Outer Surface Lipoproteins of *Borrelia burgdorferi* Activate Vascular Endothelium In Vitro

TIMOTHY J. SELLATI,^{1*} LORI DAWN ABRESCIA,¹ JUSTIN D. RADOLF,² AND MARTHA B. FURIE¹

Department of Pathology, School of Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8691,¹ and Departments of Internal Medicine and Microbiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235²

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Previously, we reported that activation of vascular endothelium by the Lyme disease pathogen *Borrelia burgdorferi* results in enhanced expression of endothelial cell adhesion molecules and promotion of the transendothelial migration of neutrophils in vitro. To investigate the role of spirochetal lipoproteins in this process, we assessed the ability of a synthetic lipohexapeptide corresponding to the N terminus of *B. burgdorferi* outer surface protein A (OspA) to activate human umbilical vein endothelial cells (HUVEC). Using a whole-cell enzyme-linked immunosorbent assay, we demonstrated that OspA lipopeptide activated endothelium in a dose-dependent fashion, as measured by upregulation of E-selectin. Near-maximal stimulation was achieved with 100 μ M lipopeptide. In addition, the lipopeptide increased expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1). Similar results were obtained with 25 nM native OspA or lipidated recombinant OspA or OspB. Incubation of HUVEC with nonlipidated OspA peptide, nonlipidated recombinant OspA or OspB, or tripalmitoyl-S-glyceryl-cysteine had little or no effect on expression of these adhesion molecules. A mutant strain of *B. burgdorferi* that lacked OspA and OspB upregulated expression of E-selectin to the same degree as its wild-type counterpart, indicating that other spirochetal components also possess the ability to activate endothelium. Conditioned medium from HUVEC incubated with OspA lipopeptide or lipidated recombinant OspA induced chemotaxis of neutrophils in Boyden chamber assays, whereas the OspA preparations alone were devoid of chemotactic activity. When HUVEC grown on connective tissue substrates were treated with OspA lipopeptide, subsequently added neutrophils migrated across the endothelial monolayers. These results implicate the outer surface lipoproteins of *B. burgdorferi* as potential effector molecules in the promotion of a host inflammatory response.

Lyme disease is caused by the arthropod-borne spirochete *Borrelia burgdorferi*. The histopathology of this chronic inflammatory disorder is characterized by variable infiltrates of neutrophils, macrophages, lymphocytes, and plasma cells (19). *B. burgdorferi* may elicit this perivascular inflammation through direct stimulation of endothelial cells lining the blood vessel walls (6, 46) and through induction of host-derived factors such as proinflammatory cytokines and chemokines (13, 26, 27, 38, 44, 52). As demonstrated by our laboratory and others, *B. burgdorferi* upregulates expression of adhesion molecules on the endothelium to promote the attachment (6, 46) and transendothelial migration (46) of leukocytes. Although these studies identified host cell surface molecules that might mediate recruitment of leukocytes into tissues infected with *B. burgdorferi*, they did not identify the spirochetal components responsible for activation of endothelium.

Given the diverse biological activities attributed to borrelial outer surface proteins (Osps) (3, 10, 11, 13, 22, 23, 33, 35, 49, 52), it seemed reasonable to hypothesize that Osps might also stimulate vascular endothelium to promote the extravascular accumulation of leukocytes. The Osps of *B. burgdorferi* are posttranslationally modified, membrane-associated lipoproteins which possess a tripalmitoyl-S-glyceryl-cysteine (3Pal-C) moiety at the N terminus (8). Two prominent lipoproteins, accounting for a significant proportion of the total protein of the organism, are OspA and OspB. OspA has been implicated

in binding of spirochetes to endothelial cells (11), can bind plasminogen (10, 23), and confers protective immunity against challenge with live organisms in animal models (22, 49). OspA and OspB also stimulate the polyclonal proliferation of murine and human B lymphocytes and induce cytokine production by human mononuclear and endothelial cells (13, 27, 35). Similar results have been obtained with a synthetic lipohexapeptide corresponding to the N terminus of OspA (14). This lipopeptide stimulates production of interleukin 1 β (IL-1 β), IL-6, IL-12, and tumor necrosis factor alpha by murine and human monocytes/macrophages (39) and causes perivascular inflammation upon intradermal injection in mice, with the initial cellular infiltrate being predominantly neutrophilic (36). The advantage to using lipohexapeptide analogs as surrogates for full-length lipoproteins lies in the ability to synthesize large quantities of these molecules under endotoxin-free conditions.

We report here that *B. burgdorferi* lipoproteins OspA and OspB, as well as a lipidated hexapeptide corresponding to the N terminus of OspA, activate monolayers of human umbilical vein endothelial cells (HUVEC) in a dose-dependent fashion. The consequences of this activation include increased expression of adhesion molecules for leukocytes, production of a chemotactic activity for neutrophils, and migration of neutrophils across the stimulated endothelium.

MATERIALS AND METHODS

Lipoproteins, LPS, and synthetic lipohexapeptides. Lipidated and unmodified hexapeptides corresponding to the N terminus of *B. burgdorferi* OspA (Cys-Lys-Gln-Asn-Val-Ser) or the N terminus of *Treponema pallidum* 17-kDa lipoprotein (Cys-Val-Ser-Cys-Thr-Thr), as well as 3Pal-C, were prepared as described (14). These peptides were stored in pyrogen-free H₂O and contained undetectable levels of endotoxin (<0.1 endotoxin unit [EU]/ml) as measured by the QCL-1000

* Corresponding author. Mailing address: Department of Pathology, SUNY at Stony Brook, Stony Brook, NY 11794-8691. Phone: (516) 444-3775. Fax: (516) 444-3419. Electronic mail address: tsellati@path.som.sunysb.edu.

quantitative, chromogenic *Limulus* amoebocyte lysate assay (BioWhittaker, Inc., Walkersville, Md.). Lipidated and nonlipidated recombinant OspA and OspB were generously provided by John J. Dunn (Brookhaven National Laboratories, Upton, N.Y.). Lipidated recombinant Osps (L-rOsps) were purified from *Escherichia coli* cells expressing the full-length B31 OspA or OspB gene, each of which included the 16-amino-acid lipoprotein signal sequence. *E. coli* lysates were subjected to Triton X-114 phase separation followed by passage of the detergent phase over columns of DEAE-Sepharose CL-6B and then S-Sepharose Fast Flow (21). Lipoproteins were stored in 50 mM NaH₂PO₄ (pH 8.0)–0.1% Triton X-100. Nonlipidated rOspA and rOspB were prepared as described previously (17) with truncated forms of the respective genes which lacked the first 17 codons specified by the wild-type structural gene. Nonlipidated proteins were purified by sequential passage over columns of Q-Sepharose, S-Sepharose, and hydroxyapatite. Nonlipidated OspA and OspB were stored in 10 mM NaH₂PO₄ (pH 6.0)–35 mM NaCl and 10 mM NaH₂PO₄ (pH 6.0)–5 mM NaCl with 0.1 mM phenylmethylsulfonyl fluoride, respectively. Native OspA (nOspA) lipoprotein, a gift from James L. Coleman (New York State Department of Health, Stony Brook, N.Y.), was affinity purified from *B. burgdorferi* B31 with monoclonal antibody (MAb) 11G1 directed against OspA, as previously described (2). Native and recombinant lipoprotein preparations contained low levels of endotoxin ranging from 0.5 to 1 EU/ml as measured by the QCL-1000 *Limulus* amoebocyte lysate assay. *E. coli* 0111:B4 LPS was obtained from Difco Laboratories (Detroit, Mich.).

Culture of spirochetes. *B. burgdorferi* strains HBD1 (46), virulent 297, and mutant 297 (30) were cultured at 33°C in serum-free Barbour-Stoenner-Kelly (BSK) medium as previously described (46). The virulent and mutant 297 strains were kindly provided by Russell C. Johnson (University of Minnesota, Minneapolis, Minn.) and underwent fewer than four passages in serum-free medium before use in experiments. Since virulence was not reassessed by our laboratory after *in vitro* culture, the virulent strain will be referred to as wild-type 297. As assessed by Western immunoblot analysis (30) with MAbs H5332, 84C, and 4B8F4, both wild-type 297 and HBD1 strains expressed OspA, OspB, and OspC, whereas the mutant 297 strain contained OspC but not OspA or OspB (data not shown).

Endothelial cell cultures. Endothelial cells were isolated from human umbilical veins by collagenase perfusion (29, 46). HUVEC were maintained in medium 199 (M199) (Life Technologies, Inc., Grand Island, N.Y.) containing 20% fetal bovine serum (FBS) (HyClone Laboratories, Logan, Utah), 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 2 µg of amphotericin B per ml at 37°C. After 3 to 5 days, cells from confluent cultures were trypsinized, pooled, and passaged onto microtiter plates or amnion for use in experiments. Second-passage endothelial cell cultures used in experiments were >97% pure, as determined by indirect immunofluorescence with an antibody to factor VIII antigen (29).

Cultures from several umbilical veins were combined to minimize variation among endothelial cells from individual donors, which may exhibit different levels of responsiveness to cytokines (4). The response of HUVEC to stimulation with 1 U of recombinant human IL-1β (Collaborative Biomedical Products, Bedford, Mass.) per ml was used as an internal positive control in the enzyme-linked immunosorbent assays (ELISAs). To normalize the data within and between experiments, results were expressed as a percentage of the response to IL-1β.

MAbs. MAbs H5332 (immunoglobulin G1 [IgG1]), 84C (IgG2b), and 4B8F4 (IgG2a), directed against OspA, OspB, and OspC, respectively, were from a panel of antibodies obtained through the Centers for Disease Control and Prevention (Fort Collins, Colo.) and were provided by Jorge L. Benach (New York State Department of Health, Stony Brook, N.Y.). MAb BB11 (IgG2b), directed against E-selectin (5), and MAb 4B9 (IgG1), directed against vascular cell adhesion molecule 1 (VCAM-1) (9), were generously provided by Roy R. Lobb (Biogen, Inc., Cambridge, Mass.). MAb R6.5 (IgG2a), directed against intercellular adhesion molecule 1 (ICAM-1) (50), was provided by Robert Rothlein (Boehringer Ingelheim Corp., Ridgefield, Conn.) and C. Wayne Smith (Baylor College of Medicine, Houston, Tex.). All MAb preparations were purified IgG.

Whole-cell ELISA. HUVEC from primary cultures were plated onto Falcon Primaria 96-well flat-bottomed microtiter plates (Becton Dickinson, Lincoln Park, N.J.) at a density of 2.5×10^4 cells per well. After 3 to 4 days, confluent monolayers were washed and incubated at 37°C with M199–20% heat-inactivated (heated for 30 min at 56°C) FBS (HIFBS) containing 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (pH 7.2), or preparations of OspA, OspB, or hexapeptide (100 µl per well) diluted in the same medium. Because of the presence of low levels of LPS contamination (≤ 1 EU/ml) in nOspA, rOspA, and rOspB preparations, these samples were treated with polymyxin B (20 µg/ml) (ICN, Cleveland, Ohio) for 1 h at room temperature before addition to HUVEC monolayers, and polymyxin B remained present during the incubation period. Levels of expression of E-selectin, VCAM-1, and ICAM-1 were determined with the MAbs BB11, 4B9, and R6.5 in a whole-cell ELISA as previously described (46). Assays for E-selectin were performed after HUVEC had been exposed to the various preparations for 4 h, whereas VCAM-1 and ICAM-1 were assessed after 12 h of treatment. These incubation periods yield maximal expression of these adhesion molecules when HUVEC are stimulated with intact, viable *B. burgdorferi* cells (46). The number and viability of HUVEC

were not significantly diminished after incubation with any of the test preparations for any length of time, as determined by direct counting of trypsinized cells with a hemacytometer and by trypan blue exclusion, respectively.

For experiments involving intact spirochetes, a BSK medium sham control was prepared by processing a volume of uninoculated BSK medium equal to the largest volume of spirochete culture used for the experiment. The sham was subjected to the same manipulations as the spirochetes themselves. This control was included to assess the possibility that exogenous LPS might account for the observed results.

Preparation of human amniotic membrane. Human placentas were obtained shortly after delivery, and the amnion was gently separated from the chorion. Teflon rings (SUNY at Stony Brook Instrument Shop) were fastened to the stromal surface of the amnion with Viton O-rings (C. E. Conover, Fairfield, N.J.). The epithelium was lysed with 0.25 N NH₄OH, and debris was removed by scraping to leave an acellular connective tissue matrix upon which endothelial cells were cultured. Before seeding of endothelial cells, the amniotic tissue was incubated with HUVEC growth medium for 2 h at 37°C (24, 25).

Neutrophil transendothelial migration assay. Neutrophils were isolated from venous blood of healthy adult volunteers by dextran sedimentation and density centrifugation with Ficoll-Hypaque gradient medium and resuspended in M199 containing either 2 or 20% HIFBS and 25 mM HEPES (pH 7.2) (46). This method yielded neutrophils that were >98% pure, as assessed by examination of stained cytospin preparations, and >95% viable, as determined by trypan blue exclusion. HUVEC-amnion cultures were prepared and maintained as previously described (46). HUVEC monolayers, cultured on amnion at a density of 1.5×10^5 cells per cm², were washed and incubated with 0.5 ml of M199–20% HIFBS containing 25 mM HEPES (pH 7.2) or various control and test preparations for 8 h at 37°C. After incubation, HUVEC were washed three times with M199–20% HIFBS. Neutrophils (0.4 ml at 2.5×10^6 cells per ml) were added and incubated with HUVEC monolayers for 30 min at 37°C. Unbound neutrophils were removed by aspiration, and tissues were fixed overnight in 10% buffered formalin at 4°C.

After fixation, samples were rinsed three times in 0.9% NaCl to remove nonadherent neutrophils. Tissues were cut out with a cork borer, stained with Wright stain, and viewed en face by light microscopy. The total number of neutrophils associated with each tissue was determined by counting nine randomly chosen, $\times 400$ fields. In addition, one replicate from each experimental group was embedded in glycol methacrylate (Polysciences, Inc., Warrington, Pa.) for cross-sectional analysis. Sections cut perpendicular to the plane of the HUVEC monolayer were examined by light microscopy to determine the percentage of neutrophils that migrated beneath the endothelium, as previously described (46). Experimental groups consisted of four to six replicates.

Boyden chamber assay. Blind-well Boyden chambers (Neuroprobe, Cabin John, Md.) were used to assess the chemotactic activity of medium conditioned by HUVEC incubated with control medium or various spirochetal preparations. Conditioned medium was prepared by incubation of HUVEC-amnion cultures (3×10^5 cells per ring) with 0.5 ml of M199–20% HIFBS in the absence or presence of 100 µM OspA peptide or OspA lipopeptide for 8 h at 37°C. Conditioned medium was also collected from HUVEC (2×10^6 cells per 60-mm-diameter plastic tissue culture plate) incubated for 24 h at 37°C with 5 ml of M199–2% HIFBS containing 25 nM nonlipidated rOspA or L-rOspA or 2×10^8 HBD1 spirochetes. OspA preparations were treated with polymyxin B (20 µg/ml) for 1 h at room temperature prior to incubation with HUVEC, and polymyxin B remained present throughout the incubation period. Medium was also harvested from an equivalent number of spirochetes cultured under the same conditions, but in the absence of endothelium. Media containing spirochetes were centrifuged at $20,000 \times g$ for 30 min, and supernatants were passed through a 0.20-µm-pore-size cellulose acetate syringe filter (Corning Glass Works, Corning, N.Y.). Aliquots were subcultured to confirm the absence of viable spirochetes.

Control or conditioned medium (115 µl) from experimental groups was placed in the lower compartment of the Boyden chamber. A 5-µm-pore-size cellulose nitrate filter (Sartorius, Edgewood, N.Y.) was placed over the lower compartment, and 50 µl of neutrophils (10^6 cells per ml) was added to the upper compartment. After incubation for 20 min at 37°C, filters were rinsed, fixed, and stained with Harris-modified hematoxylin (58). For each filter, five randomly chosen $\times 400$ light microscopic fields were examined to determine the greatest depth to which two neutrophils had simultaneously migrated. Conditioned medium from unstimulated HUVEC was used as a negative control, and the response of neutrophils to it was subtracted from the experimental values. Positive controls were prepared for experiments testing medium conditioned by spirochetes or lipopeptide by addition of the neutrophil chemoattractant C5a to M199 containing 2 or 20% HIFBS, respectively. All data from experimental groups, consisting of three replicates, are presented as the percent of the response to 1 nM C5a.

Statistics. With the InStat 2.01 statistical software package (GraphPad, San Diego, Calif.), the raw data of experimental groups from a whole-cell ELISA were subjected to an unpaired analysis of variance with the Tukey-Kramer multiple comparisons test. If the variance of the standard deviations (SD) was considered significant ($P < 0.05$), the nonparametric Kruskal-Wallis test was performed. The means $\pm$ SD of experimental groups from a Boyden chamber assay or transendothelial migration assay were subjected to an alternate Welch *t*

FIG. 1. Dose-dependent expression of E-selectin in response to OspA lipopeptide. Confluent HUVEC monolayers were incubated for 4 h with M199–20% HIFBS alone (Unstim), 200 μ M 3Pal-C, 200 μ M nonlipidated OspA hexapeptide (peptide), or lipidated OspA hexapeptide (lipopeptide) at concentrations ranging from 10 to 200 μ M. Expression of E-selectin then was measured by a whole-cell ELISA with MAb BB11. Positive controls were treated with 1 U of IL-1 β per ml for the same length of time. Samples designated No MAb were incubated with medium alone (i.e., without primary MAb) during the ELISA. Bars indicate the means $\pm$ SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. Results significantly different from those of the unstimulated (Unstim) control are shown by an asterisk ($P < 0.001$).

test to determine a one-tailed P value. $P < 0.05$ was used as the alpha value to determine statistical significance for all analyses.

RESULTS

Synthetic lipopeptides upregulate expression of E-selectin on endothelial cells. A whole-cell ELISA was used to analyze the upregulation of E-selectin on endothelial cells (46) in response to synthetic nonlipidated and lipidated hexapeptides corresponding to the N-terminal amino acid residues of *B. burgdorferi* OspA (14). Incubation of HUVEC with concentrations of lipopeptide ranging from 10 μ M (14.7 μ g/ml) to 200 μ M (294 μ g/ml) for 4 h induced expression of E-selectin in a dose-dependent fashion ($P < 0.001$) (Fig. 1). A half-maximal response was obtained with approximately 17 μ M lipopeptide, whereas 100 μ M lipopeptide was required to stimulate maximal expression of E-selectin ($76\% \pm 11\%$ of the response to 1 U of IL-1 β per ml). To determine the influence of amino acid sequence on the stimulatory activity of lipopeptides, HUVEC were incubated with a lipohexapeptide corresponding to the N terminus of the *T. pallidum* 17-kDa lipoprotein (1). At a concentration of 100 μ M (150.2 μ g/ml), this lipopeptide elevated the expression of E-selectin to $161\% \pm 8\%$ of the response to IL-1 β . In contrast, when HUVEC were incubated with nonlipidated OspA peptide (200 μ M, 132 μ g/ml) or 3Pal-C (200 μ M, 162 μ g/ml), no increase in the amount of E-selectin was observed (Fig. 1). Furthermore, simultaneous addition of nonlipidated OspA peptide and 3Pal-C (each at 100 μ M) failed to induce expression of E-selectin on endothelium (data not shown).

To examine the possibility that exogenous LPS could account for the observed results, HUVEC were incubated with peptide (100 μ M), lipopeptide (100 μ M), or *E. coli* 0111:B4 LPS (0.1 ng/ml) in the absence or presence of polymyxin B (20 μ g/ml). Whereas polymyxin B completely ablated the activity of LPS, it had no significant effect on the activity of OspA lipopeptide (Fig. 2). This result is in agreement with other reports that the activities of OspA lipopeptide are unaffected

by polymyxin B (15, 39). Moreover, peptide and lipopeptide preparations contained undetectable levels of LPS, as measured by a chromogenic *Limulus* amoebocyte assay.

Expression of E-selectin is enhanced in response to native and recombinant lipoproteins. To determine whether endothelium would respond in a similar fashion to full-length native or recombinant lipoproteins from *B. burgdorferi* B31, HUVEC were incubated with affinity-purified nOspA, L-rOspA, or L-rOspB at concentrations ranging from 1 nM (30 ng/ml) to 50 nM (1.5 μ g/ml). Near-maximal expression of E-selectin was achieved after incubation of HUVEC with 25 nM (750 ng/ml) native or recombinant lipoproteins for 4 h (Fig. 3). In contrast, when endothelium was incubated with 50 nM (1.5 μ g/ml) nonlipidated rOspA or rOspB, no increase in the expression of E-selectin was observed. As for the experiment shown in Fig. 2, nOsp and rOsp preparations were treated with polymyxin B (20 μ g/ml) prior to coincubation with HUVEC to inactivate LPS. Recombinant lipoprotein preparations contained a trace amount of detergent (0.0015% Triton X-100) after dilution. Therefore, control experiments were conducted to determine whether this concentration of detergent had any adverse effects in the whole-cell ELISA. These levels of detergent did not activate HUVEC, nor did they affect the responsiveness of endothelium to IL-1 β (data not shown).

Recent reports suggest that OspC, but not OspA or OspB, is expressed by *B. burgdorferi* in the mammalian host during the early stages of Lyme disease (20, 45). To determine whether the ability to upregulate E-selectin is limited to OspA and OspB, the stimulatory activity of mutant strain 297, which lacks OspA and OspB but expresses OspC (30), was compared with that of strains HBD1 and wild-type 297, both of which express all three of these proteins. Consistent with our previous findings (46), strain HBD1 significantly increased expression of E-selectin by HUVEC when as few as one spirochete per endothelial cell was added for 4 h (Fig. 4). The mutant 297 strain was as effective in this assay as the HBD1 and wild-type 297 strains, indicating that components of *B. burgdorferi* other than OspA and OspB may also activate endothelium.

FIG. 2. Effect of polymyxin B on the expression of E-selectin in response to OspA lipopeptide. Confluent HUVEC monolayers were incubated for 4 h with control preparations as described in the legend to Fig. 1, nonlipidated OspA hexapeptide (peptide), lipidated OspA hexapeptide (lipopeptide) (both at 100 μ M), or *E. coli* 0111:B4 LPS (0.1 ng/ml) in the absence or presence of 20 μ g of polymyxin B per ml. Expression of E-selectin then was measured by whole-cell ELISA. Bars indicate the means $\pm$ SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. NS, not significantly different. Results significantly different from those of the unstimulated (Unstim) control are indicated by asterisks ($P < 0.001$).

OspA lipopeptide and lipoprotein preparations stimulate expression of other adhesion molecules. Previously, we demonstrated that *B. burgdorferi* upregulates expression not only of E-selectin, but also of the adhesion molecules VCAM-1 and ICAM-1 (46). Therefore, we tested the ability of OspA lipopeptide to do the same. As seen in Fig. 5, incubation of HUVEC for 12 h with lipopeptide (100 μ M, 147 μ g/ml), but not with 3Pal-C or unmodified peptide, resulted in a significant

increase in the levels of VCAM-1 and ICAM-1 ($P < 0.001$). Expression of VCAM-1 and ICAM-1 was increased to $74\% \pm 3\%$ and $86\% \pm 16\%$ of the response to IL-1 β , representing a 2.3-fold increase and a 1.8-fold increase above unstimulated levels, respectively. In addition, 25 nM nOspA upregulated the expression of VCAM-1 to $85\% \pm 5\%$ and ICAM-1 to $103\% \pm 9\%$ of the response to IL-1 β , representing 1.7- and 1.8-fold increases above unstimulated levels, respectively. Comparable

FIG. 3. Upregulation of expression of E-selectin in response to nOspA, L-rOspA, or L-rOspB. Confluent HUVEC monolayers were incubated for 4 h with M199–20% HIFBS alone (U), nOspA, L-rOspA, or L-rOspB at concentrations ranging from 1 to 50 nM. In addition, HUVEC were incubated with 50 nM nonlipidated rOspA or rOspB or 0.1 ng of *E. coli* 0111:B4 LPS per ml in the absence or presence of 20 μ g of polymyxin B (PB) per ml for the same length of time. Native and recombinant lipoproteins were also incubated with polymyxin B (20 μ g/ml) for 1 h prior to addition to HUVEC monolayers. Expression of E-selectin was then measured by whole-cell ELISA. During the ELISA, samples designated C and U were incubated with medium alone or medium containing primary MAb, respectively. Bars indicate the means $\pm$ SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. Results significantly different from those of the unstimulated (U) control are indicated by asterisks ($P < 0.05$).

FIG. 4. Dose-dependent expression of E-selectin in response to *B. burgdorferi* mutant 297. Confluent HUVEC monolayers were incubated for 4 h with M199–20% HIFBS alone (U), a sham preparation made with uninoculated BSK medium (S) (see Materials and Methods), or *B. burgdorferi* HBD1, wild-type 297, or mutant 297 at ratios ranging from 1 to 100 spirochetes per endothelial cell (SP/C). Expression of E-selectin was then measured with a whole-cell ELISA. Bars indicate the means $\pm$ SD of 12 replicate samples per experimental group (4 from each of three independent experiments). Results significantly different from those of the unstimulated (U) control are indicated by asterisks ($P < 0.001$).

increases in expression were observed after incubation of HUVEC with 25 nM L-rOspA or L-rOspB (data not shown). Neither nonlipidated peptide, rOspA, nor rOspB stimulated increased expression of these adhesion molecules under similar conditions.

Activation of endothelium by OspA lipopeptide and lipoprotein results in the production of chemotactic activity for neutrophils. Upon activation by host-derived mediators of inflammation, including thrombin, cytokines, or bacterial products such as LPS (37), endothelial cells produce chemoattractants. Blind-well Boyden chamber assays were performed to determine whether incubation of HUVEC with OspA preparations resulted in release of chemotactic activity into the medium. Conditioned media were collected from HUVEC after their incubation with 3Pal-C (100 μ M, 81 μ g/ml), OspA peptide (100 μ M, 66 μ g/ml), or OspA lipopeptide (100 μ M, 147 μ g/ml) for 8 h or with rOspA or L-rOspA (both at 25 nM, 750 ng/ml) for 24 h. Neutrophils added to the upper compartment of the Boyden chamber were induced to migrate into cellulose nitrate filters in response to conditioned medium collected from HUVEC incubated with lipopeptide, which was placed in the lower compartment. However, migration of neutrophils in response to conditioned medium from HUVEC treated with 3Pal-C or unmodified peptide was not significantly greater than that in response to conditioned medium from unstimulated HUVEC (Fig. 6). Incubation of HUVEC with L-rOspA, but not its nonlipidated counterpart, also resulted in production of chemotactic activity (60% $\pm$ 2% of the response to 1 nM C5a). Neither OspA lipopeptide nor L-rOspA induced chemotaxis when tested in Boyden chamber assays at concentrations ranging from 1 nM to 100 μ M or 1 to 50 nM, respectively, indicating that the activity in conditioned medium was derived from endothelial cells. In other experiments, neutrophils migrated slightly in response to supernatant medium from *B. burgdorferi* HBD1 cultured in M199–2% HIFBS (18% $\pm$ 5% of the response to C5a) and more vigorously in response to conditioned medium from HUVEC incubated with spirochetes at a ratio of 10 organisms per endothelial cell (51% $\pm$ 3% of the response to C5a).

FIG. 5. Upregulation of expression of VCAM-1 and ICAM-1 in response to OspA lipopeptide. Confluent HUVEC monolayers were incubated for 12 h with control preparations (see legend to Fig. 1), nonlipidated OspA hexapeptide (peptide), or lipidated OspA hexapeptide (lipopeptide) (both at 100 μ M). Levels of VCAM-1 (A) and ICAM-1 (B) were assessed by whole-cell ELISA with the MAbs 4B9 and R6.5, respectively. Bars indicate the means $\pm$ SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. Results significantly different from those of the unstimulated (Unstim) control are indicated by asterisks ($P < 0.001$).

Preincubation of endothelium with OspA lipopeptide promotes the migration of neutrophils across HUVEC monolayers. Having demonstrated the ability of OspA lipopeptide to alter expression of adhesion molecules on endothelium and to

FIG. 6. Conditioned medium from HUVEC treated with OspA lipopeptide possesses chemotactic activity for neutrophils. Conditioned medium from HUVEC incubated with 3Pal-C, nonlipidated OspA hexapeptide (peptide), or lipidated OspA hexapeptide (lipopeptide) (all at 100 μ M) was tested for chemotactic activity in Boyden chambers. After addition of neutrophils, chambers were incubated for 20 min, and the extent of neutrophil migration was determined as described in Materials and Methods. Positive controls measured the response of the neutrophils to 1 nM C5a. Bars indicate the means $\pm$ SD of six replicate filters per experimental group (three from each of two independent experiments). Results significantly different from those of the unstimulated control are indicated by an asterisk ($P < 0.001$).

FIG. 7. Promotion of neutrophil transendothelial migration in response to OspA lipopeptide. HUVEC-amnion cultures were incubated with M199–20% HIFBS alone (Unstim), 3Pal-C, nonlipidated OspA hexapeptide (peptide), or lipidated OspA hexapeptide (lipopeptide) (all at 100 μ M) for 8 h and washed. Subsequently added neutrophils were incubated with the cultures for 30 min, and transendothelial migration was assessed as described in Materials and Methods. Bars indicate the means $\pm$ SD of four replicate cultures per experimental group from a representative experiment. This experiment was repeated once with similar results. Results significantly different from those of the unstimulated (Unstim) control are indicated by an asterisk ($P < 0.0001$).

stimulate the production of a chemoattractant(s) for neutrophils, we undertook experiments to determine the consequences of these phenomena. Since neutrophils do not associate with unstimulated HUVEC but will bind to and emigrate across spirochete-activated endothelium (46), we investigated whether stimulation of HUVEC grown on amniotic connective tissue with OspA lipopeptide would also promote the transendothelial migration of neutrophils. HUVEC-amnion cultures were incubated with 3Pal-C (100 μ M, 81 μ g/ml), OspA peptide (100 μ M, 66 μ g/ml), or OspA lipopeptide (100 μ M, 147 μ g/ml) for 8 h at 37°C. As seen in Fig. 7, preincubation of HUVEC-amnion cultures with lipopeptide promoted the migration of subsequently added neutrophils. In contrast, few neutrophils adhered or migrated when HUVEC cultures were incubated with growth medium alone, 3Pal-C, or nonlipidated peptide.

DISCUSSION

Our studies demonstrate that incubation of HUVEC with a synthetic, lipohexapeptide analog of *B. burgdorferi* OspA resulted in activation of the endothelium, as evidenced by the upregulation of E-selectin, VCAM-1, and ICAM-1. Increased expression of these adhesion molecules is also seen in response to intact or sonicated organisms both in vitro (6, 46) and in vivo (18, 43). Maximal levels of expression were achieved with 100 μ M lipopeptide. Similar increases in the expression of adhesion molecules were observed in response to 25 nM nOspA, L-rOspA, or L-rOspB. Whitmire and Garon (57) determined that 1 μ g (dry weight) of *B. burgdorferi* Sh-2-82 contains 0.1 μ g of protein, of which OspA and OspB constitute 0.0051 and 0.0063 μ g, respectively. On the basis of these data and the observation that 10^6 spirochetes yield approximately 1 μ g of protein (47), addition of 50 to 60 spirochetes per endothelial cell provides approximately the same amount of OspA or OspB as addition of 25 nM solutions of purified native or recombinant lipoproteins. Our previous work indicates that near-maximal expression of adhesion molecules is achieved after incubation of HUVEC with 10 organisms per endothelial cell (46). Assuming that OspA and OspB act in an additive manner, it thus appears that these two lipoproteins alone may account for a substantial portion of the stimulatory activity of intact *B. burgdorferi* in vitro. Compared with full-length OspA, considerably higher concentrations of OspA lipopeptide were required to attain comparable levels of expression of adhesion

molecules. Although the insoluble nature of the lipopeptide reagent might have resulted in diminished biological activity through aggregation, an alternative explanation is that portions of the full-length lipoprotein other than the six N-terminal amino acid residues are required to produce its full stimulatory effect.

Recent studies have demonstrated that *B. burgdorferi* in the midgut of unfed ticks expresses OspA (15, 45) and OspB (15), but not OspC (45). When ticks feed, this pattern is reversed, in that expression of OspC is induced (15, 45) and levels of OspA (15, 45) and OspB (15) are decreased. Rapid appearance of host antibodies to OspC and the absence of antibodies to OspA indicate that this pattern of antigenic expression persists in organisms during the early phase of Lyme disease (20, 45). These results raise the issue of whether the activation of endothelium by OspA and OspB that we observed in vitro is relevant to the in vivo situation. However, our data indicate that the ability to stimulate endothelium is intrinsic to a variety of spirochetal lipoproteins with diverse N-terminal sequences. Indeed, a lipohexapeptide corresponding to the N terminus of *T. pallidum* 17-kDa lipoprotein (Cys-Val-Ser-Cys-Thr-Thr) was approximately twice as potent as OspA lipopeptide (Cys-Lys-Gln-Asn-Val-Ser) in inducing expression of E-selectin. This apparent broad specificity is extended by the results of Riley et al. (42), who demonstrated increased expression of ICAM-1 on endothelium after incubation with a 47-kDa integral membrane lipoprotein purified from *T. pallidum*. That components of *B. burgdorferi* other than OspA and OspB are capable of stimulating endothelium is supported by our finding that mutant strain 297, which expresses OspC but not OspA or OspB, upregulated expression of E-selectin as efficiently as strains that possess all three of these lipoproteins. Collectively, these results raise the possibility that the stimulatory role of OspA and OspB that we have demonstrated in vitro might be assumed by other spirochetal lipoproteins, such as OspC, in the infected mammalian host.

After incubation of HUVEC with nonlipidated OspA hexapeptide, no significant increase in expression of E-selectin, VCAM-1, or ICAM-1 was observed. Similarly, whereas nOspA, L-rOspA, and L-rOspB were capable of upregulating expression of these adhesion molecules, their nonlipidated counterparts were ineffective. These results suggest that the lipid moiety of Osps is required for activation of endothelial cells. Lipid modification of OspA also enhances its immunogenic properties in vivo (21) and is essential for its B-cell mitogenic and cytokine-stimulatory activities in vitro (55). The ability of lipopeptide analogs of *T. pallidum* 17- and 47-kDa lipoproteins to activate monocytes/macrophages (1, 39) or of *B. burgdorferi* OspA or *T. pallidum* 47-kDa lipopeptides to induce dermal inflammation in mice and rabbits (36) is also dependent upon acylation. Whether the lipid portion of Osps is sufficient for activation of endothelium is uncertain. Although incubation of HUVEC with 3Pal-C failed to induce expression of E-selectin, we cannot rule out the possibility that 3Pal-C was less effective than lipidated OspA hexapeptide simply because it is less soluble. Interestingly, although 3Pal-C is incapable of stimulating endothelium or monocytes/macrophages (39) or inducing dermal inflammation in mice and rabbits (36), addition of a serine residue dramatically enhances its biological activity. Tripalmitoyl-S-glyceryl-cysteinyl-serine (Pam₃Cys-Ser) is a potent polyclonal activator of B lymphocytes, induces proliferation of the murine macrophage cell line P388D₁, and stimulates production of cytokines by human and murine monocytes/macrophages (28).

The accumulation of leukocytes at sites of inflammation is due, in part, to increased expression of endothelial adhesion

molecules. However, another important mechanism underlying this process is the production of chemotactic factors. Indeed, Boyden chamber assays demonstrated chemotactic activity for neutrophils in conditioned medium from HUVEC incubated with intact *B. burgdorferi*, OspA lipopeptide, or L-rOspA, but not with 3Pal-C, nonlipidated peptide, or rOspA. OspA lipopeptide and L-rOspA both lacked intrinsic chemotactic activity for neutrophils, implicating endothelium as a source of the chemoattractant(s). Finally, incubation of HUVEC monolayers with OspA lipopeptide promoted the transendothelial migration of subsequently added neutrophils.

The mechanism by which *B. burgdorferi* or its lipoproteins activate endothelium remains unclear. Spirochetes can bind to endothelial cells (51, 53) and have been observed within the cytoplasm (12, 34). However, a correlation between internalization of *B. burgdorferi* and stimulation of endothelial cells has not been established. Interestingly, Pam₃Cys-Ser can penetrate the plasma membrane and accumulate in the cytoplasm, the nuclear membrane, and the nucleus of bone marrow-derived macrophages (54), perhaps acting as a signaling molecule. Given their close homology to diacylglycerol, bacterial lipopeptides may perhaps stimulate cells through the activation of membrane-bound kinases. For example, Pam₃Cys-Ala-Gly promotes the translocation and activation of protein kinase C, thereby triggering proliferation of murine B cells (7). Alternatively, interaction between the lipid portion of *B. burgdorferi* lipoproteins or lipohexapeptides and the lipids of the plasma membrane may serve to anchor the bacterial molecules to the cell surface. Acylation plays such a role in the binding of Src family members to plasma membranes (41). This localization of lipoproteins may then lead to their interaction with other components involved in subsequent signaling.

This report provides evidence of a role for *B. burgdorferi* lipoproteins in activation of vascular endothelium, resulting in enhanced expression of adhesion molecules and production of chemotactic activity for neutrophils. Consequently, neutrophils adhered to and migrated across lipopeptide-stimulated endothelium, as we observed previously for endothelium incubated with viable *B. burgdorferi* (46). Neutrophilic infiltrates are present during the initial stages of an inflammatory response to *B. burgdorferi*, as seen in animal models of infection (31, 56) and in Lyme disease patients exhibiting erythema migrans (18, 19). Neutrophils are also present in the synovial fluid of patients with Lyme arthritis (19). A transition to a mixed mononuclear infiltrate occurs 24 to 48 h after deposition of spirochetes in the host skin (19, 31). Although our studies were limited to neutrophils, it is noteworthy that VCAM-1, which is a ligand for lymphocytes and monocytes but not neutrophils (37), is upregulated on HUVEC exposed to intact spirochetes (46), OspA, OspB, or lipidated OspA hexapeptide. Moreover, binding of cells of a B-lymphoma line to mouse endothelioma cells stimulated by *B. burgdorferi* is mediated largely by VCAM-1 (6). Perhaps, then, the recruitment of mononuclear cells that occurs later in infection is also a consequence of activation of endothelium by *B. burgdorferi*.

Our studies thus suggest that accumulation of leukocytes in perivascular tissues during Lyme disease may result, at least in part, from interaction of spirochetal lipoproteins or their biologically active degradation products with endothelium. Spirochetal lipopeptides may be released by the degradation of native lipoprotein within phagolysosomes, may be part of circulating immune complexes, or may be found in association with spirochetal membrane blebs (40). Membrane blebs from *B. burgdorferi* contain OspA, OspB, OspD (48), and a low-molecular-mass lipoprotein with a size of ~6 to 10 kDa (32). The B-cell mitogenic activity produced by *B. burgdorferi* is

enriched in membrane bleb preparations (57). Moreover, these extracellular vesicles adhere specifically to HUVEC in a passage number and dose-dependent manner (48). The presence of these blebs or biologically active lipopeptides in affected tissues (16) may account for the persistence of an inflammatory state in the apparent absence of cultivable organisms.

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