat anti-mouse IgG (1:2000) for 1 h. After PBS wash, the blot was developed colorimetrically with NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt) substrate.

MAMMALIAN CELL CULTURE

Human umbilical vein endothelial cells (HUVEC) and human neuroglial cells (H4) were obtained from American Type Culture Collection (Manassas, VA, USA; ATCC catalog numbers CRL-1730 and HTB-178 respectively). HUVECs were grown

in F12-K medium supplemented with 0.1 mg/ml heparin, 0.03 mg/ml endothelial cell growth supplement, and 10% fetal bovine serum. The H4 cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Both cell lines were grown at 37°C in the presence of 5% CO₂ in a humidified environment. Cells were harvested from confluent monolayers with 0.05% trypsin – 10 mM EDTA and enumerated on a hemocytometer cell counter.

BORRELIA BURGDORFERI-CELL COINCUBATION ASSOCIATION ASSAYS

HUVEC and H4 cells were seeded at a concentration of 3×10^5 cells/chamber on Lab-Tek II CC chamber slide system (Nalge Nunc International, Rochester, NY, USA), and allowed to adhere overnight. Prior to addition to cells, individual *B. burgdorferi* aliquots, at a multiplicity of infection (MOI) of 40 (1.2×10^7), were preincubated (35°C shaking at 150 rpm for 30 m) with each anti-*B. burgdorferi* mAb or control in 0.5 ml volume. Following incubation with the appropriate antibody, *B. burgdorferi* were added to each cell well. The antibody concentration used was pre-determined empirically by a series of 10-fold dilutions for each mAb to ascertain the highest dilution of antibody that produced significant reduction in cell binding. Anti-BBA64, -DbpA, and -OspA were used at 1:500, 1:5000, and 1:5000 respectively. The coincubation experiments were performed in duplicate using these dilutions. Additionally, three control *B. burgdorferi* pre-cell incubation treatments were tested: (i) mAb suspension buffer only (50% glycerol/1× PBS); (ii) a non-*Borrelia*-specific mAb (anti-*Bartonella* antibody); and (iii) no antibody (with 1× PBS added to volume).

Following overnight coincubation of treated *B. burgdorferi* with human cells, slide chambers were gently washed 3× with PBS to remove non-adherent bacteria. Cells with associated bacteria were fixed on the slide chamber with 4% paraformaldehyde for 45 m at rt. Fixed cells were washed followed by blocking with 2% BSA-PBS for 1 h. Mammalian cells were stained with Alexa 594 Phalloidin (1:200 dilution; Molecular Probes) for 10 m at rt. Samples were washed, then cells were permeabilized with 2% BSA-PBS supplemented with 0.5% Triton X-100 for 45 m at rt. *B. burgdorferi* were stained with FITC-conjugated goat anti-*B. burgdorferi* (1:75 dilution) for 1 h.

Samples were visualized on a LSM 5 Pascal confocal microscope (Carl Zeiss, USA). Micrograph images were acquired at 100× and 250× magnification. *B. burgdorferi* associated with cells were enumerated microscopically. Counts were made for (1) total human cells/field, and (2) *Borrelia* associated with cells/field, with 30 individual fields observed and counted from each slide. From the sum of the 30 fields, the average number of *B. burgdorferi* associated per cell was determined. The percent reduction in cell attachment was calculated by the difference between the antibody-treated *B. burgdorferi* and the control treatment. Assays were performed in duplicate. Comparisons of the data were performed using a two-sample *t*-test. Differences were considered significant if *p*-value was ≤ 0.05 .

RNA ISOLATION FROM *B. BURGDORFERI*-ASSOCIATED CELLS

HUVEC or H4 cells were grown to confluence in a 150 cm² cell culture flask, trypsinized, and enumerated, from which 2×10^5

cells were seeded in six well cell culture plates and were allowed to adhere for 17 h at 37°C with 5% CO₂. Log-phase *B. burgdorferi* cultures were centrifuged for 10 m at 9300 × *g*, with aliquots resuspended in F-12K or DMEM cell culture media and added to the HUVECs, H4 cells, or media alone at a MOI of 40 (7.8×10^7 bacteria). Plates were incubated at 34°C with 5% CO₂. Cell wells were inoculated concurrently in triplicate and harvested for RNA isolation at 2-, 4-, 8-, 24-, and 48-h post-inoculation. At the appropriate time, cell culture media was removed, and the plate was washed 3 × with 5 ml PBS to remove non-adherent bacteria. Cells with adherent bacteria were removed with cell scrapers in 300 μl RNeasy lysis buffer (Qiagen, Crawfordsville, IN, USA). Media from the cell-free *Borrelia* wells were centrifuged at 9300 × *g* for 5 min, washed 2 × with 5 ml PBS, and resuspended in 300 μl RNeasy lysis buffer. RNeasy lysis buffer samples were stored overnight at 4°C and then at -20°C until the RNA was purified. Prior to RNA extraction, the samples were centrifuged at 16,000 × *g* for 10 m, the RNeasy lysis buffer was decanted, followed by total RNA extraction using RNeasy spin column kit (Qiagen). Contaminating DNA was removed using the TURBO DNA-free kit (Ambion). RNA samples were tested for contaminating DNA by PCR prior to cDNA synthesis. Total RNA was quantified by spectrophotometry and RNA integrity was analyzed using an Agilent 2100 Bioanalyzer with Agilent RNA Pico Reagents (Agilent Technologies, Boulder CO).

QUANTITATIVE REVERSE TRANSCRIPTASE PCR (QRT-PCR)

Purified total RNA (100 ng) isolated from the *B. burgdorferi*-associated cells was used to synthesize cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random decamers. Real-time PCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems) using *flaB*, *bba64*, *dbpA*, and *ospA* TaqMan probe and primers described previously (Livengood et al., 2008). Real-time PCR reactions, performed in triplicate per sample, contained 1 μl cDNA, 1 μM 5' primer, 1 μM 3' primer, 0.1 μM probe, and 1 × TaqMan Universal PCR Master mixture in a total volume of 12.5 μl. Amplification parameters consisted of 1 cycle at 95°C for 10 m and 50 cycles of 95°C for 30 s and 60°C for 1 m, with data collection after each cycle in a BioRad iCycler (BioRad, Hercules, CA, USA). The iCycler software determined crossing threshold (C_T) values. *B. burgdorferi* gene expression during coinoculation with cells was determined relative to borrelial gene expression in cell-free tissue culture media using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), and was normalized against the constitutively expressed *flaB* gene.

RESULTS

PROTEIN SYNTHESIS BY *IN VITRO* CULTIVATED *B. BURGENDORFERI*

Cultured *B. burgdorferi* was assessed for the production of OspA, DbpA, BBA64, OspC, and RevA prior to antibody treatment and coinoculation with tissue culture cells. Analysis of cultured organisms by IFA demonstrated *in vitro* production of the individual proteins (Figure 1A). Immunoblot analysis of cultured borrelial lysates also showed protein production (Figure 1B).

COINCUBATION OF ANTIBODY-TREATED *B. BURGENDORFERI* WITH HUVEC AND H4 CELLS

Borrelia burgdorferi were preincubated with the mAbs for 30 m prior to placement on HUVEC and H4 cells. Following the

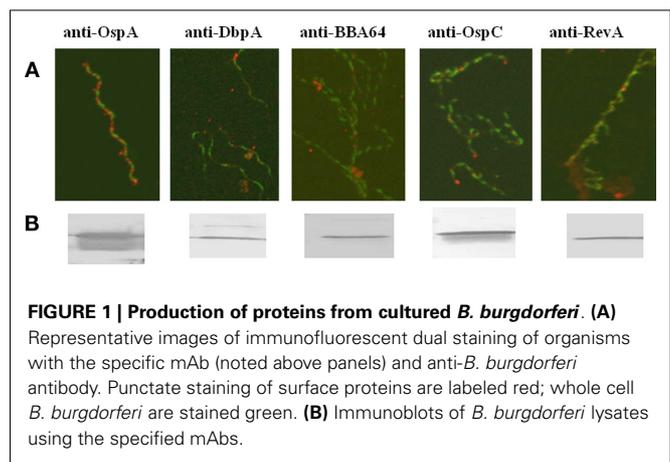


FIGURE 1 | Production of proteins from cultured *B. burgdorferi*. (A)

Representative images of immunofluorescent dual staining of organisms with the specific mAb (noted above panels) and anti-*B. burgdorferi* antibody. Punctate staining of surface proteins are labeled red; whole cell *B. burgdorferi* are stained green. (B) Immunoblots of *B. burgdorferi* lysates using the specified mAbs.

antibody incubation, a sample of the spirochetes were visualized under darkfield microscopy to determine viability. *B. burgdorferi* treated with each mAb were motile indicating that the antibody treatment was not borrelicidal.

An experiment was performed to find the least concentrated mAb solution that produced significant differences ($p < 0.05$) in cell binding from the no antibody control treatment. We considered that higher dilutions of mAb would provide less chance for steric hindrance that could lead to non-specific blocking. The optimal dilutions were determined to be 5×10^{-3} for the anti-OspA and -DbpA mAbs, and 5×10^{-2} for the anti-BBA64 antibody. Anti-RevA and anti-OspC mAbs, at all dilutions tested, demonstrated no significant inhibition of binding to HUVEC or H4 cells when compared to the no antibody control (data not shown).

Additionally, there were no significant differences between numbers of *B. burgdorferi* attached to host cells following each of the control treatments, i.e., *Borrelia* preincubated with antibody buffer only, *Borrelia* preincubated with non-specific antibody, and *Borrelia* preincubated with only PBS (data not shown). Therefore, the control data reported (as “without” or “no antibody”) in the text reflect counts obtained with the non-specific anti-*Bartonella* antibody pretreatment control.

Table 1 shows associated *B. burgdorferi*/100 cells with and without specific mAb treatment and the calculated reduction in *B. burgdorferi* cellular attachment. *B. burgdorferi* treated with anti-OspA, -DbpA, and -BBA64 antibodies demonstrated significant decreases in HUVEC and H4 cell binding when compared to *Borrelia* treated with the control. (As mentioned above, treatment with anti-OspC and anti-RevA had no effect on binding). The reduction in attachment between antibody treatments in separate experiments ranged from 30.6 to 55.2% for both HUVEC and H4 cells. The average *B. burgdorferi*/100 cells counted from 30 fields in each experiment were significantly different between antibody-treated *Borrelia* and controls ($p < 0.001$). An additive effect in attachment reduction when all three antibodies were combined was not observed. Figure 2 shows images of representative fields of *B. burgdorferi* binding to HUVECs and H4 cells respectively, with and without antibody treatment, allowing a visual inspection of the diminished attachment.

Table 1 | Reduction in *B. burgdorferi* binding to HUVEC and H4 cells following treatment with anti-*B. burgdorferi* surface protein antibodies.

Cell type/ experiment #	Average <i>B. burgdorferi</i> /100 cells ^a (SD)		Attachment reduction ^b (%)
	Antibody treatment		
	Anti-OspA	Control	
HUVEC/Exp. 1	17.6 (12.6)	37.3 (15.1)	52.8
HUVEC/Exp. 2	33.8 (13.5)	53.7 (19.8)	37.1
H4/Exp. 1	6.7 (3.2)	12.5 (4.8)	46.4
H4/Exp. 2	11.1 (4.0)	16.0 (5.9)	30.6
	Anti-DbpA	Control	
HUVEC/Exp. 1	12.7 (6.1)	26.3 (8.8)	51.8
HUVEC/Exp. 2	12.7 (4.2)	26.6 (8.8)	52.3
H4/Exp. 1	5.9 (1.7)	9.9 (3.3)	40.4
H4/Exp. 2	27.3 (7.9)	47.6 (10.6)	42.6
	Anti-BBA64	Control	
HUVEC/Exp. 1	12.5 (4.3)	27.9 (7.5)	55.2
HUVEC/Exp. 2	19.9 (6.6)	29.2 (7.4)	31.8
H4/Exp. 1	32.9 (7.9)	52.1 (9.4)	36.9
H4/Exp. 2	4.7 (1.4)	9.5 (3.4)	50.5
	Anti-BBA64, -OspA, -DbpA	Control	
HUVEC/Exp. 1	39.1 (9.6)	61.5 (9.8)	36.4
HUVEC/Exp. 2	20.4 (6.8)	53.7 (19.8)	62.0
H4/Exp. 1	37.3 (7.9)	46.7 (7.8)	20.1
H4/Exp. 2	12.6 (4.0)	16.0 (5.8)	21.2 ^c

^a30 fields counted.

^b $P < 0.0001$.

^c $P < 0.01$.

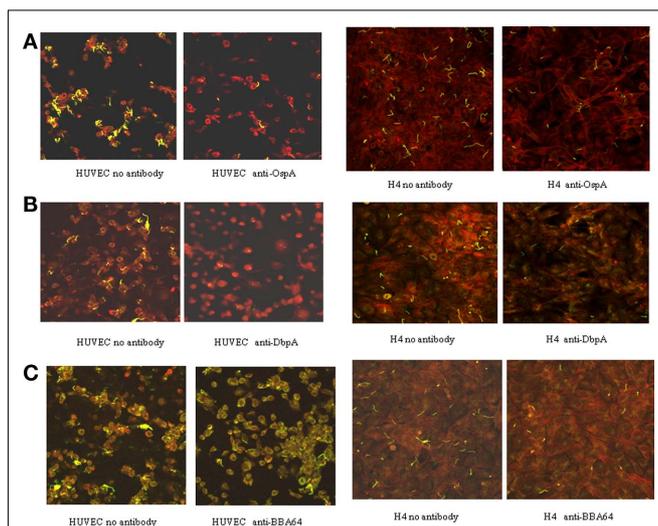


FIGURE 2 | Confocal microscopy of fluorescent-stained *B. burgdorferi* bound to HUVEC and H4 cells. Panels on the left show *Borrelia* attachment with no antibody treatment (i.e., the anti-*Bartonella* control); panels on the right show *Borrelia* attachment following incubation with anti-surface protein mAb. (A) anti-OspA; (B) anti-DbpA; (C) anti-BBA64.

OspA, DbpA, AND BBA64 GENE EXPRESSION DURING COINCUBATION WITH HOST CELLS

Antibodies directed against OspA, DbpA, and BBA64 attenuated the ability of *B. burgdorferi* to bind to host cells *in vitro* suggesting that these surface proteins may play a role in establishing infection. Therefore, we hypothesized that the genes encoding these proteins may be upregulated during cellular interaction. We employed qRT-PCR to monitor *B. burgdorferi* gene expression temporally throughout the incubation period with the tissue culture cells. We found that in both H4 and HUVEC interactions, *dbpA*, *bba64*, and *ospA* expression were generally elevated compared to *B. burgdorferi* incubation in cell-free environment.

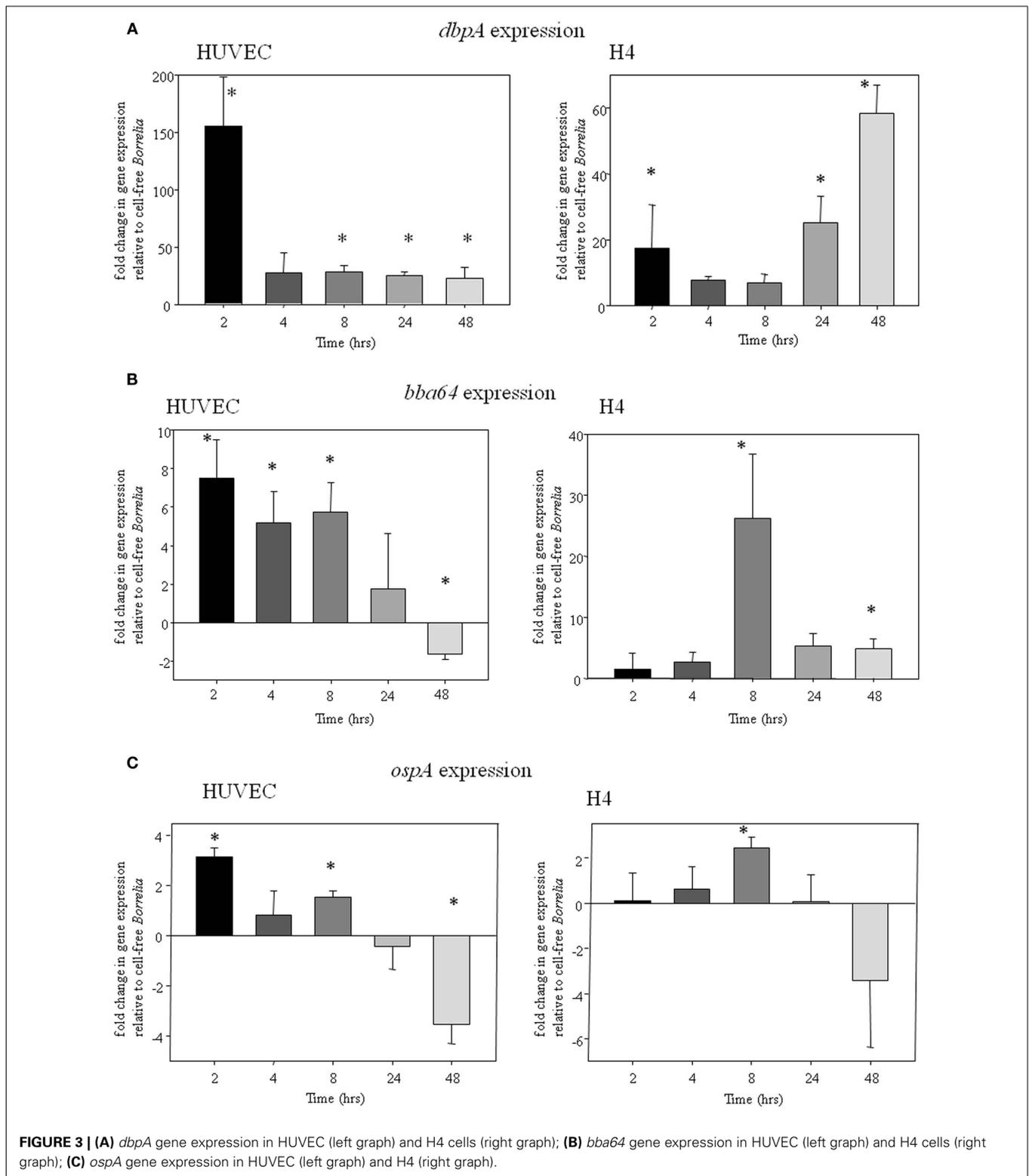
dbpA expression was the highest of the three genes in both HUVEC and H4 incubations. At 2-h post-inoculation, gene expression was approximately 150-fold higher in HUVEC than in cell-free media (Figure 3A). *dbpA* expression continued to be elevated at approximately 20-fold at 4-, 8-, 24-, and 48-h post-inoculation in HUVECs compared to the control. *dbpA* also was upregulated in H4 cells throughout the incubation period, with a peak around 60-fold at 48 h (Figure 3A).

bba64 expression measured at 2- to 8-h post-inoculation with HUVECs was between approximately five- to sevenfold higher than in cell-free media (Figure 3B). At 24-h post-inoculation, expression was detected but was not significantly increased compared to *B. burgdorferi* in cell-free media. However, by 48 h, expression had decreased below the cell-free level. In H4 cells, *bba64* expression was detected early, but not at significantly higher levels than in cell-free organisms. However, expression increased several-fold by 8-h post-inoculation, and remained elevated compared with the cell-free control at 48-h post-inoculation (Figure 3B).

ospA expression was detectable and slightly elevated in HUVECs in early time points post-inoculation when compared to expression in cell-free media. At 2 h into the incubation period, an approximate threefold increase in expression was observed. At 4-h post-inoculation, the expression level had decreased from 2 h, although a significant increase from cell-free expression was detected at 8 h. Later in the incubation, at 24 and 48 h, *ospA* expression had decreased to levels below that in cell-free media. A similar pattern was observed in H4 cells where there was no significant difference between expression in cell-free conditions, except at 8 h incubation whereby there was an approximate twofold increase (Figure 3C).

DISCUSSION

Colonization of host tissues by *B. burgdorferi* is a key factor in the pathogenesis of Lyme borreliosis. However, the mechanisms by which *B. burgdorferi* interacts with host cells to facilitate attachment, invasion, and colonization are poorly understood. To gain insight into *B. burgdorferi* genes that encode proteins required for human cell interactions, we hypothesized in an earlier study that genes upregulated during host cell infection may have roles in adherence, invasion, and colonization. In that study, we performed a global analysis of *B. burgdorferi* gene expression by microarray following coincubation of *Borrelia* with human neuroglial cells and found several genes to be upregulated during the



cellular interaction (Livengood et al., 2008). Among the upregulated genes identified by the microarray study were *ospA*, *dbpA*, and *bba64*. Other studies have shown *ospC* and *revA* to encode

products that functioned in establishing mammalian infection and in fibronectin-binding respectively (Grimm et al., 2004; Brissette et al., 2009a). Therefore, we initiated the current study to

determine if mAbs directed against these surface proteins could prevent or reduce the establishment of host cell attachment, and if so, assay expression of the genes during cellular association.

We first determined that cultured *B. burgdorferi* produced the target proteins prior to antibody treatment and cell incubation. Second, after mixing *B. burgdorferi* and the mAbs we ensured that the antibody was not bactericidal and that the spirochetes were still motile. With these requirements satisfied, *B. burgdorferi* incubated with and without specific antibody were coincubated in tissue culture with the human host cells. Three of the five anti-surface protein antibodies, anti-OspA, anti-DbpA, and anti-BBA64, significantly reduced *B. burgdorferi* binding to H4 and HUVECs (Table 1; Figure 2).

In an earlier study by Comstock et al. (1993), a mAb directed against OspA (9B3D) was shown to inhibit binding to HUVECs, therefore our anti-OspA treatment served as a positive control and allowed us to corroborate the finding with a mAb directed against OspA. The antibodies directed against RevA or OspC had no inhibitory effect on *B. burgdorferi* binding, suggesting these proteins do not play a role in attachment to HUVEC or H4 cells. RevA is expressed during mammalian infection and has recently been shown to be a fibronectin-binding protein, but its function is unknown (Gilmore and Mbow, 1998; Brissette et al., 2009a). OspC has been studied more extensively and is required by *B. burgdorferi* to establish infection in the mammalian host (Grimm et al., 2004). Because mAbs specific for a single epitope on the protein were used, we cannot discount that polyclonal antibodies directed against other determinants of OspC or RevA could provide a blocking effect. However, the anti-OspC mAb used here has been shown to protect against *B. burgdorferi* challenge when passively administered (Mbow et al., 1999). Therefore the failure of this mAb to inhibit cell adherence suggests that OspC does not function in cellular interactions (at least for these cell types) to promote infection. The lack of effect on cell binding with these two antibodies also provided evidence that the reduction in attachment measured with the anti-OspA, -DbpA, and -BBA64 antibodies was not due to non-specific inhibition.

The results of the antibody blocking experiments suggested that OspA, BBA64, and DbpA can mediate *B. burgdorferi* attachment to human cells, and that their production may be necessary for cell and tissue colonization. Therefore, we measured *B. burgdorferi* gene expression during coincubation with the host cells, and found that *dbpA*, *ospA*, and *bba64* expression were elevated during interactions with both cell types.

dbpA was highly upregulated, throughout the 48-h experiment, when *B. burgdorferi* were incubated with HUVECs and H4 cells. DbpA is an adhesin that binds decorin, a protein linked to the glycosaminoglycan (GAG) dermatan sulfate/chondroitin sulfate and is most commonly associated with collagen, but is present in many tissues (Guo et al., 1995, 1998). Proteoglycans are receptors for *B. burgdorferi* adherence to several cell types, with specific classes of GAGs playing key roles in the spirochetal binding activity (Leong et al., 1998). Fischer et al. (2003) showed that transformation of a non-adherent strain of *B. burgdorferi* lacking the *dbpBA* operon with *dbpA* could restore binding to human epithelial cells. Our observation of *dbpA* upregulation in response to interactions with host cells, combined with the reduction in cell binding by antibody

reinforces findings from earlier studies linking DbpA production with cell attachment, tissue colonization and host immune resistance (Liang et al., 2004; Blevins et al., 2008; Shi et al., 2008b; Weening et al., 2008).

bba64 was also expressed during *B. burgdorferi* interactions with HUVEC and H4 cells. Recently we demonstrated that *bba64* was necessary for mammalian infection via tick transmission, and that *bba64* is upregulated in ticks during bloodmeal ingestion (Gilmore et al., 2010; Patton et al., 2011). In addition, we have shown *bba64* expression in mouse tissues throughout persistent infection, suggesting a role in host pathogenesis, possibly by functioning in the establishment and maintenance of the organism in various tissue (Gilmore et al., 2007, 2008). The upregulation of *bba64* expression when coincubated with human cells implicates BBA64 as a multifunctional protein involved in tick transmission and mammalian host cell interactions.

Previous studies have implicated OspA in cell adherence even though *ospA* is downregulated in the tick during feeding, and is not expressed in the host in the acute phases of infection. Rupprecht et al. (2006) demonstrated that OspA from *B. garinii*, a *B. burgdorferi sensu lato* strain most frequently associated with Lyme neuroborreliosis in Europe, mediates adherence to neuronal cells *in vitro*. Also, Comstock et al. (1993) showed that a mAb against OspA could inhibit borrelial association with HUVECs. In our study, *ospA* was expressed, and significantly upregulated early in response to HUVEC interaction, and at 8-h post-inoculation in H4 cells. The early expression of *ospA* may reflect an unknown function relating to host cell attachment, with downregulation of expression later after the spirochetes have established cellular colonization. However, because *ospA* is minimally or not expressed at the time of mammalian entry, the role of OspA in mediating cellular interactions remains to be determined.

Generation of *B. burgdorferi* mutant isolates, whereby *ospA*, *dbpA*, and *bba64* have been inactivated, have led to studies to define the role of these genes in host infectivity and pathogenesis. Attenuated tissue colonization and host persistence has been shown in mice injected with mutant *B. burgdorferi* lacking *dbpA*, and decorin-deficient mouse strains demonstrated resistance to experimental infection by both needle and tick bite (Brown et al., 2001; Blevins et al., 2008; Shi et al., 2008a; Weening et al., 2008). In contrast, *ospA*- and *bba64*-knockout mutants (when introduced by needle inoculation) were not impaired in their ability to infect or persist in mice (Yang et al., 2004; Battisti et al., 2008; Maruskova et al., 2008; Gilmore et al., 2010). Despite being dispensible for mouse infection via needle inoculation of cultured organisms, *bba64* is highly expressed by wild-type organisms in several tissues during persistent infection *in vivo* (Gilmore et al., 2008). It is becoming clear that *B. burgdorferi* binding and colonization of host cell tissues is a complex process mediated by numerous adherence components produced by *B. burgdorferi*, which may contribute to functional redundancy during infection. Perhaps such redundancy provides a survival advantage to host-adapted *B. burgdorferi* to evade host immunity, or a combination of proteins are regulated to work synergistically to optimally colonize the host.

In conclusion, we have demonstrated that antibodies directed against OspA, DbpA, and BBA64 can significantly reduce the binding ability of *B. burgdorferi* to human endothelial and neuroglial

cells. Additionally, we have shown that *ospA*, *bba64*, and *dbpA* are expressed and upregulated during times of *B. burgdorferi*-host cell interactions *in vitro*. Collectively, these findings infer that these outer surface proteins contribute to borrelial attachment and host cell colonization. Future studies will address the mechanisms employed by these gene products in cellular infection by utilizing mutant strains with inactivated *dbpA*, *bba64*, *ospA* to interface with human cells in tissue culture. Additionally, we seek to identify novel *B. burgdorferi* genes that are upregulated during

interactions with human cells as a means to understand and characterize the response of this pathogen to host components during infection.

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