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Original Article

Enhanced Adhesion and OspC Protein Synthesis of the Lyme Disease Spirochete *Borrelia Burgdorferi* Cultivated in a Host-Derived Tissue Co-Culture System

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ABSTRACT

Background: The adhesion process of *Borrelia burgdorferi* to susceptible host cell has not yet been completely understood regarding the function of OspA, OspB and OspC proteins and a conflict exists in the infection process.

Aims: The adhesion rates of pathogenic (low BSK medium passaged or susceptible rat joint tissue co-cultivated) or non-pathogenic *Borrelia burgdorferi* (high BSK medium passaged) isolate (FNJ) to human umbilical vein endothelial cells (HUVEC) cultured on coverslips and the synthesis of OspA and OspC proteins were investigated to analyze the infection process of this bacterium.

Study Design: In-vitro study.

Methods: Spirochetes were cultured in BSK medium or in a LEW/N rat tibiotarsal joint tissue feeder layer supported co-culture system using ESG co-culture medium and labelled with 3H-adenine for 48 hours. SDS-PAGE, Western Blotting, Immunogold A labeling as well as radiolabeling experiments were used to compare pathogenic or non pathogenic spirochetes during the adhesion process.

Results: Tissue co-cultured *B. burgdorferi* adhered about ten times faster than BSK-grown spirochetes. Trypsin inhibited attachment to HUVEC and co-culture of trypsinized spirochetes with tissues reversed the inhibition. Also, the synthesis of OspC protein by spirochetes was increased in abundance after tissue co-cultures, as determined by SDS-PAGE and by electron microscopy analysis of protein A-immunogold staining by anti-OspC antibodies. OspA protein was synthesized in similar quantities in all *Borrelia* cultures analyzed by the same techniques.

Conclusion: Low BSK passaged or tissue co-cultured pathogenic Lyme disease spirochetes adhere to HUVEC faster than non-pathogenic high BSK passaged forms of this bacterium. Spirochetes synthesized OspC protein during host tissue-associated growth. However, we did not observe a reduction of OspA synthesis during host tissue co-cultivation *in vitro*.

Key Words: Adhesion, *Borrelia burgdorferi*, OspA, OspC, pathogenicity, cell culture, HUVEC

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Introduction

One of the most intensely studied components of *Borrelia burgdorferi* in the pathogenesis of Lyme disease is the outer surface proteins (Osp) and their role in the infection process. It has been found that the synthesis of OspA and OspC changes during the infection cycle between the tick vector and animal host. There is an increase in the synthesis of OspC and a decrease in OspA proteins during tick feeding (1-5). OspA is synthesized in Barbour, Stoenner, Kelly's medium (BSK) culture of *B. burgdorferi* except a naturally occurring mutant that lacks this protein. There is a report regarding the relationship between the infectivity of *B. burgdorferi* 297 and abundant expression of OspC in low BSK passage cultures (6). It has been found that *ospC* and *revA* genes encode products that functioned in establishing mammalian infection and in fibronectin-binding, respectively (7). Grimm et al. analyzed a *B. burgdorferi* mutant lacking OspC, an abundant Osp that spirochetes normally synthesize in the tick and down-regulate after transmission to the mammal. They demonstrated that *B. burgdorferi* strictly requires OspC to infect mice but not to localise or migrate appropriately in the tick (7). An examination of the paradigm for reciprocal regulation of *ospA* and *ospC* revealed that the heterogeneous expression of OspA and OspC by spirochete populations during the tick blood meal results from the intricate order of transcriptional and translational changes that ensue as *B. burgdorferi* transitions between the vector tick and mammalian host (8). Brisette et al. found that also RevA is produced during mammalian infection but not during colonisation of vector ticks (9). A proteome study demonstrated that the response of *B. burgdorferi* to changing *in vitro* culture conditions. Information acquired from different culture conditions indicated that systemic adaptations of the proteome occur as *B. burgdorferi* moves from one environment to another. A significant increase in the abundance of many outer surface exposed proteins (OspC, LA7, P66, P83 and P35 antigens) was observed in this study (10). The aim of our study was to investigate the

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influence of host tissues on the infection process, especially adherence of pathogenic or non-pathogenic *B. burgdorferi,* with emphasis on the synthesis of OspA and OspC. In the first part of our studies, we compared the adhesion rates of *B. burgdorferi* Freehold, New Jersey (FNJ) strain grown in BSK medium or in a rat joint-derived tissue co-culture system designed to demonstrate the effects of host tissues on the biology and pathogenesis of this spirochete (11). This co-culture system contains Lewis/N (LEW/N) rat joint tissue as a feeder layer and Ece Sen Growth medium (ESG) medium which supports the growth of the feeder layer and *B. burgdorferi* simultaneously. However, ESG medium does not support the growth of *Borrelia* without the feeder layer; the growth of *B. burgdorferi* is tissue feeder layer-dependent in the co-culture, mimicking the host tissues without inhibitory components of the immune system of the host.

Previously, we have demonstrated that *B. burgdorferi* (FNJ) cultivated in LEW/N rat joint tissue co-culture retained pathogenicity and infectivity even after multiple passages (11). Also, we have reported the complement resistance of tissue co-cultured *B. burgdorferi* 297 strain in contrast to BSK medium grown spirochetes which did not survive antibody-dependent, complement-mediated lysis after incubation with an immune serum and guinea pig complement (7). These previous findings on the pathogenesis of *B. burgdorferi* grown in association with host-derived tissues led us to compare the adhesion potential of rat joint tissue co-cultured and BSK grown spirochetes to Human Umbilical Vein Endothelium Cells (HUVEC). It has been shown that *B. burgdorferi* cultured in BSK can attach to and translocate across cultured HUVEC (12-16). In our study, we compared the differences in the attachment rates of Lyme disease spirochetes grown in a host tissue-associated environment or BSK medium to HUVEC monolayers, since adherence to endothelium is a critical step for haematogenous dissemination of *B. burgdorferi* and pathogenesis of this infection.

We also investigated the synthesis of OspA and OspC in *B. burgdorferi* during host tissue associated growth and after attachment to endothelium. According to the previous reports, *B. burgdorferi* produces OspA but not OspC in the tick vector before engorgement and the synthesis of OspC increases during blood feeding along with the shedding of OspA from the outer membrane (3). During Lyme disease, antibodies to OspA are only weakly present in the early response, but antibodies to OspC are prominent in the host (5). However, in BSK cultured spirochetes, OspC mRNA and protein expression could not be detected by reverse transcription polymerase chain reaction or indirect immunofluorescence techniques, proving that presence of host tissues may up-regulate the synthesis of this protein (13). Also, animal studies accompanied by PCR amplification of mRNA and by direct immunofluorescence have been shown that there was OspC expression of *B. burgdorferi* in the mouse model of the infection (17). We tested the hypothesis that the OspC production is stimulated if the susceptible host tissues are added into the growth environment of *B. burgdorferi.* We also studied the synthesis of OspA protein during host tissue-associated growth and searched for clues of reciprocal or simultaneous synthesis of these proteins. The results presented here extended the contention that we derived from our previous reports; the biology of Lyme disease spirochete is greatly influenced by the presence of the host tissues in the growth environment. These findings should be taken into consideration in the investigation of pathogenicity of *B. burgdorferi* and in the design of vaccines against Lyme borreliosis.

Material and Methods

LEW/N Rat Joint Tissue Feeder Layer Associated Co-Culture of *B. burgdorferi*

B. burgdorferi FNJ strain was isolated from an *Ixodes ricinus* tick and passaged in BSK medium twice before the study. A non-pathogenic form of FNJ was passaged (42 X) in BSK, having lost arthritogenicity at the 7th passage as determined by LEW/N rat bioassay (18). Preparation of LEW/N tibiotarsal joint tissue feeder layers and co-cultures was described previously; in brief, rat tibiotarsal joint tissue monolayer cells in 6% FBS complete medium were grown at 33°C, 5% CO_2, refreshed with ESG without antibiotics and sera for 7 days. Prior to inoculation, the medium was removed, 10^4 borrelia/mL was added to the flask, and the co-culture was incubated at 33°C until the spirochetes reached to late exponential phase, as determined by the use of a reference standard growth curve relating the A_{600} to the number of spirochetes counted under the microscope.

HUVEC Culture

HUVEC (Clonetics San Diego, CA, USA) was cultured in endothelial growth medium (EGM) supported with epidermal growth factor (5 ng/mL), hydrocortisone ($1.4x10^{-6}$M) (Clonetics) and 2% FBS (Sigma) on coverslips placed into flat-sided tissue culture tubes (Nunc, Denmark). $2x10^4$ HUVEC/coverslip were seeded where monolayers formed overnight at 37°C with 5% CO_2. 95% of the cells were viable, as determined by trypan blue staining.

Virulence Determinations

0.1 mL of low or high passage spirochetes grown in BSK medium or in LEW/N rat joint tissue co-cultures were I.P. injected into two day-old LEW/N rats (HSD-Indianapolis, Indiana). Swelling of tibiotarsal joints, difficulty in walking and limping 21 days post-injection were considered as the signs of experimental Lyme disease. All animal use protocols were approved by the accredited animal care and use committees at UMDNJ-RWJ Medical School.

Radiolabelling of *B. burgdorferi*

Spirochetes were grown in BSK or tissue co-cultures until they reached the mid to late log phase as determined by the corresponding bacterial numbers on the growth curve. *Borrelia* were harvested by centrifugation at 9000 x g for 30 min and washed in sterile phosphate buffered saline (PBS, pH: 7.4) (3X). Spirochetes were resuspended in PBS and the cell density was adjusted to 10^8/mL by using a reference standard curve relating the A_{600} to the number of the spirochetes, as determined by direct dark field microscopic counts.

Radiolabelled *B. burgdorferi* strains were prepared by growth in BSK medium or in tissue co-cultures supplemented with ^{3}H-Adenine (15 μCi/mL) (sp.act. :1 mCi/mL) (ICN, Costa Mesa, CA) for 48h. Briefly, spirochetes were pelleted by centrifugation, numbers were adjusted to 5X10^8 bacteria/mL and added into four mL of ^{3}H-adenine supplemented BSK and tissue co-cultures. After 48h of incubation at 33°C, cultures were washed and concentrated by centrifugation twice (15 min at 9000 rpm). *B. burgdorferi* cannot grow in ESG co-culture medium without a tissue feeder layer; to determine if ESG had an adhesion-enhancement effect, spirochetes were washed with fresh ESG medium without supplements twice, following pelleting by centrifugation. Spirochete numbers were adjusted to 5x10^8 spirochetes/mL in ESG with ^{3}H-adenine (15 μCi/mL) and incubated for 48 h at 33°C. After labelling, *Borrelia* numbers were adjusted to 1x10^8 bacteria/mL in fresh ESG and 200 mL of spirochete suspension were applied on HUVEC monolayers for adhesion assays. Vigorous motility of spirochetes observed under the phase contrast microscope was considered as a sign of viability and 99% of the cultured *Borrelia* were motile in these experiments.

Adhesion assay

Supernatant of endothelial growth medium were discarded and HUVEC monolayers were rinsed once with fresh EGM without additives, lifted with PBS and washed with 10 mM EDTA. The suspension was diluted into HBSS (Hank's Balanced salt solution), and cells were pelleted by gentle centrifugation at RT. Sterile coverslips placed into flat-sided tissue culture tubes were seeded with 2x10^4 HUVEC/coverslip. Cells formed monolayers overnight in EGM supported with epidermal growth factor (5 ng/mL), hydrocortisone (1.4x10^{-6}M) (Clonetics) and 2% FBS at 37°C with 5% CO_2. After formation of the HUVEC monolayers on coverslips, supernatants of HUVEC were discarded and cell monolayers were rinsed once with fresh EGM without supplements. Spirochetes radiolabelled with ^{3}H-adenine were pelleted by centrifugation for 15 min at 9000 x g, washed with HBSS by centrifugation, the number of spirochetes were adjusted to 1x10^8 bacteria/mL and 200 mL aliquots were applied to confluent HUVEC monolayers grown on coverslips. Also, *B. burgdorferi* were resuspended in ESG medium and labelled before the adhesion assay by using the same protocol as BSK cultured spirochetes.

Borrelia labelled in rat tissue co-cultures were harvested from the supernatant by centrifugation at 9000 x g, washed with HBSS twice, and resuspended at a density of 1x10^8 bacteria/mL in HBSS. 200 mL aliquots of *Borrelia* suspension were applied to confluent HUVEC monolayers at 37°C; media were decanted in every ten min and coverslips were rinsed with HBSS (3X) to remove non-adherent spirochetes. HUVEC monolayers with attached spirochetes were lifted with trypsin (Sigma T-4424) (2.5 mg/mL of HBSS) for five min, 200 mL of this suspension was pipetted onto filter paper strips (Millipore, MA, USA) placed into scintillation tubes (ICN); five mL of liquid scintillation fluid (ICN) was added to each filter strip and the total released radioactivity in each sample was determined in a liquid scintillation counter (LKB Instruments, Inc., Gaithersburg, MD, USA). *Borrelia* binding to identically treated coverslips without HUVEC monolayer was determined, this value was less than 1% for each assay. Adhesion assays were repeated in triplicate. Slopes of adhesion values were calculated by using the formula $m=y_1-y_0/x_1-x_0$.

Determination of the Effect of Trypsin Pre-treatment of *B. burgdorferi* on the Adherence and Reversion of the Adhesion-Inhibitory Effects of Trypsin Digestion:

Low passage or high passage *B. burgdorferi* labelled with ^{3}H-adenine were grown in BSK or tissue co-culture as described previously. Spirochetes were enumerated, pelleted by centrifugation, resuspended in HBSS, then trypsin (Type IX) (2.5 mg/mL) (Sigma) was added to each pellet and incubated at 37°C for 30 min in sterile microcentrifuge tubes (Eppendorf) with gentle agitation. After incubation, samples were washed and resuspended in HBSS (3X). Adherence of trypsinised spirochetes to HUVEC grown on microscope coverslips were determined by scintillation as described previously. For the controls, spirochetes were grown in tissue co-culture or in BSK, labelled with ^{3}H-adenine but not trypsinized before the adhesion assay, and also, sterile HBSS without trypsin was used in some of the control cultures.

We also investigated the reversion of the adhesion-inhibitory effect of trypsin. High or low passage *B. burgdorferi* were cultured in BSK medium for five days, pelleted by centrifugation at 9000 x g for 15 min, resuspended at a density of 10^8 bacteria/mL in HBSS, and trypsinized (2.5 mg/mL) for 30 min at 37°C as described previously. *Borrelia* suspensions were pelleted by centrifugation at 9000 x g, then resuspended in HBSS (3x). Rat tibiotarsal joint tissue monolayers were cultured in ESG medium with 6% FBS without antibiotics in multi-well plates (Nunc) at 33°C with 5% CO_2. After the formation of cell monolayers, tissue cultures were seeded with 100 mL of trypsinized spirochete suspensions (1X10^5 *Borrelia*/mL) and co-cultures were fed with ESG without FBS. In a parallel assay, trypsin inhibitor (Clonetics) (1 mg/1.3 mg trypsin with 10,000 BAEE units/mg protein) was added (1:1 vol/vol) into the co-cultures, incubated at 33°C with 5% CO_2 for five days. *Borrelia* were radiolabelled with ^{3}H-adenine (15 μCi/mL) for 48 hrs as described previously. Adhesion rates of trypsinized spirochetes and also *Borrelia* which was co-cultured with rat tissues post-trypsinization to HUVEC monolayers (10^{7}spirochetes/coverslip were applied) were determined by scintillation counting, as described in the adhesion assay section.

Protein A-immunogold Staining for Demonstration of the OspC Protein by Electron Microscopy

The *B. burgdorferi* FNJ strain was cultured in rat joint tissue co-culture or in BSK medium. In the parallel experiments, BSK grown spirochetes were suspended in ESG for five days before immunogold staining and electron microscopy analyses. *Borrelia* were harvested by centrifugation at 9000 x g for 15 min, pellets were resuspended in 0.03 M PBS, spun down and fixed by using 0.6% tannic acid/1% glutaraldehyde for 30 min and pelleted post-fixation. Pellets were washed in PBS for ten min 2X, embedded into Epon/Araldite mixture in disposable BEEM capsules (EM Corp., USA) and incubated at 45°C for 24 h and at 60°C for 48h. For immunostaining, grids

were transferred through 0.02 M glycine in PBS ten min (3X), pre-immune serum at 1:1000 dilution in 1% BSA in 0.02 M PBS for 15-30 min (Cappel laboratories cat # 5011-1380, whole mouse IgG, Lot # 17049), 1% BSA in PBS for one min (4X), 1:10 to 1:100 dilutions of monoclonal anti-OspC antibodies (G7 utilized in the blotting experiments) in 1% BSA in PBS for 30 min, 1% BSA in PBS one min (4X), protein A gold (EM Corp) diluted 1:10 in 1% BSA in PBS for 30 min, 0.03 M PBS for five min 4X, distilled water one min 4X, uranyl acetate oxalate for five min in distilled water for one min , 2% uranyl acetate for two minutes in distilled water for one min, 4:1 3% polyvinyl alcohol/ 2% uranyl acetate for ten min in the dark. Samples were viewed by a Philips 420 TEM and photographed.

SDS-PAGE and Western Blotting

$1x10^8$ *B. burgdorferi* grown in BSK, on HUVEC following 5 days culture in ESG medium, or in tissue co-cultures centrifuged at 9000 x g for 15 min; the pellets were lysed in dissolving buffer (62.5 mM Tris-hydrochloride, pH: 6.8, 2% beta-mercaptoethanol, 10% glycerol, 2% SDS, and 0.02% bromophenol blue), boiled for 3 minutes under reducing conditions; 150 µg protein was loaded per well on a 12% gel, and SDS-PAGE was performed (19) (Bio-Rad, NJ, USA). In Western blotting, the concentrated anti-OspA MoAb of 6D6 hybridoma supernatant was used. The gels were semi-dry transferred onto nitrocellulose membrane overnight; the membrane was blocked with 3% non-fat dry milk, incubated with primary antibody and then HRPO conjugate for 1h at RT; the blots were detected using chemoluminescence substrate(Bio-Rad, NJ, USA).

Statistical Analyses

The statistical significance of differences in *Borrelia* binding to HUVEC monolayers after BSK or tissue co-culture growth or in the trypsin-treated and untreated cultures, also tissue co-cultured versus BSK cultured spirochetes post-trypsinization, were determined by two-tailed t-test analyses with Microsoft Excel software. Standard deviations and error bars were indicated as a function of the software.

Results

Determination of Virulence of *B. burgdorferi* Used in the Adhesion Assays

FNJ strain grown in the tissue co-culture was still pathogenic at the 42nd passage. The diameter of the tibiotarsal joints of control rats was 6±0 mm, arthritic joints were 7.22±0.22 mm in diameter ($p<0.04$). The high BSK passage FNJ strain failed to produce arthritis in rats after the 7th passage and these spirochetes did not regain pathogenicity for newborn rats when they were co-cultured with the rat joint tissues. Therefore, loss of pathogenicity was irreversible for long term BSK passaged *B. burgdorferi.*

Demonstration of Increased Adhesion Rate of Tissue Co-Cultivated of *B. burgdorferi*

We tested the hypothesis that the pathogenic *B. burgdorferi* FNJ strain (spirochetes tissue co-cultivated up to 42 times) binds more avidly to HUVEC than the non-pathogenic population of the same strain spirochetes. Also, adhesion values of *Borrelia* grown in BSK medium or rat joint tissue co-culture were compared. We observed a rapid, time-dependent binding of pathogenic and non-pathogenic *B. burgdorferi* to HUVEC; the radiometric measurements of spirochete adherence increased logarithmically as indicated by the increase in radioactivity during the assay. Pathogenic spirochetes grown in BSK adhered to HUVEC at a rate two times higher than non-pathogenic spirochetes. Calculated slope values were 15.8 for m_1 (low BSK cultured FNJ), and 9.7 for m_2 (non-pathogenic, high BSK cultured FNJ) ($p<0.05$). The most striking difference in adhesion rates were observed when spirochetes were grown in tissue co-cultures; both pathogenic and *Borrelia* co-cultivated with rat joint tissues adhered to HUVEC at a rate ten times higher than BSK medium grown *B. burgdorferi.* Slope value for pathogenic, tissue co-cultured *Borrelia* (m_3) was 107.9 and for non-pathogenic, tissue co-cultured *Borrelia* (m_4) was 95.7 ($p<0.01$), respectively (Figures 1a and 1b). Adhesion of spirochetes to HUVEC did not level off during the experiment. Since *B. burgdorferi* cannot grow in ESG tissue co-culture medium, we suspended pathogenic or non-pathogenic spirochetes in this medium, and labelled and determined the adhesion rates to HUVEC; slope values were 13.6 and 8.6 for pathogenic and non-pathogenic FNJ spirochetes, respectively. These values approached the adhesion rates of the BSK grown spirochetes without tissue co-culture monolayers, which demonstrated the fact that the ESG co-culture medium alone had no adhesion-enhancing effect on the binding of spirochetes to HUVEC (Data not shown on graph).

Inhibition of adhesion by trypsinization of *B. burgdorferi*

Trypsinization of both pathogenic and non-pathogenic *B. burgdorferi* before the adhesion assay inhibited their attachment to HUVEC. Adhesion was inhibited by 96-98% in pathogenic and 99% in non-pathogenic FNJ cultures compared to the non-trypsinized controls (Table 1). Trypsin did not impair viability of *B. burgdorferi* since 99% of spirochetes were motile after trypsinization.

Reversion of the inhibitory effect of trypsin on the adhesion of *B. burgdorferi*

We observed a significant increase in the adhesion of trypsinized spirochetes to HUVEC if they were co-cultured with rat joint tissues post-trypsinization. These spirochetes started to attach to rat joint tissue monolayers 18 hours after inoculation, as determined by phase contrast microscopy. *Borrelia* attached to the feeder layers primarily by their tips, then lengthwise as they continue to grow exponentially in tissue co-cultures. The addition of a trypsin inhibitor at the time of inoculation of trypsinized *B. burgdorferi* into the rat tissue co-cultures improved their growth, and later their adhesion to HUVEC. We also observed a significant increase in the adhesion of spirochetes which were co-cultured post-trypsinization. *B. burgdorferi* grown in BSK post-trypsinization also adhered to HUVEC but the number of bound spirochetes was lower than tissue co-cultured spirochetes. In conclusion, the adhesion rates of trypsinized spirochetes were increased if *Borrelia* were tissue co-cultured post-trypsinization (Figure 2).

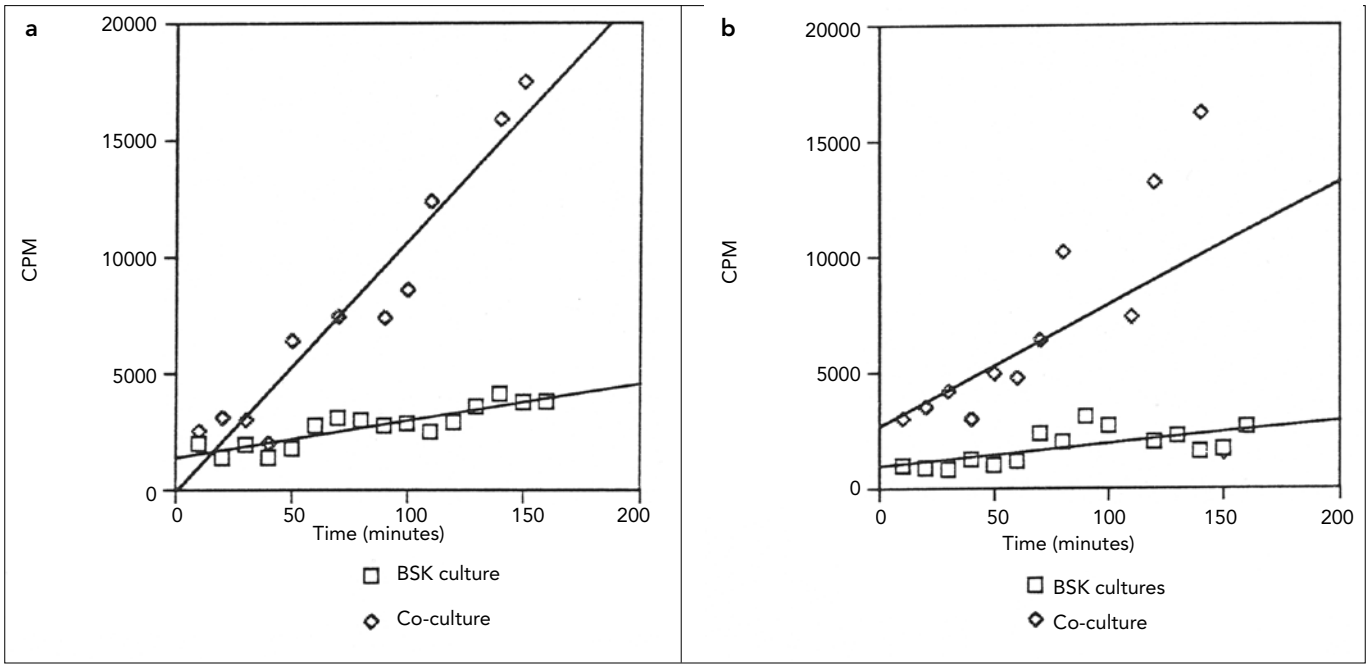

Figure 1 a, b. Adhesion rates of pathogenic B. burgdorferi FNJ (a) and non-pathogenic B. burgdorferi FNJ (b) to HUVEC

Table 1. Effects of trypsinisation on the adherence of *B. burgdorferi* to HUVEC

B. burgdorferi Culture & Treatment*	# of Bound Spirochetes**	% of Adhesion
Pathogenic, co-culture, w/o trypsin	2.4±0.18	100
Pathogenic, co-culture, trypsinized	0.096±0.007	4***
Non-pathogenic, co-culture, w/o trypsin	1.10±0.15	100
Non-pathogenic, co-culture, trypsinized	0.011±0.004	1***
Pathogenic, BSK, w/o trypsin	0.3±0.07	100
Pathogenic, BSK, trypsinized	0.006±0.00002	2***
Non-pathogenic, BSK, w/o trypsin	0.17±0.08	100
Non-pathogenic, BSK, trypsinized	0.0017±0.00003	1***

* ^{3}H-labelled B. burgdorferi (FNJ) grown in rat joint tissue co-culture or in BSK medium (with or without trypsin pre-treatment) were applied on HUVEC monolayers grown on microscope coverslips for two hours (10^{7} Borrelia/coverslip).

** Mean ± SD number of adhered spirochetes/coverslipx10-6

*** Significantly lower than control (p<0.0001).

Demonstration of OspC Protein in Tissue Co-Cultured Spirochetes by A-immunogold Staining

We observed that the material which accumulated around the spirochetes as a slime layer did not interfere with the binding of protein A gold particles onto the surface of tissue co-cultured spirochetes. OspC protein synthesis was observed only in tissue co-cultured spirochetes or *Borrelia* co-incubated with HUVEC cells for five days. In a parallel experiment, high BSK passaged non-pathogenic spirochetes were suspended in ESG medium for five days, then examined by immunogold labelling; these spirochetes did not synthesize OspC protein. These results suggest that the presence of host-derived tissues in the growth environment stimulates the synthesis of OspC protein by *B. burgdorferi*. Figure 3 demonstrates the longitudinal view of high BSK-passaged then HUVEC co-incubated *B. burgdorferi*; these spirochetes synthesized OspC protein as demonstrated by immunogold labelling with anti-OspC antibodies. We also examined the cross-sections of these spirochetes and observed 1.2 grain/section/spirochete (data not shown).

Demonstration of OspA and OspC Synthesis by SDS-PAGE and Western Blotting

The differences in the proteins of these *Borrelia* cultures were apparent at low molecular weight bands. We observed genus-specific protein OspA (31 kDa) in all of the cultures examined (Figure 4). Immunoblotting analyses done by using monoclonal antibodies also demonstrated reactivity with OspA protein in all of the cultures tested (Figure 5b). SDS-PAGE analyses confirmed the results of Protein A immunogold-staining experiments; OspC protein (23 kDa) was synthesized only in rat tissue co-cultured spirochetes (Figure 4,

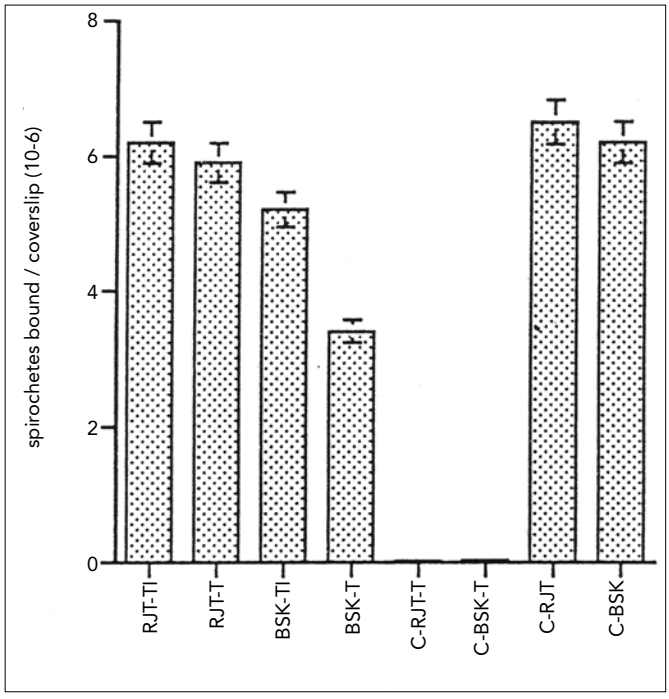

Figure 2. Reversion of the trypsin inhibition of adhesion of *B. burgdorferi* to HUVEC

(RJT-TI: rat joint tissue trypsin inhibitor; RJT-T: rat joint tissue trypsin; BSK-TI: Barbour, Stoenner, Kelly's medium trypsin inhibitor; BSK-T: Barbour, Stoenner, Kelly's medium trypsin; C-RJT-T: control rat joint tissue trypsin; C-BSK-T: control Barbour, Stoenner, Kelly's medium trypsin; C-RJT: control rat joint tissue; C-BSK: control Barbour, Stoenner, Kelly's medium)

Figure 5a). We did not observe the 23 kDa protein band in BSK cultures or spirochetes suspended in fresh ESG medium if there was no host-derived tissue culture monolayer in the cultivation environment.

Discussion

Adhesion has been considered as an important step in the pathogenesis of infectious microorganisms (20). Adherence of high passage B31 was reduced compared to low passage isolates (13, 14). We found that pathogenic FNJ had an adhesion rate twice as high compared to the non-pathogenic, of FNJ strain in our study. The decreased ability of, non-pathogenic *B. burgdorferi* to adhere to endothelium monolayers is in agreement with the premise that loss of infectivity may be due to decreased binding to host cells in that long-term *in vitro* culture in BSK medium results in the loss of infectivity in animals (21, 22). We observed the greatest difference in adhesion rates between tissue co-culture system grown and BSK medium cultured *Borrelia.* Both high and low passage spirochetes grown in the rat joint tissue co-culture system adhered to HUVEC monolayers at a rate that was ten times higher than BSK medium cultured *B. burgdorferi.* Host-derived tissues may provide necessary precursors to synthesise adhesion proteins and increase efficiency of binding of the spirochetes to HUVEC monolayers. Alderete, Baseman and Thomas have found host proteins on the surface of virulent *Treponema pallidum*;

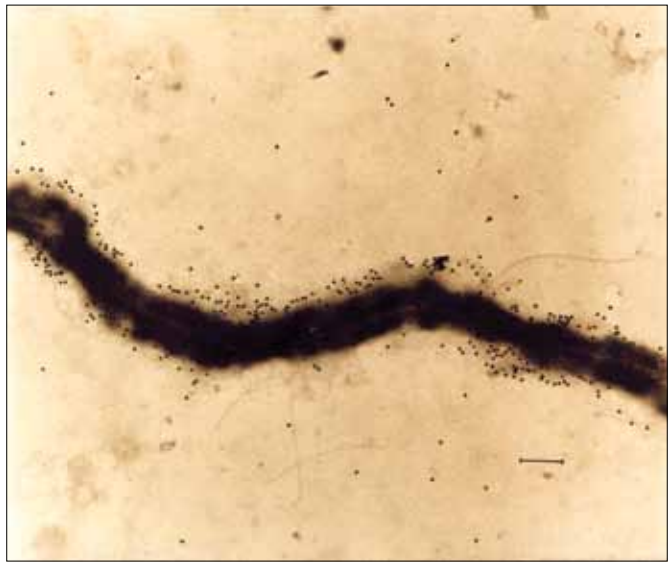

Figure 3. Protein A-immunogold labelling by using anti-OspC monoclonal antibodies (G7)

virulent *Treponemes* had the ability to adsorb host proteins on their surface (14, 23-26).

It has been found that anti-fibronectin antibodies inhibit the adhesion of *B. burgdorferi* to the matrix of endothelial cells (14). Also, in our former studies, we have found that the addition of fibronectin (75 mg/mL) into BSK medium during the growth of *B. burgdorferi* inhibited complement-mediated, antibody-dependent lysis of this spirochete, which might have resulted from coating of the surface of the spirochete by fibronectin and inhibition of complement and /or antibody binding to the surface of the spirochete before formation of the membrane attack complex (MAC) (11). Comstock and Thomas found that *B. burgdorferi* grown in BSK adhered to HUVEC and protease treatment significantly inhibited their attachment (12). In our study, we demonstrated that the adhesion of *B. burgdorferi* grown in association with the host-derived tissue layer to HUVEC was inhibited by trypsin 96-98% treatment. Also, in another study, treatment of *Borrelia* with trypsin-inhibited adherence by 65% compared to non-trypsinized spirochetes (15, 27).

The study of Kurtti et al. (28) found that the adhesion of low passage, hamster-infective strains of *B. burgdorferi* (JMNT and CD16) to RAE-25 cell line was higher than the adhesion of highly passaged, non-infectious strain B31. In our study, we tested FNJ strain of *B. burgdorferi*; in order to avoid strain-specific differences in the adhesion of different *Borrelia* strains, we examined the adhesion rates of both low or high passage stages of this strain, and found that infectious, low passage forms of these spirochete adhered to HUVEC at a rate twice as high compared to, high BSK passaged cultures.

The role of the OspC protein in the adhesion of *B. burgdorferi* to mammalian host tissues has been examined by other investigators that the synthesis of this protein was increased during the transmission of this spirochete to the host. Gilmore et al. (29) showed that *B. burgdorferi* may be transmitted to the host efficiently if this protein is synthesized by

the spirochetes. It has been demonstrated that *B. burgdorferi* isolated from ticks fed on humans can express both OspA and OspC proteins and that regulation of the expression of these proteins is phenotypically different (31-32). Human isolates of *B. burgdorferi* contain this protein in contrast to tick isolates which only synthesize OspA and OspB proteins. The up-regulation of OspC synthesis during infection in the host is in agreement with our *in vitro* experiments, which demonstrate that the host-tissue-associated growth also causes expression of the OspC protein, and this protein is synthesized during the association of the spirochetes with the endothelium before haematogenous dissemination *in vivo*. In our study, high BSK passaged spirochetes also synthesized OspC protein when they were co-cultured with host-derived tissues even though they lost pathogenicity for newborn rats. However, , high passage spirochetes did not express OspC protein when they were grown in artificial BSK medium. This protein might be important in infecting host tissues, consequently, adherence to the endothelium might be increased. However, virulence factors of *B. burgdorferi* responsible for development of the signs of the Lyme disease might be different from the factors responsible for infecting the host tissues such as adhesions of this bacterium. Masuzawa et al. (6), found a relationship between infectivity of low passage *B. burgdorferi* strains, 297, HP1, 12-92 and OspC expression. Non-pathogenic, *B. burgdorferi* infection rate in mice was very low and showed reduced expression of OspC after growth in BSK medium in contrast to low passage spirochetes. Also, isolates from the bladders of mice inoculated with high passage spirochetes expressed larger amounts of OspC compared to the BSK medium grown original inoculum, which suggests that the expression of this protein was up-regulated in *B. burgdorferi* passaged in the host. Therefore, these studies and our results indicate that both *in vivo* growth and *in vitro* host-derived tissue co-culture of *B. burgdorferi* may provide a suitable environment for up-regulation of OspC synthesis.

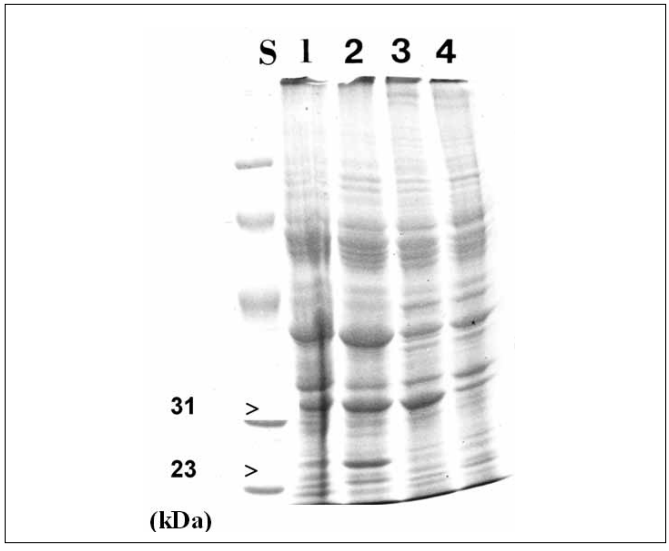

Figure 4. SDS-PAGE analyses of *B. burgdorferi* grown in tissue co-cultures or BSK medium. Lanes: 1) Rat joint tissue co-cultured spirochetes; 2) Spirochetes co-incubated with HUVEC; 3) Fresh ESG medium suspended spirochetes; 4) BSK cultured spirochetes.

Animal studies done by using the murine model of experimental Lyme borreliosis indicated that antibodies to OspC were prominent during the early course of the disease. Direct fluorescence antibody staining of uncultured spirochetes from spirochetemic mice revealed that the synthesis of OspC occurs during the infection. OspA synthesis was detected during the first two weeks and seized 30 days post-infection, suggesting that the expression of this protein is transient (13). These *ex vivo* analyses of uncultured spirochetes are in agreement with our tissue co-culture experiments; mammalian tissue co-cultured spirochetes synthesized OspC and OspA proteins similar to the initial stages of experimental infection in animal models.

Regarding to the role of OspC protein in the infection process of *B. burgdorferi* in humans and animals, there were other studies; Researchers have studied the molecular mechanisms involved in vascular interactions of the Lyme disease bacterium *in vivo (32)*. They engineered a fluorescent strain of *B. burgdorferi,* and visualised its dissemination from the microvasculature of living mice using intravital microscopy. They observed that the ability to interact with the microvascular endothelium was specific to infectious spirochetes. When mice were injected with non-infectious *B. burgdorferi* exhibiting the same fluorescence intensity as infectious spirochetes transient interactions were reduced by 94%. Furthermore, non-infectious

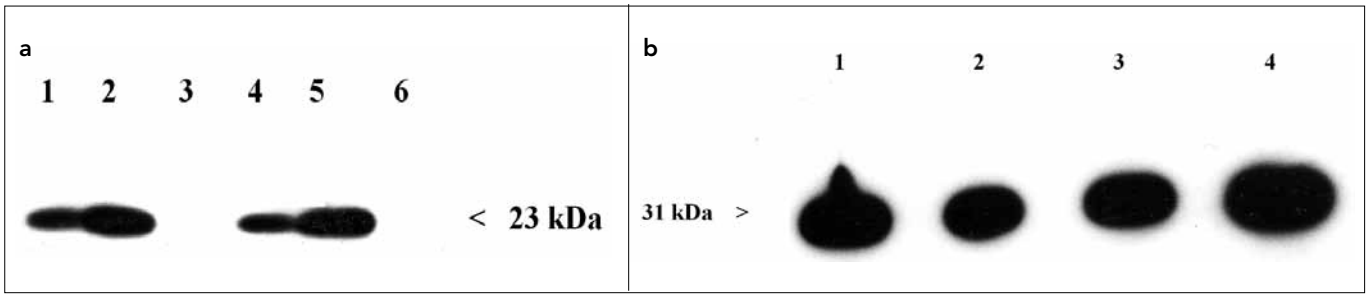

Figure 5 a, b. (a) Western blotting results of tissue co-cultivated or BSK medium grown B. burgdorferi. Lanes: 1,4) HUVEC co-cultivated spirochetes; 2,5) Rat joint tissue co-cultivated spirochetes; 3) Fresh ESG (tissue co-culture medium without tissues); 6) Spirochetes grown in BSK medium withoutthe presence of tissues. Lanes 1,2 and 3 contained high BSK, Lanes 4, 5 and 6 contained low BSK, passaged B. burgdorferi prior to HUVEC or rat joint tissue co-cultivation. (b) Western blotting results of tissue co-cultured and BSK cultivated high passage spirochetes. Lanes: 1) Rat joint tissue cocultured spirochetes; 2) Spirochetes co-incubated with HUVEC 3) Fresh ESG medium suspended spirochetes; 4) BSK cultured spirochetes.

B. burgdorferi did not form many dragging interactions and no detectable stationary adhesions; non-infectious spirochetes were not observed escaping the microvasculature. These observations indicated that early-stage interaction events were essential for sustained association and vascular escape. These observations also demonstrated that microvascular interactions were dependent on *B. burgdorferi* proteins expressed only in the infectious strain (32). These findings are in agreement with our adhesion assay; infectious spirochetes bind more avidly to HUVEC monolayers.

OspC production begins in feeding ticks (or immediately after needle inoculation), and lasts for the first couple of weeks of mammalian infection (17, 30, 33-36). Once the bacteria are established in a host, OspC production is not required for persistence (37). The OspC types of human clinical isolates have also been correlated with invasive and non-invasive phenotypes (38, 39). However, similar studies demonstrated greater diversity among invasive types than previously recognised, calling into question any causative effect of OspC sequence on invasion (40, 41). Carefully controlling for variables other than OspC has not been possible in such studies, since additional genome components of these isolates also differ. Recently, the invasive OspC protein types were shown to bind plasminogen (39), a trait shared with other *B. burgdorferi* proteins (42, 43), which was suggested to facilitate *B. burgdorferi* infection of mammals and ticks (2).

B. burgdorferi produces a number of products that allow it to colonize and persist in its natural mammalian and tick hosts. Although the functions of only a few *B. burgdorferi* products have been clearly defined, some (such as OspC) are required for the bacteria to survive the initial attack of the mammalian innate immune system (44-50). It has been found that OspC is a dissemination-facilitating factor of *Borrelia burgdorferi* during mammalian infection (51). These research findings suggest that *Borrelia burgdorferi* significantly up-regulates OspC in response to fresh bloodmeal during transmission from the tick vector to a mammal, and abundantly produces the antigen during the early infection of the mammalian host. As OspC is an effective immune target, to evade the immune system *B. burgdorferi* down-regulates the antigen once the anti-OspC humoral response has developed, suggesting an important role for OspC during early infection (51). Our data supports their findings in that the Osp C synthesis is up-regulated during the early mammalian infection. Therefore, our findings indicated that this protein is synthesized by the Lyme disease spirochete both *in vivo* and *in vitro* when they encounter the mammalian host tissues. We also observed OspA protein on the surface of the spirochete; therefore, the synthesis of OspC might be predicting the synthesis of other adhesion-related proteins during the onset of the infection to facilitate spirochete binding to the mammalian host tissues.

The duration of co-incubation of spirochetes with host tissues also has some issues; researchers analyzed *B. burgdorferi* surface proteins as determinants in establishing host cell interactions (52). In their study, pathogenic spirochetes were grown in BSK medium prior to the HUVEC adhesion assay. Spirochetes were co-incubated with HUVEC overnight and the effects of anti-Osp antibodies on the adhesion to HUVEC were determined. However, in our study, we co-cultured *B. burgdorferi* with tibiotarsal joint tissue more than 40 passages (42 weeks) or co-incubated with HUVEC for five days before the HUVEC adhesion assays; we observed avid attachment and OspC synthesis in tissue co-cultured spirochetes in contrast to BSK cultivated bacteria. *In vitro* long-term incubation or co-cultivation with susceptible host joint tissue induced OspC synthesis even in BSK-grown spirochetes, as we demonstrated in our study.

Comstock et al. (53) demonstrated that the fragment antigen binding (Fab) molecules generated from the immunoglobulin G fraction of rabbit anti-recombinant OspA serum inhibited the adherence of *B. burgdorferi* to HUVE cells by 73%. We observed OspA synthesis in rat joint tissue co-cultured or HUVEC co-incubated bacteria, as well as in BSK cultured spirochetes. Therefore, there is no conflict between our data and previous studies on adhesion of Lyme disease borrelia to HUVEC and concomitant Osp A synthesis. We could conclude these findings by stating that the synthesis of OspC protein could depend on the presence of susceptible mammalian tissues; however, OspA synthesis might occur without mammalian host tissues in the *in vitro* growth environment. Indeed, long-term artificial BSK medium grown or fresh ESG medium co-incubated *B. burgdorferi* did not display OspC on their surface in our study.

Former studies demonstrated that *B. burgdorferi* expresses OspA but not OspC in the midgut of unfed ticks. However, after ticks feed on blood, some spirochetes stop making OspA and express OspC. The work of Schwan et al. (54) examined the timing and frequency of OspA and OspC expression by *B. burgdorferi* in infected *Ixodes scapularis* nymphs as they fed on uninfected mice. Spirochetes were stained for immunofluorescence for detection of antibodies to OspA and OspC. Prior to feeding, spirochetes in nymphs expressed OspA but not OspC. During nymphal feeding, however, the proportion of spirochetes expressing OspA decreased, while spirochetes expressing OspC became detectable. In fact, spirochetes rapidly began to express OspC, with the greatest proportion of spirochetes having this protein at 48h of attachment. This study had proven the fact that the encounter of the spirochetes with human blood or tissues was necessary for the induction of OspC synthesis. Also, at least 48 hours were needed to observe maximum OspC synthesis after contact with human blood tissue. Therefore, overnight co-incubation with HUVEC cells in some studies might not be sufficient for the detectable production of OspC protein. We grew spirochetes up to 42 weeks or co-incubated them with host tissues for five days before the adhesion experiments and Osp protein analysis.

In a study performed by Ouyang et al. (55), the expression of *rpoS* and key lipoprotein genes regulated by RpoS, including *ospC*, *ospA*, and *dbpA* were examined throughout the tick-mammal infectious cycle of *B. burgdorferi*. Their data revealed that transcription of *rpoS*, *ospC*, and *dbpA* is highly induced in nymphal ticks when they take a blood meal.

Tilly et al. demonstrated that *Borrelia burgdorferi* OspC protein required exclusively in a crucial early stage of mammalian infection (56). They have also shown that spirochetes lack-

ing OspC are able to replicate in and migrate to the salivary glands of the ticks, but these spirochetes do not infect mice. They examined the timing of the requirement for OspC by using an ospC mutant complemented with an unstable copy of the ospC gene and showed that *B. burgdorferi's* requirement for OspC is specific to the mammal and limited to a critical early stage of mammalian infection when the spirochete encounters with the mammalian blood or tissues.

Bockenstedt et al. demonstrated that Osp C-mediated immunity in mice was strain-specific and differences in Osp C surface expression by spirochetes *in vivo* may account for strain-specific immunity (57). In our *in vitro* cultivations of spirochetes with susceptible host tissues, we utilised a different strain of *B. burgdorferi* (FNJ) to other researchers; the FNJ strain used in our study may continue OspC and OspA synthesis simultaneously during long-term tissue co-cultivation. Multiplicity of the parameters of growth conditions of Lyme disease spirochetes and strain-dependent variability of protein expression adds new challenges to the resolution of the issue of the sequence of synthesis of outer surface proteins as well as other adhesion-related components of *B. burgdorferi*.

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Informed Consent: *N/A.*

Peer-review: *Externally peer-reviewed.*

Author contributions: *Concept – E.Ş., L.H.S.; Design – E.Ş., L.H.S.; Supervision – E.Ş., L.H.S.; Resource – E.Ş., L.H.S.; Materials – E.Ş., L.S.; Data Collection&/or Processing – E.Ş., L.H.S.; Analysis&/or Interpretation – E.Ş., L.H.S.; Literature Search – E.Ş., L.H.S.; Writing – E.Ş., L.H.S.; Critical Reviews – E.Ş., L.H.S.*

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