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Borrelia burgdorferi Upregulates Expression of Adhesion Molecules on Endothelial Cells and Promotes Transendothelial Migration of Neutrophils In Vitro

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The accumulation of leukocytic infiltrates in perivascular tissues is a key step in the pathogenesis of Lyme disease, a chronic inflammatory disorder caused by *Borrelia burgdorferi*. During an inflammatory response, endothelial cell adhesion molecules mediate the attachment of circulating leukocytes to the blood vessel wall and their subsequent extravasation into perivascular tissues. Using cultured human umbilical vein endothelial cells (HUVEC) in a whole-cell enzyme-linked immunosorbent assay, we demonstrated that *B. burgdorferi* activated endothelium in a dose- and time-dependent fashion as measured by upregulation of the adhesion molecules E-selectin, vascular cell adhesion molecule 1 (VCAM-1), and intercellular adhesion molecule 1 (ICAM-1). As few as one spirochete per endothelial cell stimulated increased expression of these molecules. Expression of E-selectin peaked after spirochetes and HUVEC were coincubated for 4 h and returned to near-basal levels by 24 h. In contrast, expression of VCAM-1 and ICAM-1 peaked at 12 h and remained elevated at 24 h. HUVEC monolayers cultured on acellular amniotic tissue were used to investigate the consequences of endothelial cell activation by spirochetes. After incubation of HUVEC-amnion cultures with *B. burgdorferi*, subsequently added neutrophils migrated across the endothelial monolayers. This process was mediated by E-selectin and by CD11/CD18 leukocytic integrins. The extent of migration depended on both the number of spirochetes used to stimulate the HUVEC and the length of the coincubation period. These results raise the possibility that *B. burgdorferi* induces a host inflammatory response and accompanying perivascular damage through activation of vascular endothelium.

The spirochete *Borrelia burgdorferi* is the most prevalent arthropod-borne pathogen in the United States and the etiologic agent of Lyme disease. Spirochetes are deposited in the skin of the host by the bite of the deer tick, *Ixodes scapularis* (30). Following a brief period of spirochetemia, *B. burgdorferi* disseminates hematogenously to sites of secondary infection including the central and peripheral nervous systems, joints, and heart (18).

Adherence of spirochetes to the endothelium of the vascular wall is thought to be an important first step in the dissemination of Lyme disease (15, 52). Spirochetal infiltration of stromal tissue and the extravascular accumulation of leukocytes may be key steps in initiating the vascular damage associated with this chronic inflammatory disorder (19). Spirochetes are sometimes found adjacent to areas of vascular damage, which ranges from perivasculitis to endarteritis obliterans (32). The histopathology of such lesions is characterized by increased vascular permeability, extravascular deposition of fibrin, and occlusion of the vascular lumen due to hyperproliferation of smooth muscle cells and expansion of connective tissue (19). In addition, leukocytic infiltrates consisting of various mixtures of neutrophils, macrophages, eosinophils, lymphocytes, and plasma cells have been observed and exhibit clinical stage and tissue specificity (17, 19).

The formation of leukocytic infiltrates is mediated, at least in part, by specific adhesion molecules on the vascular endothelium (41). E-selectin, formerly known as endothelial-leukocyte adhesion molecule 1, is upregulated upon stimulation of endothelium with interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α), or bacterial lipopolysaccharide (LPS) but is not present on unstimulated endothelial cells (7). Sialyl-Lewis X-related carbohydrates on the surface of leukocytes act as ligands for E-selectin (39). E-selectin mediates the loose attachment of leukocytes to the endothelium, allowing them to roll along the blood vessel wall (51). Following activation of integrins on their surface, leukocytes adhere more tightly to the endothelium through interaction with members of the immunoglobulin superfamily of adhesion molecules. Vascular cell adhesion molecule 1 (VCAM-1) mediates binding of monocytes and lymphocytes via the β_1 integrin very late antigen 4 (VLA-4) (10, 12, 20). Intercellular adhesion molecule 1 (ICAM-1) binds to the β_2 or CD11/CD18 leukocytic integrins (35, 48). Constitutively expressed on endothelial cells only at low levels, the expression of VCAM-1 and ICAM-1 is upregulated in response to cytokines, LPS, and bacterial lipoproteins (11, 33, 45). After binding tightly to the endothelium, leukocytes migrate across the vessel wall to take up residence in the perivascular tissues and carry out their effector functions.

Whether *B. burgdorferi* possesses LPS that might serve to augment expression of endothelial adhesion molecules has been a matter of controversy (3, 53). Nonetheless, it seemed reasonable to hypothesize that *B. burgdorferi* may facilitate extravascular accumulation of leukocytes via such upregulation. To test this premise, we used an enzyme-linked immunosorbent assay (ELISA) that allows detection of adhesion molecules on human umbilical vein endothelial cells (HUVEC) following their incubation with spirochetes. In addition, we

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used an in vitro model of the vascular wall, which consists of HUVEC monolayers cultured on amniotic tissue, to study the migration of leukocytes across spirochete-stimulated endothelium. Our results indicate that *B. burgdorferi* increases expression of endothelial adhesion molecules in a time- and dose-dependent fashion. Furthermore, this upregulation results in migration of neutrophils across the stimulated HUVEC monolayers.

MATERIALS AND METHODS

Culture of spirochetes. *Borrelia anserina* and *Borrelia hermsii* were generously provided by Jorge L. Benach and were cultured in standard Barbour-Stoenner-Kelly (BSK) II medium (2) containing 10% rabbit serum. *B. burgdorferi* was cultured at 33°C in serum-free BSK medium that was modified to minimize the content of LPS. The medium consisted of 19 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 2 mM sodium citrate, 23 mM glucose, 6 mM sodium pyruvate, 1.5 mM *N*-acetylglucosamine, 22 mM sodium bicarbonate, 8.3% (vol/vol) 10× CMRL Medium-1066 (Gibco Laboratories, Grand Island, N.Y.), 5% (vol/vol) EX-CYTE VLE (a low-endotoxin bovine lipid-lipoprotein supplement from Miles, Kankakee, Ill.), 4.2% (wt/vol) bovine serum albumin (BSA) (bovine albumin fraction V, catalog no. A9056, from Sigma Chemical Co., St. Louis, Mo.), 4.2% (wt/vol) Neopeptone (Difco Laboratories, Detroit, Mich.), and 1.2% (wt/vol) gelatin (Difco). All constituents of the medium were of tissue culture grade except for the Neopeptone, and low-endotoxin water from a Milli-Q UF system (Millipore, Bedford, Mass.) was used for its preparation. All glassware was baked at 180°C for 4 h before use to destroy LPS.

Since some preparations of BSA contain substantial amounts of LPS, attempts were made to identify batches that were low in endotoxin. Unfortunately, batches of BSA that contained little or no LPS were often incapable of supporting the growth of spirochetes in the absence of rabbit serum. Therefore, BSA that was optimized for the growth of *B. burgdorferi* (see above) was depleted of LPS as follows. A 12% BSA solution in pyrogen-free water was passed through a Minisart-N 0.2-μm-pore-size prefilter (Sartorius, Edgewood, N.Y.) and then through a Q-15 membrane adsorber (Sartorius), which is a strongly basic anion exchanger. The Q-15 filter was equilibrated before use with 50 mM sodium acetate–20 mM sodium chloride (pH 4.7). The filtered BSA was devoid of any LPS-like stimulatory activity, as demonstrated by its inability to promote migration of neutrophils across HUVEC monolayers.

For use in experiments, a human blood isolate of *B. burgdorferi* (HBD1) (4), having undergone 38 passages before storage in liquid nitrogen, was thawed and passaged an additional 2 to 15 times in the low-endotoxin BSK medium. Spirochetes were harvested during late-log-phase growth, centrifuged at 5,000 × *g* for 20 min at room temperature (RT), and gently resuspended in Medium 199 (M199) (Gibco) containing 20% heat-inactivated fetal bovine serum (HIFBS; heated for 30 min at 56°C) (HyClone Laboratories, Logan, Utah) and 25 mM HEPES, pH 7.2. In some experiments, spirochetes were disrupted by sonication for 5 min on ice or killed by heating for 1 h at 56°C. Aliquots of sonicated spirochetes were also subjected to heating for 1 h at 56°C. To control for the possible introduction of LPS during sonication, a sham was prepared by sonicating a volume of uninoculated M199–20% HIFBS equivalent to the volume of spirochete suspension that was sonicated. Loss of viability was confirmed by direct observation with dark-field microscopy and by attempting to subculture aliquots of treated bacteria.

Endothelial cell cultures. Endothelial cells were isolated from human umbilical veins by a modification (29) of the method of Jaffe et al. (31). Briefly, umbilical cords were perfused with a solution of 0.035% collagenase (Worthington Biochemical Corp., Freehold, N.J.) and incubated for 12 min at 37°C. Endothelial cells were flushed from the cord and plated onto 1.5% gelatin-coated (21) 60-mm plastic tissue culture plates (Corning Glass Works, Corning, N.Y.). HUVEC were maintained in growth medium consisting of M199 containing 20% FBS, 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. Cultures from several umbilical veins were combined to minimize variation among endothelial cells from individual donors, which may exhibit varying responsiveness to cytokines (5). The response of HUVEC to stimulation with 1 U of recombinant human IL-1β per ml (Collaborative Biomedical Products, Bedford, Mass.) was used as an internal positive control in the ELISAs. To normalize the data within and between experiments, results were expressed as a percentage of the response to IL-1β.

Second-passage endothelial cell cultures used in experiments were >97% pure as determined by indirect immunofluorescence with an antibody to von Willebrand factor (29). However, contaminating cells of potential leukocytic origin were detected by indirect immunofluorescence with monoclonal antibodies (MAbs) PD7/26 and 2B11 directed against the leukocytic common antigen, CD45 (Accurate Chemical and Scientific Corp., Westbury, N.Y.). To assess the contribution of these contaminants to observed results, HUVEC were passaged five times in the presence of endothelial cell growth factor (Sigma), which effectively eliminated these contaminants.

MAbs. MAb BB11 (immunoglobulin G2b [IgG2b]), directed against E-selectin (6), and MAb 4B9 (IgG1), directed against VCAM-1 (12), were generously provided by Roy R. Lobb (Biogen Inc., Cambridge, Mass.). MAb R6.5 (IgG2a), directed against ICAM-1 (49), was provided by Robert Rothlein (Boehringer Ingelheim Corp., Ridgefield, Conn.) and C. W. Smith (Baylor College of Medicine, Houston, Tex.). MAb IB4 (IgG2a), directed against the common β chain (CD18) of the CD11/CD18 leukocytic integrins (54), was the gift of Samuel D. Wright (The Rockefeller University, New York, N.Y.). The MAbs used in inhibition studies were shown previously to block functional interactions of these adhesion molecules with their ligands (6, 54). MOPC-141 and UPC 10 were used as nonbinding isotype-matched control MAbs. MOPC-141 (Sigma) is an IgG2b MAb of unknown specificity obtained from a mineral oil-induced plasmacytoma. UPC 10 (Sigma) is an IgG2a MAb, isolated from a pristane-induced plasmacytoma, which recognizes β-2-6-linked fructosan. MAb MCB1 (IgG3) is directed against the 41-kDa endoflagellar antigen of *B. burgdorferi* B31 (13). MCB1 was used as a hybridoma supernatant. All other MAb preparations were purified IgG.

Whole-cell ELISA. The whole-cell ELISA was performed essentially as described by McCarron et al. (36) with the following modifications. HUVEC from primary cultures were plated into 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 with media lacking antibiotics and were incubated with M199–20% HIFBS containing 25 mM HEPES (pH 7.2), BSK medium controls, or spirochetal preparations (100 μl per well) for various lengths of time at 37°C. Following coincubation, HUVEC were washed three times with M199–5% HIFBS containing 25 mM HEPES, pH 7.2 (used for all subsequent washes and as diluent for antibodies and enzyme), and incubated with MAb BB11, R6.5, or 4B9 (0.1 μg/100 μl per well) for 1 h. HUVEC were then washed and incubated with biotinylated goat anti-mouse IgG (0.013 μg/100 μl per well) (Pierce, Rockford, Ill.) for 1 h. After washing, HUVEC were incubated with avidin-horseradish peroxidase (0.2 μg/100 μl per well) (Pierce) for 1 h. HUVEC were then washed four times and incubated with *o*-phenylenediamine (50 μg/100 μl per well) in 1× stable peroxide buffer (Pierce) for 30 min. All labeling steps were performed at RT. The reaction was quenched by addition of 4 N HCl (100 μl per well), and the wells were read immediately at 490 nm with an MR700 microplate reader (Dynatech Laboratories, Chantilly, Va.). The cell number and viability of HUVEC were not significantly diminished following 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.

MAbs directed against adhesion molecules were tested for their ability to cross-react with spirochetal antigen. *B. burgdorferi* HBD1 was sonicated as described above, and spirochetal antigen (2.5 μg/100 μl per well) was added to untreated microtiter wells. The amount of antigen added to each well was equivalent to adding a ratio of 100 spirochetes per endothelial cell in the whole-cell ELISA. Following incubation for 4 h at 37°C, wells were washed three times with M199–5% HIFBS containing 25 mM HEPES, pH 7.2. MAb BB11, 4B9, R6.5, or MCB1 (0.1 μg/100 μl per well) was added, and an ELISA was performed as just described above.

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 with Viton O-rings (C. E. Conover, Fairfield, N.J.). The epithelium was lysed with 0.25 N NH_4OH, and debris was removed by scraping to leave an acellular connective tissue matrix upon which endothelial cells were cultured. Tissues were washed extensively and stored at 4°C in HEPES-buffered saline. Before seeding endothelial cells, the amniotic tissue was incubated with HUVEC growth medium for 2 h at 37°C (24, 26).

Isolation of human neutrophils. Blood from healthy adult volunteers was collected in heparinized syringes for the isolation of neutrophils by the method of Anderson et al. (1). In brief, after sedimentation of erythrocytes with 0.6% dextran (Pharmacia, Piscataway, N.J.) for 45 min at RT, the leukocyte-rich plasma was centrifuged at 300 × *g* for 5 min at RT to pellet the neutrophils. Cells were resuspended in Dulbecco's phosphate-buffered saline containing 0.2% glucose and applied to a cushion of Ficoll-Hypaque gradient medium (specific gravity = 1.077). Ficoll Type 400 was obtained from Sigma, and Hypaque 50% was obtained from Sanofi-Winthrop Pharmaceuticals, New York, N.Y. After centrifugation at 675 × *g* for 20 min at RT, neutrophils were washed and resuspended in M199–20% HIFBS. 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.

Neutrophil transendothelial migration assay. HUVEC-amnion cultures were prepared by passaging HUVEC from primary cultures onto amnion at a density of 1.5×10^5 cells per cm^2. Within 7 to 10 days, endothelial cells form confluent monolayers characterized by a transendothelial electrical resistance (29). HUVEC monolayers were washed with medium lacking antibiotics and incubated with 0.5 ml of M199–20% HIFBS containing 25 mM HEPES (pH 7.2), BSK medium controls, or spirochetal preparations for various lengths of time at 37°C. Following 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. For MAb inhibition experiments, HUVEC-amnion cultures were incu-

bated with medium controls or with *B. burgdorferi* HBD1 at 10 organisms per endothelial cell for 5 h and washed. Cultures were then incubated with 10 μg of MAb BB11 to E-selectin per ml, 10 μg of MOPC-141 per ml (an isotype-matched control MAb), or control medium for 45 min. Neutrophils were preincubated on ice with MAb IB4 to CD18, isotype-matched control MAb UPC 10 (both at 10 μg/ml), or control medium. After warming, neutrophils were added to cultures, and the migration assay was performed as described above. After coincubation of *B. burgdorferi* and HUVEC, the motility of spirochetes in the bathing medium was assessed by dark-field microscopy, and endothelial cell viability was determined by trypan blue exclusion.

Following fixation, samples were rinsed three times in 0.9% saline 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 random, 400× fields. Because it is difficult to determine the disposition of neutrophils (above versus below the endothelial monolayer) by viewing specimens en face, one replicate from each experimental group was embedded in glycol methacrylate for cross-sectional analysis (Polysciences Inc., Warrington, Pa.). Sections (2.2 μm thick) were cut perpendicular to the plane of the HUVEC monolayer and stained with Giemsa stain or toluidine blue. The percentage of neutrophils that migrated beneath the endothelium was calculated by determining the disposition of 100 neutrophils with respect to the endothelium. To correct for possible loss of neutrophils attached to the apical surfaces of HUVEC during preparation for embedding, the number of cells on a portion of one replicate was counted en face following the embedding procedure. The number of cells remaining was compared with the en face counts prior to embedding. These counts did not differ significantly, thus ruling out the possibility that loss of neutrophils attached to the apical surface biased the percentage of migrated neutrophils observed. Experimental groups consisted of four to six replicates.

Statistics. With the InStat 2.01 statistical software package, 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 ± SD of experimental groups from a transendothelial migration assay were subjected to an alternate Welch t 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

Incubation of HUVEC with *B. burgdorferi* results in increased expression of adhesion molecules. A whole-cell ELISA was employed to analyze the upregulation of the adhesion molecules E-selectin, VCAM-1, and ICAM-1 on endothelial cells in response to *B. burgdorferi*. As few as one spirochete per endothelial cell was capable of stimulating expression of these adhesion molecules (E-selectin, $P < 0.001$; VCAM-1, $P < 0.01$; ICAM-1, $P < 0.05$), but maximal effects were seen with 10 spirochetes per endothelial cell (all three adhesion molecules, $P < 0.001$) (Fig. 1). Upregulation of E-selectin in response to 10 organisms per endothelial cell was comparable to that obtained with 0.1 U of IL-1β per ml (data not shown). With a ratio of 10 spirochetes per endothelial cell, time course experiments were undertaken to determine when maximal levels of expression were achieved. Levels of E-selectin peaked at 4 h and returned to near-basal amounts by 24 h (Fig. 2A). In contrast, expression of both VCAM-1 and ICAM-1 peaked at 12 h and remained elevated at 24 h (Fig. 2B and 2C). The temporal patterns of expression of adhesion molecules in response to spirochetes were similar to those induced by IL-1β (Fig. 2). To ensure the specificity of recognition of the adhesion molecules, the MAbs to E-selectin, VCAM-1, and ICAM-1 were tested for their ability to cross-react with spirochetal antigens. Recognition of spirochetal preparations by these MAbs was minimal. In contrast, a MAb directed against the 41-kDa endoflagellar antigen of *B. burgdorferi* produced a strong signal (Fig. 3).

Macrophages secrete IL-1β and TNF-α in response to *B. burgdorferi* (16, 28), and our second-passage HUVEC cultures contained contaminating cells that expressed a leukocytic marker, CD45. To examine the possibility that production of cytokines by these contaminants explained our results, HUVEC were passaged five times in the presence of endothelial cell growth factor, which eliminated all $CD45^+$ cells. Incubation with one spirochete per endothelial cell upregulated expression of E-selectin on second- and fifth-passage HUVEC to 39 and 56% of the response to IL-1β, respectively (data not shown). Similarly, 10 spirochetes per endothelial cell stimulated expression of E-selectin to 75 and 69% of the response to IL-1β on second- and fifth-passage HUVEC, respectively (data not shown). Therefore, the contaminating leukocytic cells contributed little or nothing to activation of HUVEC by *B. burgdorferi*.

To determine whether the source of the spirochetal isolate or its number of passages in culture influenced the ability of *B. burgdorferi* to stimulate endothelium, the activity of the HBD1 human blood-derived isolate was compared with that of BEP4, a tick isolate (14). Strains HBD1 and BEP4 had undergone >38 passages and <20 passages in culture, respectively, before being used in experiments. There were no significant differences in the ability of either strain to upregulate expression of

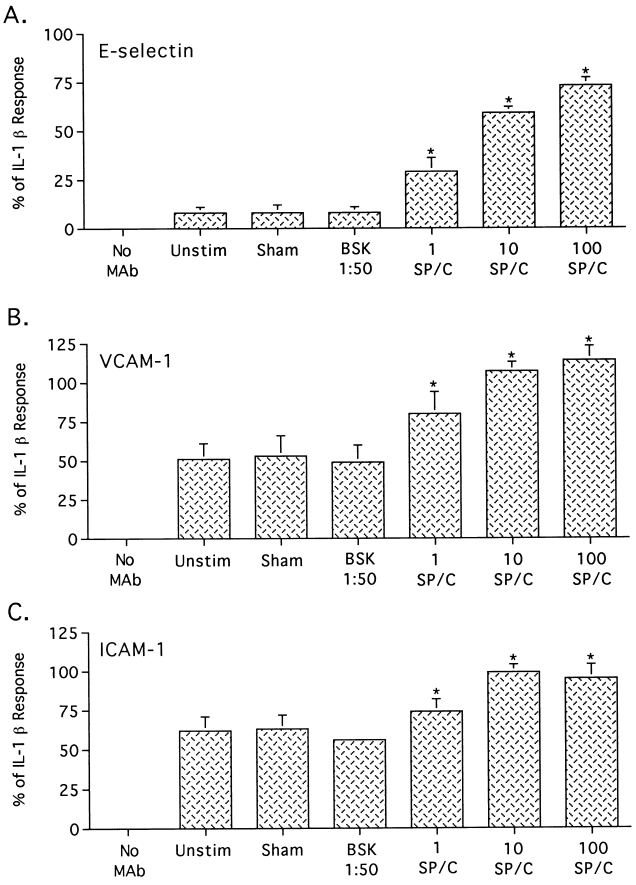

FIG. 1. Dose-dependent expression of HUVEC adhesion molecules in response to *B. burgdorferi*. Confluent HUVEC monolayers were incubated with M199–20% HIFBS alone (Unstim), a sham preparation made with uninoculated BSK medium (Sham) (see Materials and Methods), BSK medium diluted 1:50 with M199–20% HIFBS (BSK 1:50), or *B. burgdorferi* HBD1 at ratios of 1, 10, or 100 spirochetes per endothelial cell (SP/C). Expression of E-selectin was measured by a whole-cell ELISA with MAb BB11 after 4 h of incubation (A). Levels of VCAM-1 (B) and ICAM-1 (C) were assessed at 12 h of incubation with MAbs 4B9 and R6.5, respectively. Positive controls were treated with 1 U of IL-1β per ml for the same lengths of time. Samples designated "No MAb" were incubated with 100 spirochetes per HUVEC, but medium without primary MAb was used during the ELISA. Bars represent the mean ± SD of eight replicate samples per experimental group (four from each of two independent experiments). *, significantly different from sham control ($P < 0.05$).

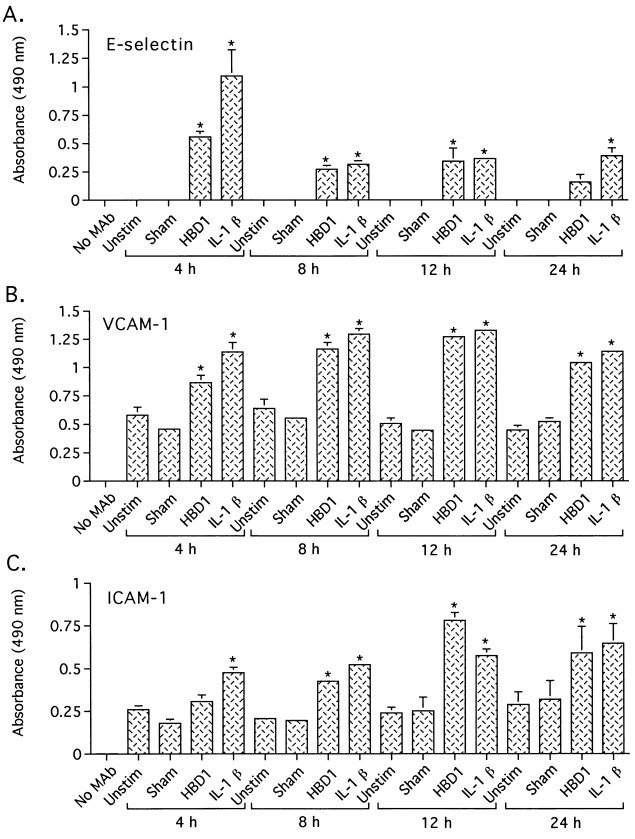

FIG. 2. Temporal patterns of expression of HUVEC adhesion molecules in response to *B. burgdorferi*. Confluent HUVEC monolayers were incubated with *B. burgdorferi* HBD1 at 10 organisms per endothelial cell for the indicated lengths of time or with control preparations as described in the legend for Fig. 1. After washing of the cultures, expression of E-selectin (A), VCAM-1 (B), and ICAM-1 (C) was assessed by a whole-cell ELISA. Bars represent the means ± SD of four replicate samples per experimental group. *, significantly different from sham control ($P < 0.01$).

adhesion molecules on HUVEC at any ratio of spirochete to endothelial cell tested (data not shown). To investigate whether the stimulatory activity observed was specific to *B. burgdorferi*, HUVEC were incubated with *B. anserina* and *B.*

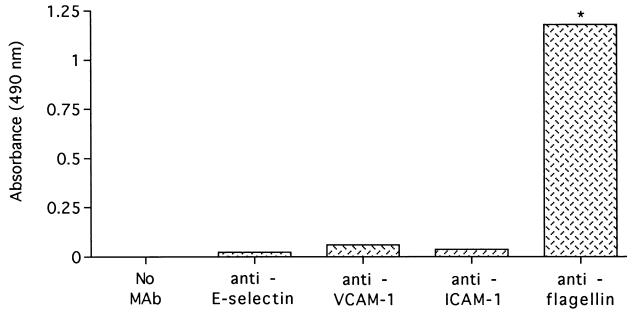

FIG. 3. Lack of cross-reactivity between MAbs to cell adhesion molecules and spirochetal antigens. Microtiter wells containing spirochetal antigen were incubated without primary MAb (No MAb) or with MAb BB11 to E-selectin, 4B9 to VCAM-1, R6.5 to ICAM-1, or MCB1 to the 41-kDa endoflagellar antigen of *B. burgdorferi*. The extent of binding of the various antibodies was determined by ELISA. Bars represent the means ± SD of three replicate samples per experimental group. *, significantly different from the control lacking primary MAb ($P < 0.001$).

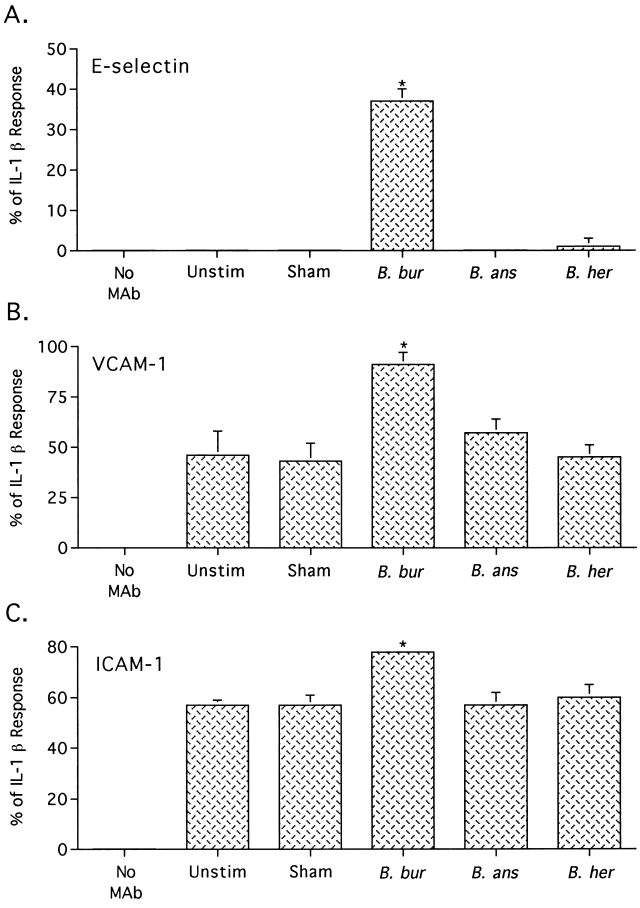

FIG. 4. Stimulatory activity of other *Borrelia* species. Confluent HUVEC monolayers were incubated with control preparations (see legend for Fig. 1) or with *B. burgdorferi*, *B. anserina*, or *B. hermsii* at 10 spirochetes per endothelial cell. After 4 h of incubation, expression of E-selectin was assessed by a whole-cell ELISA (A). Expression of VCAM-1 (B) or ICAM-1 (C) was measured following 12 h of incubation. Bars represent the means ± SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. *, significantly different from sham control ($P < 0.001$).

hermsii at a ratio of 10 spirochetes per endothelial cell (Fig. 4). Neither of these species was capable of inducing expression of E-selectin, VCAM-1, or ICAM-1. In other experiments, as many as 100 organisms per endothelial cell were also ineffective (data not shown).

Upregulation of adhesion molecules is not due to contaminants from medium. Bacterial LPS activates vascular endothelium to promote adhesion (44) and transendothelial migration (25) of neutrophils. To assess the possibility that exogenous LPS might account for our results, a sham was prepared by processing a volume of uninoculated BSK medium equal to the volume of spirochete culture used for the experiment. The sham was subjected to the same manipulations as the spirochetes themselves. In no instance did the sham preparations significantly upregulate expression of adhesion molecules (Fig. 1, 2, 4, and 5), discounting exogenous LPS as the source of stimulatory activity. A second concern was that spirochetes might adsorb LPS or another stimulatory activity from the BSK medium, thereby concentrating its effects. Therefore, as shown in Fig. 1, a 1:50 dilution of BSK medium was tested for its ability to stimulate HUVEC. The volume of BSK medium used to make this dilution was equal to the volume of culture me-

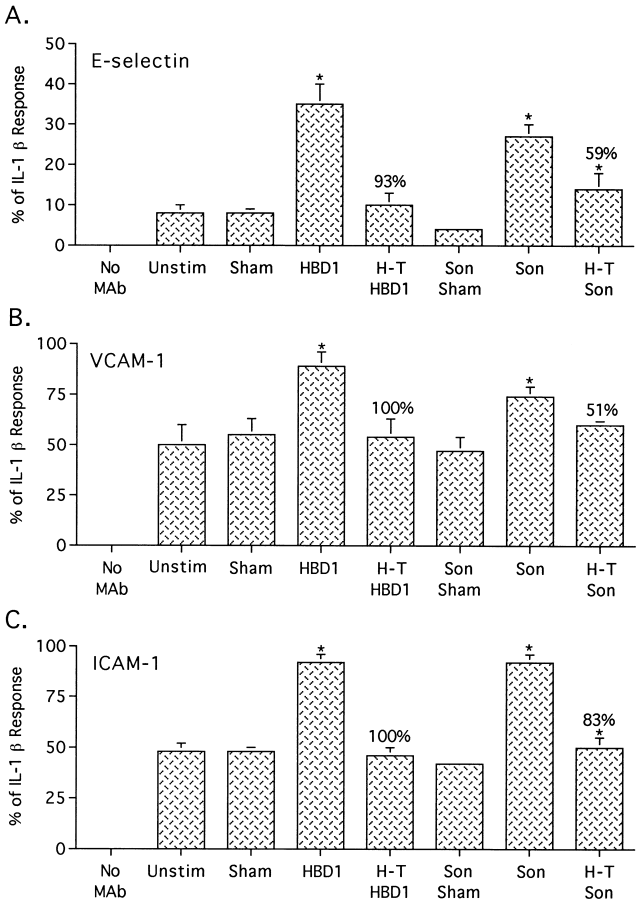

FIG. 5. Effect of heat treatment and sonication on the stimulatory activity of *B. burgdorferi*. Confluent HUVEC monolayers were incubated with control preparations (see legend for Fig. 1), strain HBD1 of *B. burgdorferi* (10 spirochetes per endothelial cell), an equivalent number of spirochetes disrupted by sonication (Son), or a sonication sham prepared by sonicating uninoculated M199–20% HIFBS immediately prior to sonication of spirochetes (Son Sham) (see Materials and Methods). Aliquots of intact and sonicated organisms were heat treated for 1 h at 56°C (H-T HBD1 and H-T Son, respectively). Expression of E-selectin was measured by a whole-cell ELISA after 4 h of incubation (A). Expression of VCAM-1 (B) or ICAM-1 (C) was measured following 12 h of incubation. Bars represent the means ± SD of four replicate samples per experimental group from a representative experiment. This experiment was repeated once with similar results. Percentages above the error bars indicate the level of inhibition compared with corresponding unheated samples. *, significantly different from sham control ($P < 0.05$).

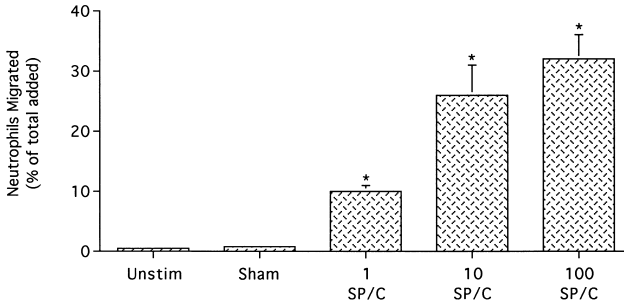

FIG. 6. Dose-dependent promotion of neutrophil transendothelial migration in response to *B. burgdorferi*. HUVEC-amnion cultures were incubated with M199–20% HIFBS alone (Unstim), with a sham preparation made with uninoculated BSK medium (Sham) (see Materials and Methods), or with the indicated number of *B. burgdorferi* HBD1 spirochetes per endothelial cell (SP/C) for 6 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 represent the means ± SD of four replicate cultures per experimental group from a representative experiment. This experiment was repeated once with similar results. *, significantly different from sham control ($P < 0.0001$).

dium from which spirochetes were derived for addition to each microtiter well. This volume thus represented the maximum amount of BSK medium from which a stimulatory activity could be adsorbed. The diluted BSK medium was without effect, indicating that adsorption of factors from the medium is unlikely to account for our results.

Upregulation of adhesion molecules is reduced by treatments that kill spirochetes. To assess whether viability was necessary for the stimulatory activity of *B. burgdorferi*, HUVEC were incubated with organisms that were killed by several means. Heating for 1 h at 56°C nearly eliminated the ability of spirochetes to induce expression of E-selectin (Fig. 5A) and completely abolished their ability to upregulate VCAM-1 and ICAM-1 (Fig. 5B and C). Spirochetes that were fixed in 3% buffered paraformaldehyde for 30 min at 4°C and then washed three times in M199–20% HIFBS were also devoid of stimulatory activity, as were spirochetes subjected to five rounds of freezing and thawing (data not shown). In contrast, preparations of sonicated spirochetes retained 87% ± 11% of the activity of an equivalent number of viable organisms (Fig. 5). Heating the sonicated preparations reduced, but did not eliminate, the stimulatory activity.

***B. burgdorferi* promotes the transendothelial migration of neutrophils.** Having demonstrated the ability of *B. burgdorferi* to alter expression of adhesion molecules on endothelium, we undertook experiments to determine the consequences of this phenomenon. Previous studies have shown that neutrophils do not associate with unstimulated HUVEC but will bind to and emigrate across cytokine-activated endothelium (23, 25, 48). We investigated the ability of *B. burgdorferi* to promote migration of neutrophils across monolayers of HUVEC cultured on amniotic connective tissue. HUVEC-amnion cultures were incubated with spirochetes at a ratio of 1, 10, or 100 bacteria per endothelial cell for 6 h at 37°C. As seen in Fig. 6, preincubation of HUVEC-amnion cultures with spirochetes promoted the migration of subsequently added neutrophils in a dose-dependent manner ($P < 0.0001$). Although as few as one spirochete per endothelial cell was capable of promoting neutrophil migration, a near-maximal effect was observed with 10 organisms per endothelial cell. Very few neutrophils were adherent to the apical surface of the HUVEC monolayer, indicating that the leukocytes were not merely binding to spirochetes attached to the endothelium (Fig. 7). Few neutrophils adhered or migrated when HUVEC cultures were incubated with growth medium or a sham preparation of spirochetes. The latter control discounts exogenous LPS as an explanation for spirochete-induced migration.

Time course experiments identified the duration of coincubation required to achieve maximal transmigration of neutrophils. Maximal migration occurred after incubation of *B. burgdorferi* with HUVEC for 8 h (Fig. 8). Longer coincubation periods were significantly less effective, with an incubation period of 24 h resulting in only 12% of maximal migration.

Experiments were performed to determine whether viable spirochetes were required to promote transmigration of neutrophils. Viable, heat-treated, or sonicated spirochetes were added to HUVEC-amnion cultures at 10 organisms per endothelial cell and incubated for 8 h at 37°C. As shown in Fig. 9, viable organisms produced expected stimulation of neutrophil migration, whereas heat-killed *B. burgdorferi* retained little ac-

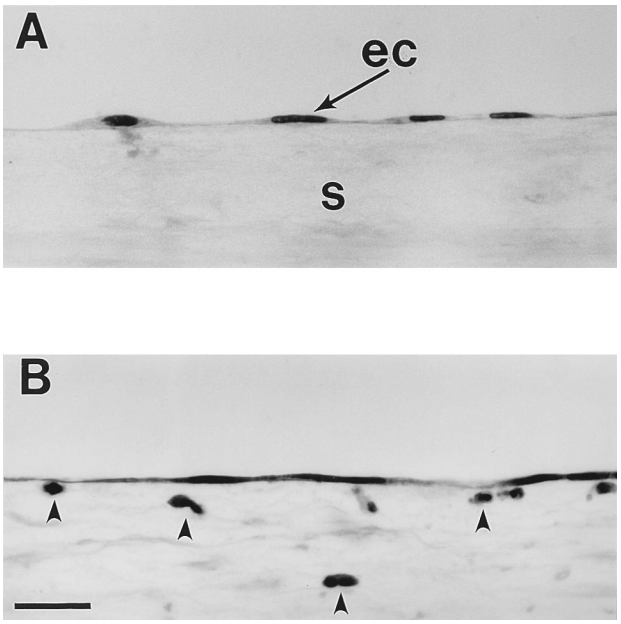

FIG. 7. Light micrographs of cross-sections of HUVEC-amnion cultures. HUVEC-amnion cultures were incubated with a sham preparation made with uninoculated BSK medium (A) or with *B. burgdorferi* HBD1 at 10 spirochetes per endothelial cell (B) for 5 h and washed. Subsequently added neutrophils were incubated with the cultures for 30 min, and tissues were fixed, embedded, and sectioned perpendicular to the plane of the endothelial monolayer as described in Materials and Methods. In the control preparation, no neutrophils were found associated with the unstimulated HUVEC monolayer (ec) or within the underlying amniotic stroma (s) (A). In contrast, neutrophils migrated beneath the spirochete-stimulated endothelium (B). Some of these neutrophils are indicated by arrowheads. Bar = 30 μm.

tivity. Sonicated spirochetes were capable of promoting a significant amount of neutrophil transmigration, although their activity was reduced by 38% in comparison with an equivalent number of live spirochetes. Upon heating the spirochetal sonicate for 1 h at 56°C, stimulatory activity was completely eliminated.

Spirochete-stimulated migration of neutrophils involves E-selectin- and CD18-dependent adhesion pathways. Since our results indicated that spirochetes promote transmigration of neutrophils, additional studies were undertaken to identify the cellular adhesion molecules involved. Spirochete-induced migration of neutrophils was inhibited 39% ($P < 0.05$) by a MAb to E-selectin and 83% ($P < 0.001$) by a MAb to the CD11/CD18 family of leukocytic integrins (Fig. 10). A combination of the two MAbs blocked the migration of neutrophils nearly completely. In contrast, no inhibition of migration was observed in the presence of equal concentrations of isotype-matched MAbs UPC 10 and MOPC-141 directed against irrelevant antigens. These data demonstrate that functional E-selectin and CD11/CD18 are required to support maximal migration of neutrophils across spirochete-stimulated HUVEC monolayers.

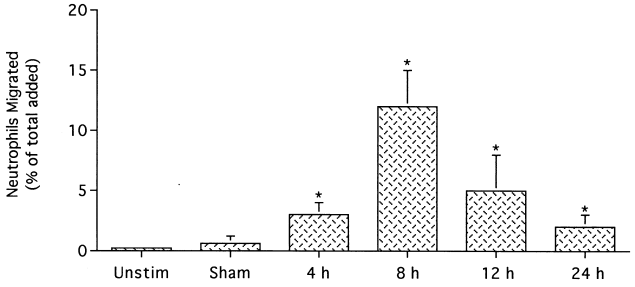

FIG. 8. Time course for promotion of neutrophil transendothelial migration in response to *B. burgdorferi*. HUVEC-amnion cultures were incubated with control preparations (see legend for Fig. 6) or with *B. burgdorferi* HBD1 at 10 spirochetes per endothelial cell for the indicated lengths of time and washed. Subsequently added neutrophils were incubated with the cultures for 30 min, and the extent of migration was determined as described in Materials and Methods. Bars represent the means ± SD of four replicate cultures per experimental group. *, significantly different from sham control ($P < 0.001$).

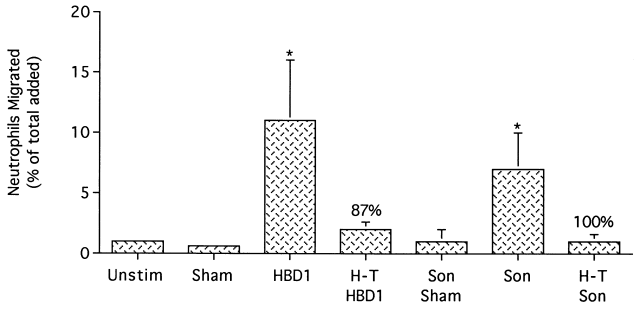

FIG. 9. Effects of heat treatment and sonication upon the ability of *B. burgdorferi* to promote neutrophil migration. HUVEC-amnion cultures were incubated with control preparations (see legend for Fig. 6), strain HBD1 of *B. burgdorferi* (10 spirochetes per endothelial cell), an equivalent number of spirochetes disrupted by sonication (Son), or a sham prepared by sonicating uninoculated M199–20% HIFBS immediately prior to sonication of spirochetes (Son Sham) (see Materials and Methods) for 8 h at 37°C and washed. Aliquots of intact or sonicated organisms were heat treated for 1 h at 56°C (H-T HBD1 and H-T Son, respectively). Subsequently added neutrophils were incubated with cultures for 30 min, and the extent of migration was determined as described in Materials and Methods. Bars represent the means ± SD of four replicate cultures per experimental group. Percentages above the error bars indicate the level of inhibition compared with equivalent unheated samples. *, significantly different from sham control ($P < 0.001$).

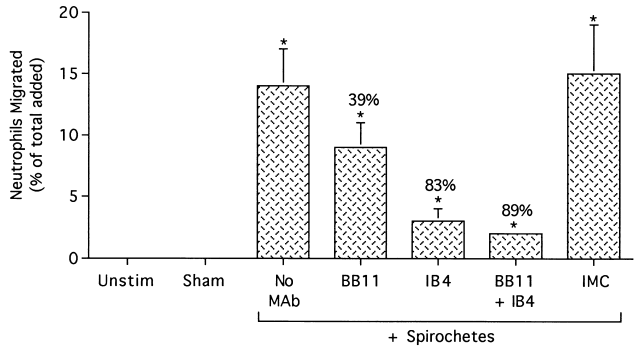

FIG. 10. Neutrophil migration across spirochete-activated HUVEC-amnion cultures is inhibited by MAbs to CD11/CD18 and E-selectin adhesion molecules. HUVEC-amnion cultures were incubated with control preparations (see legend for Fig. 6) or with *B. burgdorferi* HBD1 at 10 organisms per endothelial cell for 5 h and washed. Cultures were then incubated with MAb BB11 to E-selectin, MOPC-141 (an isotype-matched control MAb), or control medium for 45 min. Neutrophils were preincubated on ice with MAb IB4 to CD18, isotype-matched control MAb UPC 10, or medium without MAb (No MAb). After warming, neutrophils were added to cultures for 30 min, and migration was quantitated as described in Materials and Methods. Bars represent the means ± SD of four replicate cultures per experimental group from a representative experiment. This experiment was repeated once with similar results. "IMC" designates cultures that included both MOPC-141 and UPC 10 isotype-matched MAbs. Percentages above the error bars indicate the level of inhibition compared with the isotype-matched MAb control. *, significantly different from sham control ($P < 0.05$).

DISCUSSION

Results presented here demonstrate that *B. burgdorferi* activates endothelium in a dose- and time-dependent manner. The consequences of this activation include increased expression of adhesion molecules on the endothelial cell surface and promotion of the transendothelial migration of neutrophils. These effects were obtained with as few as one spirochete per endothelial cell. Our results correlate well with the observation that there is an association between upregulated expression of E-selectin on vascular endothelium and the perivascular accumulation of neutrophils in the cutaneous acute erythema migrans of a Lyme disease patient (18). Schaible et al. (46) have demonstrated elevated expression of E-selectin, P-selectin, VCAM-1, and ICAM-1 on blood vessels of affected joint and heart tissue from severe combined immunodeficient mice and immunocompetent susceptible AKR/N mice infected with *B. burgdorferi*. The overall staining intensity on tissue sections increased with the time after infection and was also associated with the presence of spirochetes in the tissues. In addition, Koch et al. (34) observed upregulated expression of E-selectin, VCAM-1, and ICAM-1 in synovial tissues from patients with rheumatoid arthritis and osteoarthritis. This result indicates that E-selectin, which is usually associated with acute inflammatory reactions (33), also may be present in the context of chronic inflammatory disease.

The temporal pattern of adhesion molecule expression in response to *B. burgdorferi* parallels those produced by stimulation of HUVEC with cytokines in vitro (40, 42, 43) and varies from those produced by administration of cytokines or LPS in vivo (9, 22, 37, 38). In general, expression of endothelial adhesion molecules in vivo peaks earlier and remains elevated for a longer period of time. The more complex milieu of an inflammatory lesion may account for these differences. Böggemeyer et al. (8) have studied expression of adhesion molecules on a mouse endothelioma cell line (bEnd3) in response to a European tick isolate of *B. burgdorferi* (ZS7). In agreement with our results, levels of E-selectin, VCAM-1, and ICAM-1 were increased. Upregulation of the adhesion molecule P-selectin was also observed. However, maximal upregulation of these molecules was not observed until 50 h of incubation or later. These results are inconsistent with expression of endothelial adhesion molecules seen in response to cytokines or LPS either in vitro or in vivo (9, 22, 37, 38, 40–43). An explanation for these differing results may lie in the properties of geographically distinct isolates of *B. burgdorferi* or in the responsiveness of secondary human endothelial cell cultures, versus a murine endothelioma cell line, to spirochetes.

The stimulatory activity of *B. burgdorferi* appears to be intrinsic to the organism. The lack of activity of BSK medium controls excluded the possibility that exogenous LPS might be responsible for the activation. Moreover, heating the spirochetes at 56°C diminished the activation of HUVEC. The activity of LPS is stable to boiling and would not have been reduced under these conditions (25). This result also implies that spirochetes do not use an endogenous classical LPS for stimulating the endothelium. Finally, stimulation of HUVEC by spirochetes was observed in the absence of any contaminating cells, precluding secretion of cytokines by leukocytic cells as the mechanism of activation of endothelium.

Although Böggemeyer et al. (8) found that viable spirochetes and organisms disrupted by sonication or repetitive freezing and thawing exhibited similar activities, our results suggest that viability is important for the stimulation of HUVEC by *B. burgdorferi*. This conclusion is derived from the observation that heating at 56°C, repetitive freezing and thawing, or fixation with 3% paraformaldehyde completely eliminated the stimulatory activity of spirochetes. However, results with sonicated spirochetes, which retained partial activity, are at odds with this conclusion. It may be that the stimulatory activity is associated with internal components of the spirochete that require intact, viable organisms for secretion. However, preliminary data suggest that spirochete-conditioned HUVEC culture medium does not activate endothelium (47a). Alternatively, the stimulatory activity may be a surface-associated molecule, such as an outer surface lipoprotein, that is destroyed by some physical treatments but not by sonication. Experiments are under way in our laboratory to clarify the requirement for viability and to examine the potential role of spirochetal outer surface lipoproteins in activation of HUVEC.

Riley et al. (45) have demonstrated activation of vascular endothelium in response to intact *Treponema pallidum* or a 47-kDa integral membrane lipoprotein purified from this spirochete. Expression of ICAM-1 on the surface of treponeme-stimulated HUVEC is induced following 24 h of coincubation with ratios of 100 to 1,000 organisms per endothelial cell. Heating the treponemes at 56°C for 30 min ablates this activity. Activation is not observed in response to 10 treponemes per endothelial cell, indicating that HUVEC are more sensitive to stimulation by *B. burgdorferi* than by *T. pallidum.*

Activation of HUVEC by *B. burgdorferi* promoted the transendothelial migration of neutrophils. Optimal migration of neutrophils occurred after stimulation of endothelium with spirochetes for 8 h. Interestingly, this period of stimulation coincided with diminishing expression of E-selectin and near-maximal levels of VCAM-1 and ICAM-1, as measured by ELISA. In contrast, Munro et al. (38) observed that peak accumulation of neutrophils and maximal expression of E-selectin occur concomitantly 2 h after intracutaneous injection of baboons with *Escherichia coli* LPS. In agreement with our results, accumulation of neutrophils in baboons injected with TNF-α reaches a plateau between 6 and 9 h after treatment. This plateau occurs shortly after maximal expression of E-selectin is achieved and before expression of ICAM-1 peaks. In addition, increased expression of ICAM-1 at 9 h coincides with the onset of accumulation of mononuclear cells, which reach maximal levels at 24 h postinjection (37). In our study, the reduced capacity of HUVEC that were stimulated with spirochetes for 24 h to support transmigration of neutrophils may reflect the transition, seen in inflammatory lesions, from an infiltrate that is composed largely of neutrophils to one that is predominantly mononuclear. An alternative explanation may be that upregulation of adhesion molecules alone is insufficient to stimulate extravasation of leukocytes. Other factors, such as chemoattractants, may be required and perhaps are lacking after coincubation of spirochetes with HUVEC for 24 h.

Evidence for the involvement of spirochete-stimulated expression of adhesion molecules in extravasation of neutrophils is provided by the results of our blocking MAb studies. Pretreatment of spirochete-stimulated HUVEC with MAb against E-selectin or incubation of neutrophils with MAb against CD11/CD18, a ligand for ICAM-1, inhibited migration of neutrophils across HUVEC monolayers. Anti-E-selectin partially inhibited and anti-CD18 profoundly inhibited transmigration, with a combination of the two MAbs blocking migration nearly completely. A similar pattern of inhibition is seen when these MAbs are used to suppress migration of neutrophils across HUVEC stimulated with low concentrations (0.1 U/ml) of IL-1β (23). Upregulation of E-selectin also plays a role in binding of neutrophils to HUVEC infected with the obligate intracellular endothelial cell pathogen *Rickettsia rickettsii* (50). Expression of E-selectin peaks at 4 to 8 h postinfection. At-

tachment of neutrophils to HUVEC is maximal 6 to 8 h after infection and diminishes by 24 h, at which time E-selectin has returned to near basal levels. When HUVEC that have been infected with *R. rickettsii* for 8 h are incubated with MAb BB11 to E-selectin, adhesion of neutrophils is inhibited by 61%.

Our results indicate that *B. burgdorferi* is capable of directly stimulating endothelial cells to promote recruitment of leukocytes. However, it should be recognized that indirect mechanisms, involving host-derived factors, also may be important in vivo. For example, *B. burgdorferi* stimulates human monocyte/macrophages to produce IL-1β and TNF-α (16, 27, 28), both of which upregulate the expression of adhesion molecules and chemotactic cytokines by endothelial cells (11, 47). Further study will be required to delineate the relative contributions of direct and host-mediated activation of endothelium during infection with *B. burgdorferi*.

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