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Production of Interleukin-8 (IL-8) by Cultured Endothelial Cells in Response to *Borrelia burgdorferi* Occurs Independently of Secreted IL-1 and Tumor Necrosis Factor Alpha and Is Required for Subsequent Transendothelial Migration of Neutrophils

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Previous studies have shown that *Borrelia burgdorferi*, the spirochetal agent of Lyme disease, promotes inflammation by stimulating endothelial cells to upregulate adhesion molecules for leukocytes and to produce a soluble agent that is chemotactic for neutrophils. We determined that interleukin-8 (IL-8) was the chemotactic agent for neutrophils present in conditioned media from cultured human umbilical vein endothelial cells stimulated with *B. burgdorferi*. As few as one spirochete per endothelial cell stimulated production of IL-8 within 8 h of coincubation. When 10 spirochetes per endothelial cell were added, IL-8 was detected after 4 h of coculture. Production of IL-8 continued in a linear fashion for at least 24 h. Neutralizing antibodies against IL-8 reduced migration of neutrophils across spirochete-stimulated endothelial monolayers by 93%. In contrast, pretreatment of neutrophils with antagonists of platelet-activating factor did not inhibit migration. Increases in production of IL-8 and expression of the adhesion molecule E-selectin by endothelial cells in response to *B. burgdorferi* were not inhibited by IL-1 receptor antagonist or a neutralizing monoclonal antibody directed against tumor necrosis factor alpha, used either alone or in combination. These results suggest that activation of endothelium by *B. burgdorferi* is not mediated through the autocrine action of secreted IL-1 or tumor necrosis factor alpha. Rather, it appears that *B. burgdorferi* must stimulate endothelium either by a direct signaling mechanism or by induction of a novel host-derived proinflammatory cytokine.

To enter tissues during inflammation, leukocytes must first bind to and then traverse the endothelial monolayer that lines the blood vessel wall. The endothelium itself plays a critical role in regulating this process. Treatment of endothelium with the proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) upregulates the expression of adhesion molecules for leukocytes, such as vascular cell adhesion molecule 1, intercellular adhesion molecule 1, and E-selectin (39). Moreover, IL-1 and TNF-α stimulate endothelial cells to produce several members of the chemokine family of chemotactic cytokines. These include IL-8 and GRO proteins, which are chemotactic for neutrophils, as well as monocyte chemoattractant protein 1, which is chemotactic for monocytes (2).

Accumulation of leukocytes in infected tissues is a prominent histopathological feature of Lyme disease (12). Recent evidence suggests that activation of endothelium by the causative organism of Lyme disease, *Borrelia burgdorferi*, contributes to formation of these inflammatory infiltrates. Incubation of cultured endothelial cells with *B. burgdorferi* stimulates expression of adhesion molecules for leukocytes (6, 36) and production of a soluble chemotactic agent for neutrophils (35). Consequently, neutrophils adhere to and migrate across monolayers of human umbilical vein endothelial cells (HUVEC) that have been previously exposed to *B. burgdorferi* (36). *B. burgdorferi* does not contain classical lipopolysaccharide (LPS) (41), which is a potent activator of endothelial cells (2). Instead, stimulation of endothelium by this spirochete is mediated, at least in part, by its lipidated outer surface proteins (Osps) (35).

The phenotype of endothelium incubated with *B. burgdorferi* is thus quite similar to that of endothelium treated with IL-1 or TNF-α. Despite this similarity, we show herein that activation of HUVEC by *B. burgdorferi* is not dependent on secretion of either of these cytokines by the endothelium. We further identify IL-8 as the chemoattractant for neutrophils that is produced by HUVEC in response to *B. burgdorferi.*

MATERIALS AND METHODS

Culture of spirochetes. *B. burgdorferi* HBD1, originally isolated from human blood (3), was cultured at 33°C in serum-free Barbour-Stoenner-Kelly medium modified to minimize the content of LPS (36). Spirochetes (passages 40 to 53) were harvested during late-log-phase growth, centrifuged, and resuspended in medium 199 (M199; Life Technologies Inc., Grand Island, N.Y.) containing 20% heat-inactivated (heated for 30 min at 56°C) fetal bovine serum (HIFBS; HyClone Laboratories, Logan, Utah) and, in conditioned medium and enzyme-linked immunosorbent assay (ELISA) experiments, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.2). To control for the introduction of exogenous LPS, a sham preparation was made by subjecting a volume of uninoculated growth medium equal to the largest volume of spirochete culture used in each experiment to the same manipulations as the spirochetes themselves.

Endothelial cell cultures. Endothelial cells were isolated from human umbilical veins by collagenase perfusion and maintained in M199–20% FBS supplemented with 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 2 μg of amphotericin B per ml at 37°C (20, 36). After 3 to 5 days, cells from confluent cultures were trypsinized, pooled, and passaged onto tissue culture plates or amnion for use in experiments.

Antibodies and proteins. Monoclonal antibody (MAb) BB11, directed against E-selectin (5), was provided by Roy R. Lobb (Biogen Inc., Cambridge, Mass.). Neutralizing MAb to human TNF-α and goat neutralizing polyclonal antibody to human IL-8 were purchased from R & D Systems (Minneapolis, Minn.). Ac-

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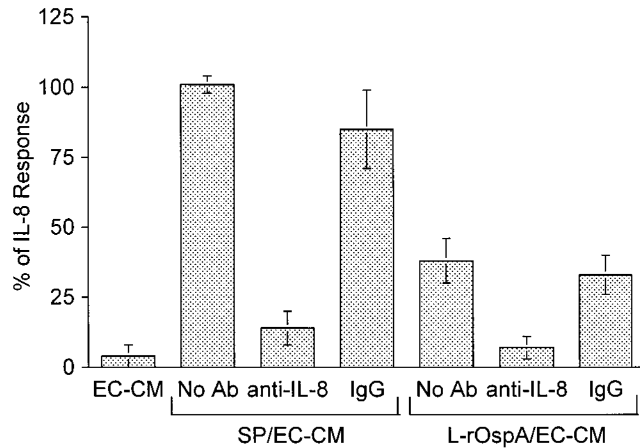

FIG. 1. IL-8 is the major soluble chemoattractant for neutrophils produced by HUVEC in response to *B. burgdorferi*. Conditioned media were collected from unstimulated HUVEC (EC-CM), HUVEC incubated with *B. burgdorferi* for 24 h at a ratio of 10 spirochetes per EC (SP/EC-CM), or HUVEC incubated for 24 h with 25 nM lipidated recombinant OspA from *B. burgdorferi* (L-rOspA/EC-CM). SP/EC-CM and L-rOspA/EC-CM were either untreated (No Ab) or incubated with 200 μg of anti-IL-8 antibody or control goat IgG per ml. Conditioned media were tested for chemotactic activity toward neutrophils in Boyden chambers. Results are presented as the percentage of the response to 5 nM IL-8. Bars represent the means ± SD of three replicate samples. This experiment was repeated once with similar results.

cording to the manufacturer, the anti-human IL-8 antibody neutralizes the activity of recombinant human IL-8, as measured by secretion of myeloperoxidase by neutrophils. Moreover, it prevented chemotaxis of neutrophils in response to recombinant human IL-8 but not the unrelated chemoattractant C5a (Sigma Chemical Co., St. Louis, Mo.) in Boyden chamber assays performed in our laboratory (data not shown). In ELISAs performed by the manufacturer, this antibody did not cross-react with any of 116 chemokines and other cytokines tested. All antibody preparations were purified immunoglobulin G (IgG). Purified goat IgG, obtained from Sigma, was used as a control. Recombinant human IL-6, IL-8, TNF-α, and IL-1 receptor antagonist (IL-1ra) were obtained from R & D Systems. Recombinant human IL-1β was supplied by Collaborative Biomedical Products (Bedford, Mass.). Lipidated recombinant OspA from *B. burgdorferi* B31 (13) was the gift of John J. Dunn (Brookhaven National Laboratories, Upton, N.Y.).

Quantitation of IL-8 and E-selectin. HUVEC plated at 2×10^5 cells per well in 24-well tissue culture plates were grown to confluence and incubated at 37°C with 1.0 ml of growth medium or test preparations for various times. Conditioned media were collected and centrifuged at $20{,}000 \times g$ for 30 min. Amounts of IL-8 in supernatants were measured with a commercial ELISA kit (R & D Systems). Levels of expression of E-selectin on HUVEC following experimental treatments were determined by using MAb BB11 in a whole-cell ELISA (36). To normalize the data within experiments, results were expressed as a percentage of the response to 0.1 U of IL-1β per ml.

To examine the roles of IL-1 and TNF-α in stimulation of endothelium by *B. burgdorferi*, some HUVEC monolayers were treated with IL-1ra and/or neutralizing MAb to TNF-α for 30 min at 37°C before the addition of aliquots of spirochetes, which also contained the inhibitors. IL-8 and E-selectin were then quantitated as described above. The inhibitors did not affect the number or viability of HUVEC.

Chemotaxis assay. Conditioned media collected from HUVEC (2×10^6 cells per 60-mm-diameter tissue culture plate) incubated with 5.0 ml of control medium or various spirochetal preparations for 24 h at 37°C were tested for their ability to induce chemotaxis of neutrophils in Boyden chamber assays (35). Neutrophils were isolated from venous blood of healthy adults by dextran sedimentation and density centrifugation with Ficoll-Hypaque gradient medium (36) and resuspended in M199–20% HIFBS. Conditioned medium from unstimulated HUVEC was used as a negative control, and the response of neutrophils to it was subtracted from the experimental values. Results are presented as the percentage of the response to 5 nM IL-8.

Neutrophil transendothelial migration assay. HUVEC were plated at a density of 1.5×10^5 cells per cm^2 on acellular connective tissue substrates prepared from human amnion and cultured for 7 to 10 days (15, 20). Confluent monolayers were washed, incubated with M199–20% HIFBS or various preparations for 8 h at 37°C, and again washed. Neutrophils (5×10^5 cells per cm^2) were added to the HUVEC monolayers for 30 min at 37°C. The cultures were then fixed in 10% buffered formalin, rinsed in saline, and stained with Wright stain. The total number of neutrophils associated with each tissue and the percentage of neutrophils that migrated beneath the endothelium were determined by light microscopy with whole mounts viewed en face and sections cut perpendicular to the plane of the HUVEC monolayer, respectively (36).

In some experiments, antibody to IL-8 was added both above and beneath HUVEC-amnion cultures, which were elevated on silicone rubber supports (17) to allow for better access of the antibody. To test the role of platelet-activating factor (PAF) in transmigration, neutrophils were pretreated with 0.1 mM of the PAF antagonists 1-*o*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (Calbiochem, La Jolla, Calif.) or PCA-4248 (Biomol, Plymouth Meeting, Pa.) for 10 min at 22°C. Because 95% ethanol was used to reconstitute the PAF antagonists, control samples in each experiment included a volume of 95% ethanol equal to the volume of antagonist added to treated neutrophils. Preliminary experiments demonstrated that the 95% ethanol did not affect transmigration of neutrophils. Migration of neutrophils across HUVEC in response to a chemotactic gradient of PAF (Sigma) was measured essentially as described previously (17). HUVEC-amnion cultures were elevated on silicone rubber supports, neutrophils were added to the apical sides of the cultures, and 10^{-7} M PAF in M199–20% HIFBS was placed below the cultures. Migration was assessed after 20 min of incubation.

Statistics. Raw data of experimental groups from ELISAs were subjected to an unpaired analysis of variance with the Tukey-Kramer multiple-comparison test. The means ± standard deviations (SD) of experimental groups from a Boyden chamber assay or transendothelial migration assay were subjected to an alternate Welch *t* test to determine a one-tailed *P* value. A P of <0.05 was used as the alpha value to determine statistical significance for all analyses.

RESULTS

IL-8 is the major soluble chemoattractant for neutrophils produced by spirochete-stimulated endothelium. As we reported previously, conditioned medium collected from HUVEC incubated with *B. burgdorferi* contains chemotactic activity, as measured by its ability to induce neutrophils to migrate into a microporous filter in a Boyden chamber apparatus (35). Experiments were performed to determine if IL-8 contributed to this chemotactic activity. Treatment of the conditioned medium with polyclonal antibody to IL-8 inhibited the migration of neutrophils by 87%, whereas control goat IgG had no significant effect. In the same experiment, the anti-IL-8 antibody also reduced migration of neutrophils in response to conditioned medium from HUVEC incubated with lipidated recombinant OspA from *B. burgdorferi* by 82% (Fig. 1).

To determine the kinetics of production of IL-8, conditioned media were collected from HUVEC incubated with *B. burgdorferi* at a ratio of 1 or 10 spirochetes per endothelial cell (EC) after 4, 8, 12, or 24 h (Fig. 2). As few as 1 spirochete per EC stimulated production of IL-8 following 8 h of coculture. When

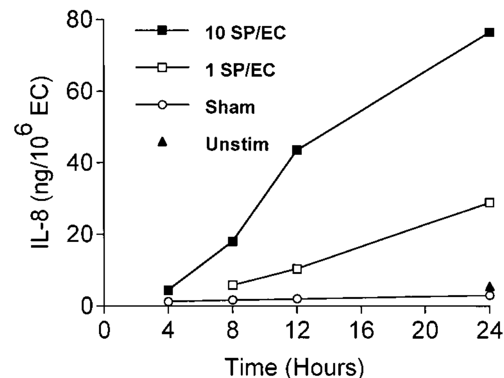

FIG. 2. Time- and dose-dependent production of IL-8 by HUVEC in response to *B. burgdorferi*. Conditioned media were collected from HUVEC incubated with medium alone (Unstim), sham preparations (Sham), or *B. burgdorferi* at a ratio of 1 or 10 spirochetes per EC (SP/EC) for the indicated times. Amounts of IL-8 were measured by ELISA. Data points represent the average value of two replicate samples. Replicates differed by an average of 10% and never by more than 25%. Time course studies were repeated twice with 10 spirochetes per EC and once with 1 spirochete per EC, yielding results similar to those shown in this figure.

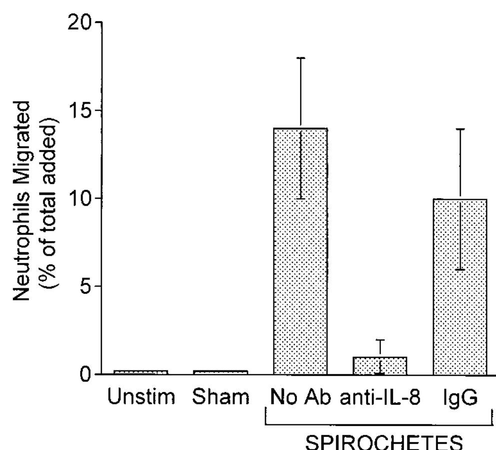

FIG. 3. Migration of neutrophils across spirochete-stimulated HUVEC is dependent on IL-8. HUVEC were incubated with M199–20% HIFBS (Unstim), sham preparations (Sham), or *B. burgdorferi* at a ratio of 10 spirochetes per EC (SPIROCHETES) for 8 h. *B. burgdorferi* was incubated with HUVEC either alone (No Ab), with 200 μg of anti-IL-8 antibody per ml, or with 200 μg of control goat IgG per ml. 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 samples. This experiment was repeated once with similar results.

10 spirochetes per EC were added, IL-8 was detected after 4 h of coculture. An additional experiment using a greater number of replicate samples confirmed that HUVEC incubated with 1 spirochete per EC produced significantly more IL-8 than HUVEC treated with sham preparations after 8 h of stimulation ($P = 0.0086$). When 10 spirochetes per EC were added, secretion of IL-8 was elevated compared to that of sham-treated control HUVEC after 4 h of incubation in two additional experiments. However, the difference was significant ($P = 0.0001$) in only one of these studies. In three separate experiments, HUVEC produced approximately threefold more IL-8 in response to 10 spirochetes per EC than they did in response to 1 spirochete per EC. HUVEC continued to produce IL-8 in response to spirochetes for at least 24 h. Production of IL-8 was variable among experiments. Of 10 experiments in which IL-8 levels were measured, one resulted in levels of secretion of IL-8 that were not more than double the levels in untreated HUVEC at any time point. In the other experiments, following 8 h of coculture at a ratio of 10 spirochetes per EC, amounts of IL-8 averaged 13.5 ± 8 ng per 10^6 EC and ranged from 4.8 to 33.2 ng per 10^6 EC. HUVEC incubated with either sham preparations or M199–20% HIFBS alone produced little IL-8 (Fig. 2).

Migration of neutrophils across spirochete-stimulated HUVEC monolayers is dependent on the production of IL-8 but not of PAF. Since incubation of HUVEC with *B. burgdorferi* promotes the transendothelial migration of neutrophils (36), the role of IL-8 in this process was investigated. Coincubation of HUVEC and spirochetes for 8 h resulted in the migration of 14% ± 4% of subsequently added neutrophils. When HUVEC were coincubated with spirochetes and antibody to IL-8, migration of neutrophils was inhibited by 93%. In contrast, treatment with control goat IgG did not significantly inhibit migration (Fig. 3).

The potential role of the lipid chemoattractant PAF was investigated by using specific receptor antagonists. The extent of migration of neutrophils treated with the PAF antagonist 1-*o*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine across spirochete-stimulated HUVEC did not differ significantly from that of untreated neutrophils. However, transendothelial migration of neutrophils in response to a chemotactic gradient of PAF was nearly completely inhibited by pretreatment of neutrophils with the PAF antagonist (Fig. 4). In an additional experiment, similar results were obtained with another PAF antagonist, PCA-4248 (data not shown).

Inhibition of IL-1 and TNF-α does not affect activation of HUVEC by *B. burgdorferi*. To determine if stimulation of HUVEC by spirochetes was mediated by the autocrine actions of IL-1 or TNF-α, we used a naturally occurring IL-1ra which binds to the same cellular receptors as IL-1 but does not result in subsequent signaling (11) and a neutralizing anti-TNF-α MAb. Production of IL-8 and expression of E-selectin were used as measures of activation of the HUVEC. The inhibitors, used alone or in combination, had no effect on the production of IL-8 in response to *B. burgdorferi* after 8 h of coincubation (Fig. 5A). However, in the same experiment, the IL-1ra inhibited secretion of IL-8 in response to 0.1 U of IL-1β per ml by 98% and the anti-TNF-α MAb inhibited its production in response to 0.1 ng of TNF-α per ml by 96%. When HUVEC were stimulated with a mixture of IL-1β and TNF-α at these concentrations, a combination of both inhibitors reduced production of IL-8 by 99% (Fig. 5B). The experiment illustrated in Fig. 5 was repeated, but the incubation time was extended to 24 h. Although the cytokine inhibitors were as effective against IL-1 and TNF-α as they were after 8 h of incubation, they again did not significantly reduce the amount of IL-8 produced by spirochete-stimulated HUVEC (data not shown).

Similarly, the IL-1ra and the anti-TNF-α MAb, used either alone or in combination, did not significantly inhibit the upregulation of E-selectin by HUVEC in response to spirochetes (Fig. 6). In the same experiment, upregulation of E-selectin by HUVEC in response to 0.1 U of IL-1β per ml was inhibited by 99%, and the anti-TNF-α MAb inhibited increased expression

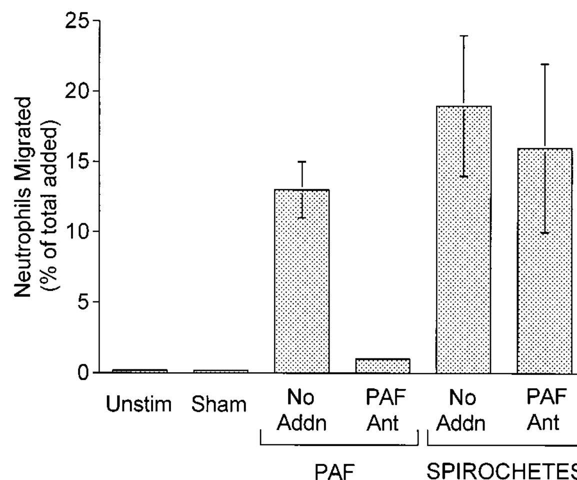

FIG. 4. The migration of neutrophils across spirochete-stimulated HUVEC is not the result of chemotaxis in response to PAF. HUVEC were incubated with M199–20% HIFBS (Unstim), sham preparations (Sham), or *B. burgdorferi* at a ratio of 10 spirochetes per EC (SPIROCHETES) for 8 h. Neutrophils were either untreated (No Addn) or treated with 100 μM PAF antagonist 1-*o*-hexadecyl-2-acetyl-*sn*-glycero-3-phospho-(*N*,*N*,*N*-trimethyl)-hexanolamine (PAF Ant) for 10 min prior to their addition to the *B. burgdorferi*-stimulated HUVEC. To test the efficacy of the PAF antagonist, untreated or treated neutrophils were also added to unstimulated HUVEC-amnion cultures that were then placed above medium containing 10^{-7} M PAF. Transendothelial migration was assessed as described in Materials and Methods. Bars represent the means ± SD of four to six replicate samples. This experiment was repeated with another PAF antagonist, PCA-4248, with similar results.

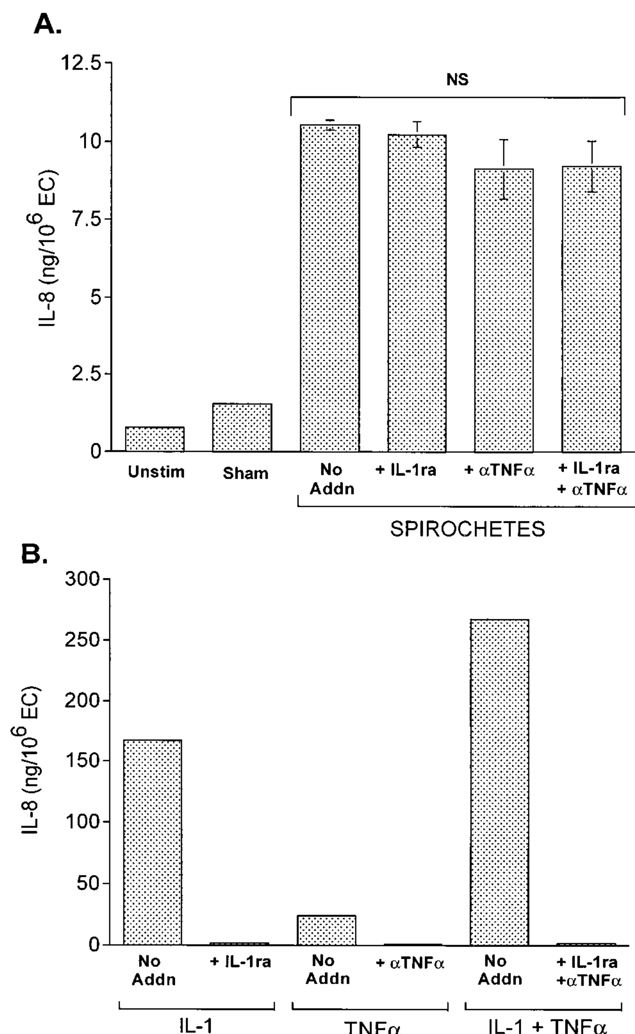

FIG. 5. Production of IL-8 by HUVEC in response to *B. burgdorferi* is not inhibited by IL-1ra or a MAb against TNF-α. (A) HUVEC were incubated with M199–20% HIFBS alone (Unstim), with sham preparations (Sham), or with *B. burgdorferi* at a ratio of 10 spirochetes per EC (SPIROCHETES) either alone (No Addn) or in the presence of 200 ng of IL-1ra per ml (+IL-1ra), 10 μg of anti-TNF-α neutralizing MAb per ml (+αTNFα), or a combination of both. (B) In the same experiment as that described for panel A, HUVEC were incubated with media containing IL-1, TNF-α, or a combination of both cytokines either alone or in the presence of inhibitors. After 8 h, conditioned media were collected, and IL-8 was measured by ELISA. Note the difference in the *y*-axis scale between panels A and B. The four rightmost bars in panel A represent means ± SD of three replicate wells. All other bars represent the average value of two replicate wells. NS, no significant differences among bracketed groups. IL-1ra (four experiments) and anti-TNF-α MAb (two experiments) were tested individually for their effect on production of IL-8 by HUVEC in response to *B. burgdorferi*, yielding similar results.

of E-selectin by HUVEC treated with 0.1 ng of TNF-α per ml by 91%. A combination of the two inhibitors reduced upregulation of E-selectin by HUVEC after incubation with both IL-1β and TNF-α by 90% (Fig. 6). Use of insufficient amounts of inhibitors cannot account for their inability to suppress activation of endothelium by *B. burgdorferi*, because secretion of IL-8 and upregulation of E-selectin by HUVEC in response to recombinant IL-1β and/or TNF-α were always greater than that seen in response to spirochetes (Fig. 5 and 6).

HUVEC produce IL-6 when stimulated with either sonicated preparations of *B. burgdorferi* or Osps purified from *B. burgdorferi* (29). Ma and Weis (29) found that approximately 2×10^5 HUVEC produce less than 0.5 ng of IL-6 after 24 h of incubation with 5 μg of sonicated *B. burgdorferi*. We therefore considered the possibility that IL-6 mediates activation of endothelium by these spirochetes. We found that conditioned medium collected from 2×10^5 HUVEC incubated with 10 ng of IL-6 per ml for 24 h did not promote chemotaxis of neutrophils in Boyden chamber assays. In addition, IL-6 at 10 or 100 ng per ml did not stimulate production of IL-8 by HUVEC as measured by ELISA (data not shown).

DISCUSSION

In this study, we show that the chemokine IL-8 is the predominant soluble chemoattractant produced by endothelium in response to *B. burgdorferi*. Moreover, production of IL-8 is required to promote the migration of neutrophils across spirochete-stimulated endothelium in vitro, which suggests that endothelium-derived IL-8 may play an important role in formation of the neutrophilic infiltrates that appear early during infection with *B. burgdorferi* in vivo (12, 21). Neutrophils are also seen in the synovial fluid of patients with Lyme arthritis (12). Although synovial fluid of such patients is chemotactic for neutrophils, IL-8 is not detected (18). In the joints, then, infection with *B. burgdorferi* apparently results in production of a chemoattractant for neutrophils that is distinct from IL-8. Production of IL-8 in response to *B. burgdorferi* has also been observed in cultures of human mononuclear cells, although exogenous LPS was not ruled out as a possible explanation for activation (34).

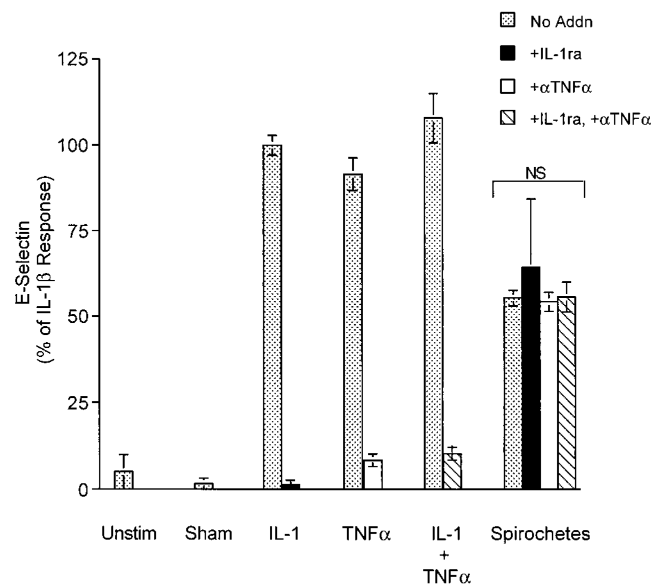

FIG. 6. Upregulation of E-selectin by HUVEC in response to *B. burgdorferi* is not inhibited by IL-1ra or a MAb against TNF-α. HUVEC were incubated with M199–20% HIFBS alone (Unstim), with sham preparations (Sham), or with *B. burgdorferi* at a ratio of 10 spirochetes per EC (SPIROCHETES) either alone (No Addn) or in the presence of 200 ng of IL-1ra per ml (+IL-1ra), 10 μg of anti-TNF-α neutralizing MAb per ml (+αTNFα), or a combination of both. To test the efficacy of the inhibitors, HUVEC were also incubated with media containing IL-1, TNF-α, or a combination of both cytokines either alone or in the presence of inhibitors. After 4 h, upregulation of E-selectin was measured by whole-cell ELISA. Bars represent means ± SD of four to eight replicate wells. NS, no significant differences among bracketed groups. IL-1ra (four experiments) and anti-TNF-α MAb (two experiments) were tested individually for their effect on upregulation of E-selectin in response to *B. burgdorferi*, yielding similar results.

We previously reported that the number of neutrophils migrating across HUVEC is maximal when the endothelium has been preincubated with *B. burgdorferi* for 8 h. After 24 h of preincubation, the number of emigrating neutrophils is greatly reduced. We had speculated that the reduction in migration might be due to lack of sufficient chemoattractants at later time periods (36). However, data presented in this study indicated that HUVEC exposed to spirochetes secreted IL-8 at a fairly constant rate for at least 24 h. Therefore, the inability of HUVEC stimulated with *B. burgdorferi* for 24 h to support migration of neutrophils is more likely due to temporal changes in expression of adhesion molecules (36) or to production of an inhibitor of IL-8-mediated chemotaxis. Such an inhibitor most probably would be bound to the endothelial amnion cultures rather than soluble, since conditioned media collected from spirochete-HUVEC cocultures after 24 h contained demonstrably active IL-8. Interestingly, the ability of HUVEC-amnion cultures treated with low concentrations (e.g., 0.1 U per ml) of IL-1 to promote traversal of neutrophils is also transient (14), suggesting that reduction of neutrophil migration across endothelium that has been stimulated for long time periods may be a general phenomenon.

HUVEC stimulated with IL-1 or TNF-α produce membrane-associated PAF, which has been implicated as a mediator in subsequent transmigration of neutrophils (25). However, antibody to IL-8 suppressed migration of neutrophils across spirochete-treated HUVEC nearly completely, indicating that no insoluble chemoattractants bound to the endothelium or matrix can substitute for IL-8. In addition, the ability of neutrophils to traverse HUVEC incubated with *B. burgdorferi* was not diminished by pretreating the leukocytes with antagonists of PAF, which demonstrated that PAF was not acting as a chemoattractant in our in vitro system. Nonetheless, a recent study indicates that PAF may play a role in Lyme disease, since a PAF antagonist reduces the inflammatory response in skin lesions of mice infected with *B. burgdorferi* (22).

Inhibitors of IL-1 and TNF-α did not prevent activation of HUVEC by *B. burgdorferi*, as measured by production of IL-8 and expression of the adhesion molecule E-selectin. Although we cannot rule out possible involvement of intracellular IL-1α (30), our results show that stimulation of endothelium by spirochetes occurs independently of secreted IL-1 and TNF-α. This finding was unexpected, given that endothelial cells are capable of producing both IL-1 (28) and TNF-α (1, 8) and respond to these cytokines in much the same way as they do to *B. burgdorferi* (2, 16, 36). Therefore, it seemed likely that the autocrine actions of IL-1 and TNF-α would mediate stimulation of HUVEC by *B. burgdorferi*. Furthermore, endothelium-derived IL-1 is at least partially responsible for activation of HUVEC infected with the spotted fever agents *Rickettsia rickettsii* (38) and *Rickettsia conorii* (23). However, stimulation of endothelium by a mechanism that is exclusive of IL-1 and TNF-α is not without precedent, since neither cytokine is an intermediary in LPS-induced expression of intercellular adhesion molecule 1 by HUVEC (27). Like *B. burgdorferi*, LPS also stimulates endothelial cells to secrete IL-8 (2). To our knowledge, the participation of IL-1 and TNF-α in this process has not been tested. However, when unfractionated blood or purified neutrophils are treated with LPS, IL-8 is secreted in two distinct stages: an initial phase, lasting up to 12 h after stimulation, that is independent of IL-1 and TNF-α, and a secondary phase, from 12 to 24 h, that involves both of these cytokines (7, 10). In contrast, our in vitro studies demonstrated that IL-1 and TNF-α did not contribute significantly to production of IL-8 by HUVEC in response to spirochetes during the first 24 h of coincubation. Nevertheless, we cannot rule out an autocrine role for these cytokines during later stages of infection in vivo.

Since HUVEC produce IL-6 in response to components of *B. burgdorferi* (29), we investigated the potential role of this cytokine in stimulation of HUVEC by spirochetes. We found that incubation of HUVEC for 24 h with levels of IL-6 approximately 20- to 200-fold higher than those reported to be produced in response to *B. burgdorferi* (29) did not result in the activation of HUVEC, as measured by production of IL-8. Although IL-1, TNF-α, and IL-6 share a number of biological activities (11), treatment of HUVEC with IL-6 does not promote adhesion of neutrophils (37). Together, these observations rule out a role for IL-6 in activation of HUVEC by *B. burgdorferi*. Rather, the production of IL-6 may serve to amplify inflammatory responses associated with Lyme disease through the activation of T and B lymphocytes (11).

How *B. burgdorferi* effects stimulation of HUVEC is an open question. Perhaps *B. burgdorferi* elicits production of an as-yet-unidentified host cytokine with functions that overlap those of IL-1 and TNF-α. Alternatively, direct binding of *B. burgdorferi* to endothelial cells (9, 40) might initiate the same signaling mechanisms that are used by IL-1 and TNF-α. Genes that are induced by IL-1 and TNF-α are regulated in part by nuclear factor (NF)-κB (26). In HUVEC, activation of NF-κB is necessary, but not sufficient, to induce expression of adhesion molecules in response to IL-1, TNF-α, or LPS (4, 31). The promoter for the IL-8 gene contains binding sites for both NF-κB and the transcription factor NF-IL-6. Experiments in which reporter gene constructs under the control of the IL-8 promoter were transfected into a human fibrosarcoma cell line suggest that activation of both these factors in response to IL-1 and TNF-α is required for expression of IL-8 (24, 32). It is therefore probable that treatment of HUVEC with *B. burgdorferi* results in activation of NF-κB as well as other transcription factors. Such activation of NF-κB has been recently demonstrated in human monocytic cells stimulated either with OspA purified from *B. burgdorferi* or with synthetic analogs of Osps (33).

B. burgdorferi induces production of IL-1 and TNF-α by monocytes/macrophages (19), and both of these cytokines have been detected in mice infected with this organism (21). Activation of endothelium by IL-1 and TNF-α is thus likely to contribute to the inflammation associated with spirochetal infection. However, our results indicate that stimulation of endothelium by a mechanism that is independent of secreted IL-1 and TNF-α may also be important in the pathogenesis of Lyme disease. Our data further suggest that endothelium-derived IL-8 plays a role in recruitment of neutrophils early in infection (12, 21). Whether other chemokines secreted by endothelium induce extravasation of mononuclear cells during the later stages of Lyme disease (12, 21) is under investigation in our laboratory.

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