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Borrelia burgdorferi Induces Chemokines in Human Monocytes

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Lyme disease is clinically and histologically characterized by strong inflammatory reactions that contrast the paucity of spirochetes at lesional sites, indicating that borreliae induce mechanisms that amplify the inflammatory response. To reveal the underlying mechanisms of chemoattraction and activation of responding leukocytes, we investigated the induction of chemokines in human monocytes exposed to *Borrelia burgdorferi* by a dose-response and kinetic analysis. Lipopolysaccharide (LPS) derived from *Escherichia coli* was used as a positive control stimulus. The release of the CXC chemokines interleukin-8 (IL-8) and GRO-α and the CC chemokines MIP-1α, MCP-1, and RANTES was determined by specific enzyme-linked immunosorbent assays, and the corresponding gene expression patterns were determined by Northern blot analysis. The results showed a rapid and strong borrelia-inducible gene expression which was followed by the release of chemokines with peak levels after 12 to 16 h. Spirochetes and LPS were comparably effective in stimulating IL-8, GRO-α, MCP-1, and RANTES expression, whereas MIP-1α production preceded and exceeded chemokine levels induced by LPS. Unlike other bacteria, the spirochetes themselves did not bear or release factors with intrinsic chemotactic activity for monocytes or neutrophils. Thus, *B. burgdorferi* appears to be a strong inducer of chemokines which may, by the attraction and activation of phagocytic leukocytes, significantly contribute to inflammation and tissue damage observed in Lyme disease.

Lyme borreliosis is a multisystem disease caused by infection with the spirochete *Borrelia burgdorferi* sensu lato (17, 25, 35, 43). Although spirochetes frequently can be isolated from various affected tissues, suggesting that the clinical symptoms and histopathological signs of inflammation are associated with the presence of borreliae at lesional sites (14), the low number of spirochetes clearly contrasts the strong local inflammatory reaction (10, 30, 31, 43). In Lyme arthritis, a vigorous synovial mononuclear infiltration and hypertrophy which is histologically indistinguishable from synovitis found in rheumatoid arthritis has been observed, but only few organisms were detectable (43). In addition, predominantly polymorphonuclear granulocytes (PMNs) are found in the joint fluid (2, 42). Thus, a *B. burgdorferi* infection appears to induce an effective chemotactic response which augments the local inflammation.

The site-directed immigration of leukocytes into inflamed tissue is provoked by gradients of chemokines that contribute to vascular adhesion, direct transendothelial migration, and movement through the extracellular matrix (16). Chemokines are potent chemoattractant cytokines (1, 26) that appear to be the main factors responsible for the recruitment of distinct effector cells during inflammatory diseases. Members of the CC chemokine subfamily, such as MIP-1α (macrophage inflammatory protein 1α), MCP-1 (monocyte chemotactic protein 1), and RANTES (regulated upon activation, normal T-cell expressed and secreted) preferentially attract monocytes and lymphocytes (33). The CXC chemokines which contain an ELR motif preceding the first cysteine, such as IL-8 (interleukin-8), NAP-2 (neutrophil-activating protein 2), and GRO-α (melanoma growth-stimulatory activity), are major neutrophil chemoattractants (22, 34). Among the many different cell types that have been shown to synthesize chemokines, activated monocytes and macrophages are the most efficient producer cells. Previously, we demonstrated that an infection of monocytes with influenza A virus or coxsackievirus virus B3 induced tumor necrosis factor alpha, IL-1, and IL-6 production (5, 13, 15, 24, 36) as well as a strong expression of mononuclear cell-attracting chemokines (40). Expression and release of proinflammatory cytokines and chemokines was strongly enhanced by lipopolysaccharide (LPS), which is known to be a powerful inducer of a wide variety of immunological mediators (6, 8). More specifically, *Treponema pallidum*- and *B. burgdorferi*-derived lipoproteins have been shown to be potent macrophage activators and inducers of proinflammatory cytokines (28). These observations indicate that the interaction of bacterial molecules and cells as well as the infection of host cells may induce the production and release of chemotactic activities such as chemokines.

The mechanisms by which leukocytes are attracted to lesional sites in *B. burgdorferi* infections are still incompletely understood. In particular, little attention has been directed to the chemokine family in the pathogenesis of Lyme disease. This study was designed to examine possible *B. burgdorferi*-derived intrinsic chemotactic factors and the induction of chemokines in borrelia-exposed human monocytes. The results demonstrate the stimulation of a strong chemokine response in monocytes, while spirochete-derived molecules were not chemotactic.

MATERIALS AND METHODS

Exposure of human monocytes to *B. burgdorferi*. Human monocytes were prepared from the buffy coat of healthy blood donors. After separation of the mononuclear cells by Ficoll-Hypaque density gradient centrifugation, the monocytes were enriched by elutriation to a purity of >90% as determined by nonspecific esterase staining or fluorescence-activated cell sorting analysis using fluorescein isothiocyanate-labeled anti-CD14 (Immunotech, Hamburg, Germany) (40). T cells were eliminated by rosetting to sheep erythrocytes (19, 20). Neutrophils were separated from erythrocytes by a 3% dextran sulfate sedimentation and a subsequent hypotonic lysis of the remaining erythrocytes as previously described (38, 39).

B. burgdorferi sensu stricto strain LW 2 was maintained in a modified Barbour-

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Stoenner-Kelly's medium (Sigma, Munich, Germany) (14). Spirochetes were washed in phosphate-buffered saline (PBS) and enumerated by dark-field microscopy. Human monocytes (0.5×10^6/ml) were exposed or mock exposed to *B. burgdorferi* at different monocyte/borrelia ratios (10:1 to 1:10) for 2, 4, 8, 16, and 24 h in RPMI 1640 culture medium (Life Technologies, Eggenstein, Germany) supplemented with 2 mM L-glutamine, 15 mM HEPES, and 10% pooled human AB serum (Sigma). LPS (10 ng/ml) from *Escherichia coli* O127:B8 (Difco, Detroit, Mich.) was used as a positive control stimulus for chemokine induction. Culture supernatants were collected at the above-mentioned time points and stored in aliquots at −70°C until further use.

Chemotaxis assay. Cell migration was assessed in quadruplicate, using a 48-well microchemotaxis chamber (Neuro Probe, Bethesda, Md.) consisting of two chambers separated by a porous polycarbonate filter (12). Culture supernatants from human monocytes exposed to *B. burgdorferi* at a ratio of 1:10 for 16 h and cell-free *B. burgdorferi* culture supernatants (mock control) were investigated for chemotactic activities. Aliquots (25 μl) of the test samples were placed into the lower chamber. fMLP (formyl-Met-Leu-Phe; 10^{-8} M; Sigma), or recombinant MCP-1 or IL-8 (50 ng/ml; IC Chemikalien, Ismaning, Germany) in RPMI 1640 medium was used as a positive control to assess monocyte or neutrophil chemotaxis, respectively. The upper chamber was filled with 50 μl of a freshly prepared monocyte or neutrophil cell suspension (2×10^6/ml). Both compartments were separated by a polycarbonate filter (Costar, Bodenheim, Germany) with 5-μm pores for examining monocyte chemotaxis. After incubation at 37°C for 90 min, the filter was removed, fixed in methanol, and stained with hematoxylin (Sigma). The total number of migrated monocytes per well was densitometrically evaluated on stained filters by a computer-assisted imaging system (Vilber Lourmat; distributed by Fröbel, Wasserburg, Germany). For neutrophil chemotaxis assays, a polyvinylpyrrolidone-containing polycarbonate filter with 3-μm pores was used to prevent adherence of the migrated cells. The number of neutrophils attracted into the medium of the lower chamber was quantitated enzymatically by determining β-glucuronidase activity after lysis of the cells (conversion of *p*-nitrophenyl-β-D-glucuronide; Sigma).

Determination of chemokines. Chemokine levels were determined by specific sandwich enzyme-linked immunosorbent assays (ELISAs) developed in our laboratory (41). Briefly, 96-well microtiter plates (Maxisorp; Nunc, Wiesbaden, Germany) were coated with a monoclonal antibody in PBS specific for IL-8 (IC Chemikalien), MCP-1, GRO-α, RANTES (all from R&D Systems, Wiesbaden, Germany), or MIP-1α (Promega; distributed by Serva, Heidelberg, Germany), respectively. Plates were blocked with 1% bovine serum albumin in PBS and stored sealed at 4°C. Samples (100 μl per well) were incubated at room temperature for 1 h. After three washes with 0.1% Tween 20 in PBS, a specific polyclonal antibody of goat or rabbit origin was added in the same buffer and incubated at room temperature for another hour. All polyclonal antibodies were from R&D Systems except those for IL-8, which were purchased from IC Chemikalien. Detection was performed with a peroxidase-conjugated third antibody (donkey anti-goat or donkey anti-rabbit, both from Dianova, Hamburg, Germany) and subsequent conversion of *o*-phenylenediamine dihydrochloride substrate. The optical density of the samples was determined photometrically at 490 nm and plotted against a standard curve performed with the respective recombinant chemokines (purchased from IC Chemikalien). The sensitivities of the established ELISAs were <10 pg/ml for IL-8 and GRO-α, 20 pg/ml for RANTES and MIP-1α, and 50 pg/ml for MCP-1. Intra- and interassay variances were less than 5% (41).

Statistics. The results of the chemotaxis assays and ELISAs were expressed as means ± standard deviations (SD) of three to four independently performed cultures. They were compared with control values by the Student's *t* test and considered significantly different at *P* values of <0.05.

RNA preparation and Northern blot analysis. Total RNA was prepared by a modified guanidine thiocyanate method as previously described in detail (37). Five micrograms of total RNA was denatured by glyoxal-dimethyl sulfoxide treatment and separated on 1% agarose gels. The RNA was capillary blotted by 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany). After UV crosslinking, hybridization was performed under continuous rotation in a hybridization oven (Biometra, Göttingen, Germany). The membranes were hybridized with digoxygenin (DIG)-labeled antisense riboprobes overnight under highly stringent conditions in 50% formamide at 68°C and finally washed in 0.1× SSC–0.1% sodium dodecyl sulfate at the same temperature. Bound DIG-labeled riboprobes were visualized nonradioactively by using a DIG nucleic acid detection kit (Boehringer Mannheim) and CDP-Star chemiluminescence substrate (Tropix, Bedford, Mass.; distributed by Serva).

Generation and labeling of the riboprobes. Probes, 300 to 400 bp long, corresponding to human MIP-1α, MCP-1, GRO-α, and IL-8, were generated by reverse transcription-PCR and subsequent cloning of the respective PCR products. Briefly, 1 μg of total RNA from LPS-stimulated human monocytes was oligo(dT) primed and reverse transcribed with Superscript II reverse transcriptase (Life Technologies). The cDNA was amplified by specific forward and reverse primers containing artificial restriction sites at their 5′ ends by SuperTaq DNA polymerase (Stehelin, Basel, Switzerland). The amplified DNA was gel purified, extracted by Qiaex (Qiagen, Hilden, Germany), digested with *Bam*HI and *Eco*RI, and finally site-directed cloned into the respective sites of pBluescript SK$^-$ (Stratagene, La Jolla, Calif.). The specificity of the inserts was confirmed by sequencing. DIG-labeled sense and antisense riboprobes were generated with T3 or T7 RNA polymerase by using a DIG-RNA labeling kit (Boehringer Mannheim) with 1 μg of linearized vector as a template. Labeling efficiency was examined by dot blot analysis.

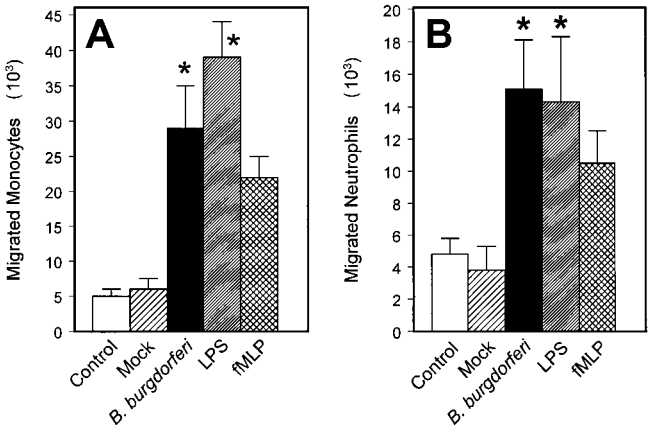

FIG. 1. Monocyte and neutrophil chemotaxis to supernatants of human monocytes exposed to *B. burgdorferi*. Human monocytes (0.5×10^6) were untreated (control), stimulated with LPS (10 ng/ml), or exposed to *B. burgdorferi* at a ratio of 1:10. The supernatants were harvested after 16 h and analyzed for monocyte- or neutrophil-specific chemotactic activities in a microchemotaxis assay. Borrelia culture supernatants (Mock) were used in parallel to test for intrinsic bacterium-derived chemotactic activities. fMLP (10^{-8} M) was used as a positive control for chemotaxis. The number of migrated cells was determined densitometrically (monocytes [A]) or enzymatically (neutrophils [B]) as described in Materials and Methods. Values represent the means ± SD of identically prepared quadruplicate cultures. *, significantly different from the untreated control ($P < 0.01$).

RESULTS

Chemoattraction of leukocytes by factors released from human monocytes exposed to *B. burgdorferi*. Chemotaxis assays were performed to screen for borrelia-derived or borrelia-induced chemotactic factors and to identify the leukocyte populations that were responsive to the factors. Both freshly prepared monocytes and granulocytes were strongly attracted by factors released from *B. burgdorferi*-activated monocytes (Fig. 1). Totals of $(29 \pm 6) \times 10^3$ monocytes and $(15.1 \pm 3) \times 10^3$ granulocytes (means ± SD of four independently performed experiments) migrated through the polycarbonate filters of the chemotaxis chambers (spontaneous migration was in the range of $[5 \pm 1] \times 10^3$ cells from a stock of 100×10^3 per well). These results were comparable to those obtained with LPS-stimulated monocyte supernatants and clearly exceeded the number of cells chemoattracted by fMLP. In contrast, *B. burgdorferi* culture supernatants did not induce chemotaxis, and the number of migrating cells did not differ from that in experiments performed with medium alone, thus excluding that *B. burgdorferi*-derived factors acted as chemoattractants for monocytes or neutrophils. To distinguish between directional chemotaxis and random chemokinesis, equal concentrations of borrelia-induced supernatants were added to both sides of the polycarbonate filter. Under these conditions, the migration of leukocytes was arrested, indicating that chemotaxis and not random chemokinesis had taken place.

Kinetics of neutrophil-attracting chemokine production. The positive results of the neutrophil chemotaxis assays (Fig. 1B) indicated the production of CXC chemokines. Therefore, the kinetic release of IL-8 and GRO-α was investigated by ELISA after exposure of human monocytes to *B. burgdorferi* at a ratio of 1:10 for 2, 4, 8, 16, and 24 h. A rapid and strong

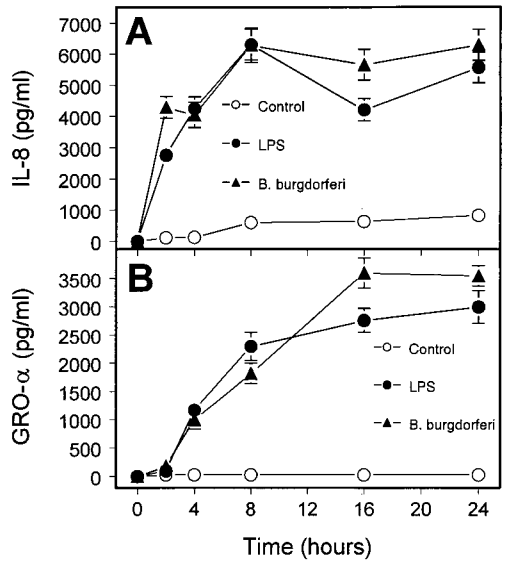

FIG. 2. Kinetics of CXC chemokine production by human monocytes. Monocytes (0.5×10^6) were either untreated, stimulated with LPS (10 ng/ml), or exposed to *B. burgdorferi* at a ratio of 1:10. IL-8 (A) and GRO-α (B) production was determined by specific ELISAs at the indicated time periods. Values represent the means ± SD of three identically performed experiments.

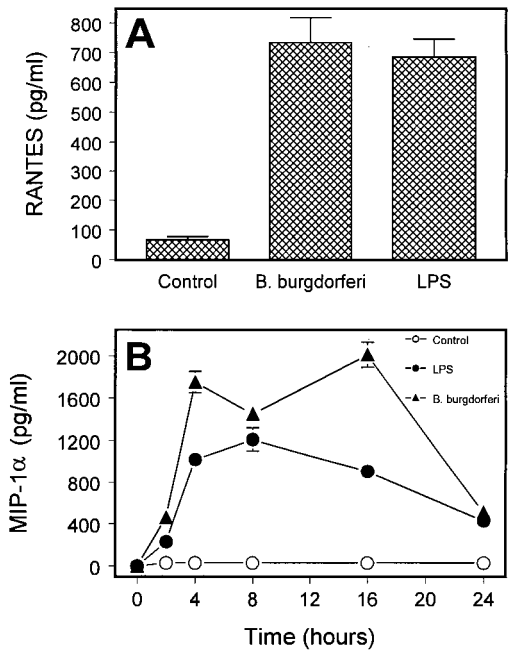

FIG. 3. CC chemokine release after exposure of human monocytes to *B. burgdorferi*. The experiments were performed as described for Fig. 2. RANTES levels (A) were determined by specific ELISA and are shown at 16 h poststimulation. The kinetic release of MIP-1α is shown in panel B. Values represent the means ± SD of three identically performed cultures.

release of both neutrophil chemoattractants was observed (Fig. 2). As soon as 2 h after exposure to *B. burgdorferi*, significantly elevated levels of IL-8 were found in the supernatants compared with the unexposed control monocytes ($P < 0.01$). GRO-α levels of comparable significance were first detected 4 h after stimulation. Peak levels were found for IL-8 8 h after stimulation, while GRO-α levels peaked after 16 to 24 h. As already indicated by the chemotaxis assays, the borrelia-inducible IL-8 and GRO-α release was similar to that after stimulation of human monocytes with LPS (Fig. 2). We obtained comparable results when the monocytes were exposed to lower doses of *B. burgdorferi* (monocyte/borrelia ratio of 5:1).

Induction of CC chemokines. The finding that monocytes migrated in response to factors released from borrelia-exposed cells (Fig. 1A) prompted the specific analysis of MIP-1α, MCP-1, and RANTES release. The results for the induction of the monocyte and lymphocyte chemoattractants MIP-1α and RANTES are shown in Fig. 3. A significant induction of RANTES with maximum concentrations of 800 pg/ml was found 16 h after exposure of human monocytes to *B. burgdorferi* (Fig. 3A; $P < 0.01$ in comparison with the untreated control). Significantly elevated levels of MIP-1α were detected after 2 h (Fig. 3B; $P < 0.01$ compared with the untreated control) and reached maximum values of more than 2,000 pg/ml after 4 h. The onset was earlier and the total amount of released MIP-1α was higher than after stimulation of monocytes with LPS ($P < 0.05$ after 2, 4, and 16 h). MCP-1 release was delayed, starting 8 to 12 h after exposure, and peak levels of 5,000 pg/ml were reached after 16 h. In contrast to MIP-1α, the optimal dose for an efficient induction of MCP-1 was found at a monocyte/borrelia ratio of 5:1 (Fig. 4).

Dose-dependent chemokine release. Human monocytes were exposed to graded ratios of *B. burgdorferi* (monocyte/*B. burgdorferi* ratios of 10:1, 5:1, 1:1, 1:2, and 1:10) for 16 h, and the dose-dependent release of the chemokines MCP-1, MIP-1α, and IL-8 was determined by ELISA (Fig. 4). Very low ratios (10:1 to 5:1) were sufficient to induce the release of high MCP-1 and IL-8 levels ($P < 0.001$ compared with the control), while the dose of *B. burgdorferi* required to stimulate a significant production of MIP-1α was higher (1:2 to 1:10; $P < 0.001$). Interestingly, stimulation of monocytes with higher doses of *B. burgdorferi* (1:10 [Fig. 4]) resulted in the production of only low amounts of MCP-1 that were in the range of background levels. IL-8 release was also considerably lower, while the release of MIP-1α peaked at the highest monocyte/borrelia ratios.

Chemokine gene expression in borrelia-exposed monocytes. To elucidate the underlying molecular mechanisms upregulating chemokine production, we studied chemokine gene expression by Northern blot analysis 8 h after exposure of human monocytes to *B. burgdorferi* at a ratio of 1:10. We found a strongly inducible MIP-1α, IL-8, and GRO-α mRNA accumulation (Fig. 5). The borrelia-induced MCP-1 expression was only slightly higher than in the untreated control. However,

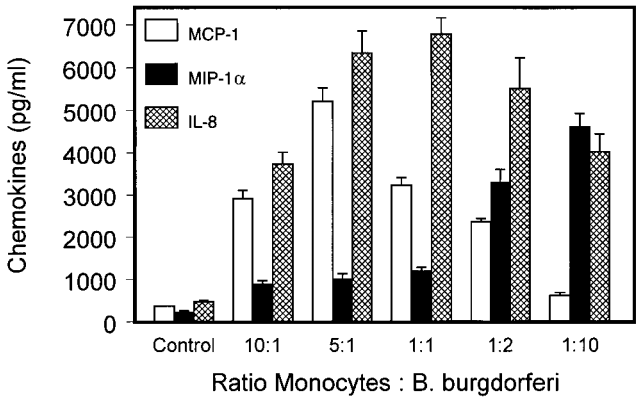

FIG. 4. Dose-dependent release of chemokines. Human monocytes (0.5×10^6/ml) were exposed to graded ratios of *B. burgdorferi* (monocyte/*B. burgdorferi* ratios of 10:1, 5:1, 1:1, 1:2, and 1:10) for 16 h, and the dose-dependent release of the chemokines MCP-1, MIP-1α, and IL-8 was determined by ELISA. Values represent the means ± SD of three identically prepared cultures.

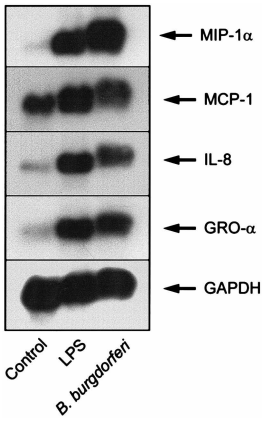

FIG. 5. Chemokine gene expression after exposure of human monocytes to *B. burgdorferi*. Monocytes (10^7) were either untreated (Control) or exposed to 10 ng of LPS per ml or to *B. burgdorferi* at a monocyte/*B. burgdorferi* ratio of 1:10. After 8 h, 5 μg of total RNA was analyzed for MIP-1α, MCP-1, IL-8, GRO-α, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression by DIG-labeled riboprobes. One representative Northern blot analysis of three is shown.

this was due to a relatively strong constitutive MCP-1 expression in the untreated control, which can be occasionally found as a donor-specific variation (9). This observation parallels the results shown in Fig. 4, where the highest doses of *B. burgdorferi* failed to induce a maximal MCP-1 release. LPS as a strong inducer of chemokine gene expression increased chemokine mRNAs to similar levels, suggesting that *B. burgdorferi* alone was capable of inducing an almost maximal expression of chemokine genes.

DISCUSSION

A hallmark of inflammatory tissue reactions is the recruitment and activation of leukocytes. Cytokines with chemotactic activities (chemokines) play a pivotal role in mediating these events. Chemokines are produced by various cells, including epithelial cells, endothelial cells, fibroblasts, and monocytes (1, 26). In infectious diseases, the interaction of microorganisms with the host cells can directly induce the release of cytokines and chemokines (5, 13, 15, 24, 36, 40). One of the strongest bacterium-derived stimulators of cytokine expression in monocytes is LPS.

In Lyme borreliosis, heavy inflammatory infiltrates dominated by mononuclear cells are typically found at lesional sites (35). Even in SCID (severe combined immunodeficiency) mice, infection with *B. burgdorferi* leads to arthritic symptoms similar to those seen in normal mice (32), suggesting a lymphocyte-independent pathogenesis in which cells of the innate response and proinflammatory mediators play a major role. In contrast to the strong inflammatory response, only a few spirochetes are detectable in affected tissues (7, 10, 30, 43). Although it is tempting to assume that the spirochetes induce mechanisms that amplify the local inflammatory response, the underlying mechanisms are not yet identified. Previous in vitro studies by our group and by others have demonstrated that *B. burgdorferi* strongly activates monocytes which consecutively form cell aggregates and release proinflammatory cytokines including IL-1β, IL-6, and tumor necrosis factor alpha (28, 30). The outer surface lipoprotein A (OspA) was identified as a main borrelia-derived factor with cytokine-inducing activity. However, since these cytokines lack chemotactic properties, they can contribute directly neither to the generation of mononuclear infiltrates in the synovium nor to the immigration of predominantly PMNs into the joint fluid as found in vivo after *B. burgdorferi* infection. To investigate the pathogenetic role of chemoattractants in Lyme borreliosis, the present in vitro study addressed the question of whether *B. burgdorferi* produced factors with chemotactic activity or otherwise was able to induce the release of chemokines from human monocytes. Monocytes were of particular interest, since their increasing accumulation and activation may provide a self-perpetuating source for the release of chemotactic activities. The results showed that culture supernatants from *B. burgdorferi* LW 2 were chemotactically inactive (Fig. 1). Thus, unlike a wide range of bacteria which produce fMLP-like chemotactic factors, for which granulocytes and monocytes bear specific fMLP receptors (18, 23), *B. burgdorferi* does not release such factors.

Monocytes exposed to *B. burgdorferi* strongly expressed chemokine mRNA and released high levels of both CC and CXC chemokines. The highest levels of MCP-1 were inducible by the lowest applied doses (Fig. 4), suggesting a differential, dose dependently regulated chemokine gene expression. In addition, this finding suggests MCP-1 as an early borrelia-inducible mononuclear cell attracting stimulus at the onset of the disease. The strong borrelia-inducible production of neutrophil- as well as mononuclear cell-attracting chemokines may offer an explanation for cellular recruitment to lesional sites and the consecutive inflammatory reaction. Our results are supported by a recently published PCR analysis demonstrating the upregulation of chemokine gene expression in human endothelial cells after stimulation with *B. burgdorferi* ultrasonicates (11). However, in contrast to our findings, MIP-1α was not induced in borrelia-activated endothelial cells, which may reflect tissue-specific differences of chemokine expression. In our hands, no differentially inducible chemokine pattern was identified, which is consistent with the accumulation of mononuclear cells in the synovium and PMNs in the joint effusions during human Lyme arthritis (2, 42). In addition to its potent neutrophil-attracting activity, IL-8 may act as a T-cell chemoattractant (21, 27, 45). The predominant attraction of neutrophils into the joint fluid may play a significant role in the acute phase of the disease. At present, the molecular mechanisms responsible for the different localization of mononuclear cells and PMNs in Lyme arthritis are unknown. Immunohistochemical analyses are in progress to investigate a possible compartmentalized differential chemokine expression. Mice with defective neutrophil functions develop more severe arthritic symptoms and more spirochetes are present (3), which points to an important role of PMNs in innate antispirochete immunity.

The mechanisms by which *B. burgdorferi* activates monocytes to produce cytokines have not been fully clarified; however, since *B. burgdorferi* lacks LPS (44), then LPS cannot be responsible for the inflammatory response in Lyme borreliosis. Other bacterial molecules or the activation of monocytes by phagocytosing the spirochetes are probably involved. Many proinflammatory cytokine genes are targets for the transcription factor NF-κB, which seems to play a pivotal role in the concerted regulation of an immune response (4). Recently, Wooten et al. demonstrated that in human endothelial cells, the *B. burgdorferi*-specific lipoprotein OspA was capable of inducing the nuclear translocation of NF-κB and the production of cytokines whose transcription is NF-κB dependent, such as IL-6 or IL-8 (46). These findings are in line with our own experiments demonstrating that not only *B. burgdorferi* but also OspA induced in vitro clustering of monocytes, though less effectively than the whole bacteria (29). However, a receptor for OspA has not yet been defined. Thus, it is tempting to speculate that borrelia lipoproteins may be involved in the direct induction of chemokines. Studies are currently in progress to address this issue.

In conclusion, our data demonstrate that *B. burgdorferi* is a potent inducer of a wide variety of chemokines which may significantly contribute to leukocyte recruitment, inflammation, and tissue damage observed in Lyme disease.

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