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Anaplasma phagocytophilum-Borrelia burgdorferi Coinfection Enhances Chemokine, Cytokine, and Matrix Metalloprotease Expression by Human Brain Microvascular Endothelial Cells[▽]

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***Borrelia burgdorferi* and *Anaplasma phagocytophilum* coinfect and are transmitted by *Ixodes* species ticks. Clinical indicators suggest that *A. phagocytophilum* coinfection contributes to the severity, dissemination, and, possibly, sequelae of Lyme disease. Previous in vitro studies showed that spirochete penetration through human brain microvascular endothelial cells of the blood-brain barrier is facilitated by endothelial cell-derived matrix metalloproteases (MMPs). *A. phagocytophilum*-infected neutrophils continuously release MMPs and other vasoactive biomediators. We examined *B. burgdorferi* infection of brain microvascular barriers during *A. phagocytophilum* coinfection and showed that coinfection enhanced reductions in transendothelial electrical resistance and enhanced or synergistically increased production of MMPs (MMP-1, -3, -7, -8, and -9), cytokines (interleukin 6 [IL-6], IL-10, and tumor necrosis factor alpha), and chemokines (IL-8 and macrophage inflammatory protein 1α) known to affect vascular permeability and inflammatory responses.**

Lyme disease is the most frequently reported arthropod-borne infection in North America and Europe (12, 35). The bacteria which are transmitted to humans by the bites of infected *Ixodes persulcatus* complex ticks can spread to the skin, heart, joints, eyes, and, in addition, the peripheral and central nervous systems (CNS) (40, 51). As the diversity of clinical presentations for Lyme disease has been recognized, some have suggested that concurrent infections by other tick-borne pathogens could influence the natural course of disease, leading to more severe infection, persistence, and even refractoriness to effective therapies (3). A prime candidate as a potential influence on the clinical manifestations of Lyme disease is *Anaplasma phagocytophilum*, the causative agent of human granulocytic anaplasmosis (HGA). Like *Borrelia burgdorferi*, *A. phagocytophilum* is transmitted by *I. persulcatus* complex tick bites, and increasing amounts of data show that coinfection is not infrequent (16, 48). Coinfection that results in simultaneous clinical manifestations is well documented (31). At least five clinical studies provide evidence that coinfections contribute to enhanced morbidity and clinical manifestations lengthier than those observed with Lyme disease or HGA alone (4–6, 28, 36, 41).

Experimental coinfections in the mouse model reveal modified immunological responses to both pathogens associated with higher bacterial burdens, longer persistence, and worsened disease (23, 47, 52). As penetration into and out of the bloodstream are obligatory events for the dissemination of *B. burgdorferi* and *A. phagocytophilum*, their interactions at the level of the blood-endothelial cell interface are likely to be critical (20). We recently showed that, for the human cerebrospinal fluid isolate *B. burgdorferi* 297 (30), penetration through endothelial cells is facilitated by the actions of endothelial cell-derived matrix metalloproteases (MMPs) (20, 22). Moreover, we showed that *A. phagocytophilum*-infected neutrophils protractedly produce biologically active molecules, including chemokines, cytokines, and MMPs (14, 15). With the concept that *A. phagocytophilum*-infected neutrophil-derived products would increase spirochete spread, we found that *A. phagocytophilum*-infected neutrophils augment the trans-endothelial cell migration of *B. burgdorferi*, suggesting that increased blood and tissue spirochete loads also occur by a mechanism not dependent on adaptive immune response (32).

Since the major candidates as biological mediators for enhanced *B. burgdorferi* penetration of human brain microvascular endothelial cell (BMEC) barriers include MMPs, cytokines, and chemokines, we examined whether in vitro coinfection with *B. burgdorferi* and *A. phagocytophilum*-infected human neutrophils would (i) induce MMPs and cytokines known to affect endothelial barrier integrity or (ii) enhance in vitro vascular permeability, measured by transendothelial electrical resistance (TEER) since permeability in human BMECs (and epithelial cells) is inversely proportional to TEER (1, 24, 34, 50).

MATERIALS AND METHODS

The spirochetes. Low-passage (less than five in vitro passages) *B. burgdorferi* was cultured at 34°C in Barbour-Stoenner-Kelly II medium containing 10% rabbit serum as described by Barbour (2). In our study, we used *B. burgdorferi* 297, a strain originally isolated from human cerebrospinal fluid (30). The bacteria were examined for motility with a dark-field microscope to verify their viability and that the organisms were thoroughly dispersed at the start of all the assays. *Borrelia burgdorferi* quantification was performed by using quantitative real-time PCR targeting the single-copy chromosomal *flgB* (29). Amplifications were performed using a Bio-Rad iCycler iQ5 multicolor real-time PCR detector (20).

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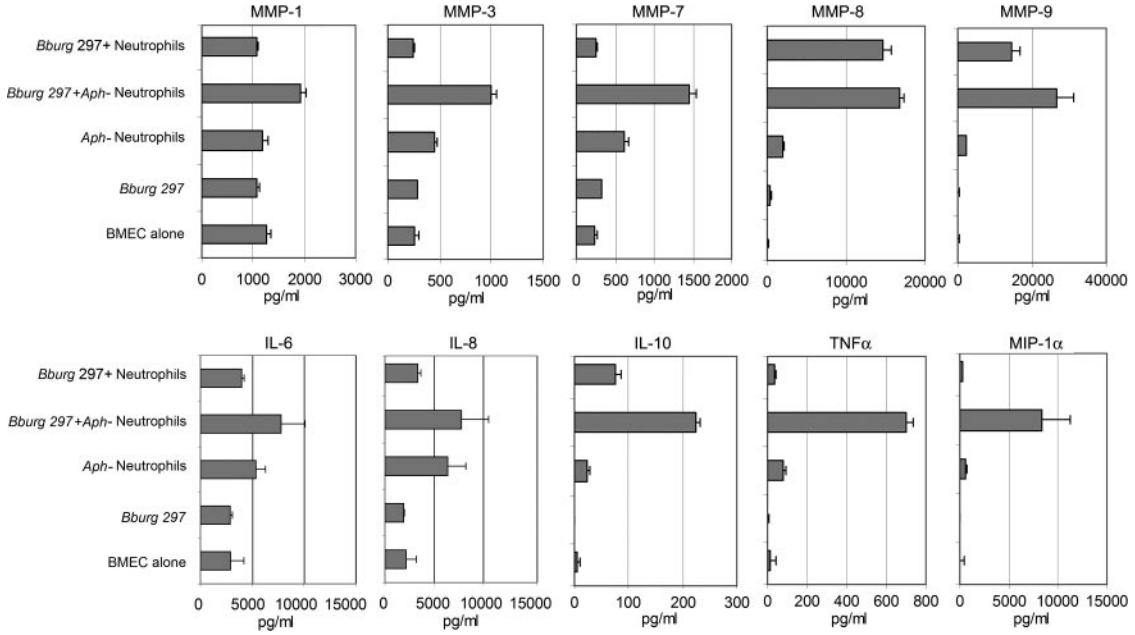

FIG. 1. MMP, cytokine, and chemokine expression. In the presence of both *A. phagocytophilum* and *B. burgdorferi*, MMP-1, -3, -7, -8, and -9 and IL-10, TNF-α, and MIP-1α were sometimes synergistically induced compared to their levels of induction with each pathogen alone, while the effects on IL-6 and IL-8 secretion were additive. The synergism for MMP-8 and MMP-9 with uninfected neutrophils and *B. burgdorferi* was significantly less than under conditions of coinfection. Comparisons of expression levels, including for *A. phagocytophilum*-infected neutrophils (*Aph*-Neutrophils) versus *B. burgdorferi* 297 (*Bburg* 297); both *A. phagocytophilum*-infected neutrophils and *B. burgdorferi* 297 (*Bburg* 297 +*Aph*-Neutrophils) versus both uninfected neutrophils and *B. burgdorferi* 297; and both *A. phagocytophilum*-infected neutrophils and *B. burgdorferi* 297 versus *B. burgdorferi* 297 alone, resulted in statistically significant differences ($P < 0.02$, one-sided Student's t test) except for MMP-1 (*A. phagocytophilum*-infected neutrophils versus *B. burgdorferi* 297) and MMP-2 (both *A. phagocytophilum*-infected neutrophils and *B. burgdorferi* 297 versus both uninfected neutrophils and *B. burgdorferi* 297 and both *A. phagocytophilum*-infected neutrophils and *B. burgdorferi* 297 versus *B. burgdorferi* 297 alone). The results shown (means ± standard deviations) are representative of the results of duplicate studies.

***Anaplasma phagocytophilum*-infected neutrophils.** Neutrophils, obtained from the peripheral blood of healthy donors under a protocol approved by the Johns Hopkins School of Medicine institutional review board, were isolated and infected overnight with *A. phagocytophilum* Webster strain (13). Romanowsky staining (Hema-3; Fisher, Middletown, VA) was used to confirm that >90% of the neutrophils were infected (13).

The human BMECs. A human BMEC cell line whose phenotypic expression was stabilized by immortalizing the cells with pSVT, a pBR322-based plasmid containing the DNA sequence encoding the simian virus 40 large-T antigen (44), was used in these studies. Similar to the primary human BMEC cell line (XIII) from which they were derived, the transfected human BMECs are positive for FVIII-Rag, carbonic anhydrase IV, and *Ulex europeus* agglutinin I; take up acetylated low-density lipoprotein; and express gamma glutamyl transpeptidase (43, 44). Human BMECs were cultured at 37°C in medium 199 (GIBCO) supplemented with 20% heat-inactivated fetal bovine serum and 1× Glutamax (GIBCO) in a humidified environment of 95% air, 5% CO_2.

In vitro coinfection. Human BMECs were grown to confluence on 24-well tissue culture plates or 8-well electrode arrays (8W10E; Applied Biophysics, Troy, NY) (~8×10^5/well) and were then incubated alone and with uninfected neutrophils (2×10^5) or *A. phagocytophilum*-infected neutrophils (2×10^5) with and without *B. burgdorferi* strain 297 (2×10^5) (20, 32). The approximate multiplicity of infection (MOI) of *Borrelia* and of *A. phagocytophilum*-infected neutrophils to human BMECs was 1:4; each *A. phagocytophilum*-infected neutrophil was estimated to contain 10 to 50 bacteria.

Cytokine, chemokine, and protease expression. The human BMECs grown on culture plates were incubated for 5 h in triplicate or quadruplicate, and the culture medium (50 μl) was then examined for MMP, cytokine, and chemokine secretion. To do this, we took advantage of multiplex Luminex technology (Luminex Corporation, Austin, TX) to monitor a panel of human MMPs consisting of MMP-1 (collagenase 1), MMP-2 (gelatinase A; 72-kDa gelatinase or type IV gelatinase), MMP-3 (stromelysin-1, proteoglycanase), MMP-7 (matrilysin or PUMP), MMP-8 (neutrophil collagenase), MMP-9 (gelatinase B), MMP-12 (macrophage metalloproteinase), and MMP-13 (collagenase 3) using a Fluorokine MAP multiplex human MMP panel (R&D Systems, Minneapolis, MN) which measures the total concentrations of pro-, mature, and TIMP-1-complexed forms. The same multiplex Luminex technology was used to monitor a panel of human cytokines/chemokines consisting of interleukin 1α (IL-1α), IL-5, IL-6, IL-8 (CXCL8), IL-10, IL-12 (P70), MCP-1 (CCL2), macrophage inflammatory factor 1α (MIP-1α) (CCL3), gamma interferon (IFN-γ), and tumor necrosis factor alpha (TNF-α) (LINCO Research, Inc., St. Charles, MO).

Assessment of barrier function by ECIS. *E*lectric *c*ell substrate *i*mpedance *s*ensing (ECIS) (Applied BioPhysics, Troy, NY) measures the resistance and impedance of small gold electrodes that serve as substrates for cell attachment and growth (25). In our study, human BMECs were grown in 8-well electrode arrays (8W10E) until stable resistances of >1,400 Ω were reached (19, 25). Resistances were recorded every 80 s for 17 h postinfection, and the results for duplicate samples were averaged. The same five experimental groups as described above, using an MOI of 1:4 of *Borrelia* and *A. phagocytophilum*-infected neutrophils to human BMECs, were tested.

RESULTS

MMP, cytokine, and chemokine expression during coinfection. Cultures were sampled after a 5- to 6-h incubation period determined in previous experiments to correspond to a time of significant spirochete transmigration (20, 32). At this point, little production of cytokines and chemokines was stimulated by infection of human BMECs by *B. burgdorferi* alone (Fig. 1). Nor did spirochetes alone cause induction of or increased expression of MMP-2, MMP-9, or MMP-1. Compared to the results with *B. burgdorferi* and neutrophils alone, coinfection with both *A. phagocytophilum*-infected neutrophils and *B. burgdorferi* resulted in increased, sometimes synergistic release of

MMP-1 (1,064 ± 23 [mean ± standard deviation] versus 1,917 ± 112 pg/ml), MMP-3 (244 ± 11 versus 1,000 ± 51 pg/ml), MMP-7 (247 ± 18 versus 1,458 ± 93 pg/ml), MMP-8 (14,670 ± 1,128 versus 16,712 ± 610 pg/ml), and MMP-9 (14,393 ± 2,490 versus 26,706 ± 4,608 pg/ml), as well as of IL-10 (76 ± 10 versus 225 ± 7 pg/ml), MIP-1α (236 ± 85 versus 8,330 ± 2,892 pg/ml), and TNF-α (34 ± 8 versus 700 ± 39 pg/ml) (all P values were <0.002) (Fig. 1). The secretion of cytokines IL-6 and IL-8 with coinfection was also greater than that with *B. burgdorferi* and neutrophils alone, but the results were additive ($P < 0.02$). The remaining cytokines/chemokines and MMPs were unaffected or minimally affected by coinfection (data not shown). Our finding is in accord with the results of a recent study showing that mouse brain endothelial cells can secrete granulocyte-macrophage colony-stimulating factor, IL-1α, IL-6, IL-10, and IL-12 but that, in the absence of lipopolysaccharide or amyloid-β, only IL-6 was spontaneously secreted in high levels (49). In addition, no IL-2, IL-4, or IFN-γ secretion was found. While synergistic release of MMP-8 and MMP-9 was also observed with uninfected neutrophils and *B. burgdorferi*, the quantities were statistically less than under conditions of coinfection (Fig. 1).

Assessment of human BMEC monolayer barrier function. When human BMECs were incubated with *B. burgdorferi* 297 alone, the BMEC monolayer integrity initially became compromised approximately 5 h after spirochete addition, reached a nadir by 11 h, and then recovered to control levels by 17 h (Fig. 2A). That human BMECs remained viable throughout the process was also shown by transient changes in human BMEC TEER that occurred in the presence of continuous spirochete infection. Interestingly, while the drop in TEER with coinfection also reached a nadir at 11 h, it was more dramatic with coinfection than with *B. burgdorferi* or with *A. phagocytophilum*-infected neutrophils alone (Fig. 2B). Also, while TEERs for human BMECs exposed overnight to either *B. burgdorferi* alone or *A. phagocytophilum*-infected neutrophils alone had essentially recovered, TEER for human BMEC exposed to both pathogens remained compromised even as late as 17 h, at the conclusion of the study (Fig. 2B). The presence of neutrophils with or without *B. burgdorferi* had little effect on the overall TEER changes relative to TEER for human BMEC baseline controls (Fig. 2A).

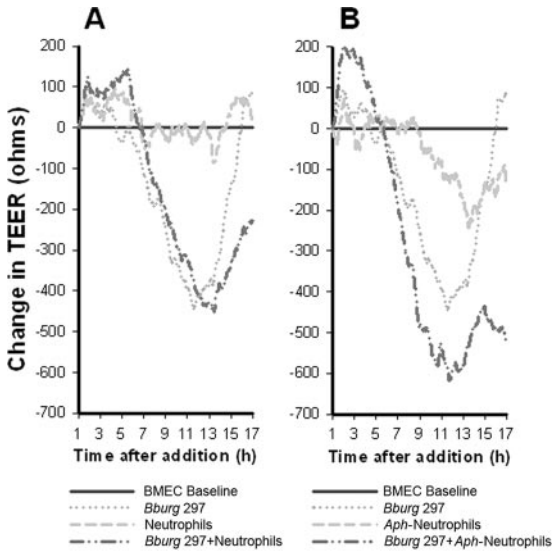

FIG. 2. Real-time changes in human BMEC monolayer integrity analyzed by ECIS. Human BMECs alone as control (BMEC Baseline) or with *B. burgdorferi* strain 297 (*Bburg* 297), with *A. phagocytophilum*-infected neutrophils (*Aph*-Neutrophils), with both bacteria (*Bburg* 297+*Aph*-Neutrophils), and with both uninfected neutrophils and *B. burgdorferi* (*Bburg* 297+Neutrophils) were incubated (MOI, 0.2) for 16 h. Temporal changes in human BMEC TEER relative to TEER in the controls from 1 to 16 h after addition of the pathogens and/or neutrophils are shown. (A) Shows changes in TEER for human BMECs incubated alone, with *B. burgdorferi* (*Bburg* 297), with uninfected neutrophils, or with both *B. burgdorferi* and uninfected neutrophils. (B) Shows changes in TEER for human BMECs incubated with *B. burgdorferi* alone, *A. phagocytophilum*-infected neutrophils alone, or with both *B. burgdorferi*- and *A. phagocytophilum*-infected neutrophils. Shown are the representative traces of changes in monolayer electrical resistance relative to monolayer electrical resistance of the human BMEC controls in ohms over time, based on the averages of the results of duplicate runs.

DISCUSSION

It is reasonable to speculate that these data imply that MMPs and/or cytokines and chemokines induced during coinfection could promote the enhanced transmission and perhaps greater dissemination of *B. burgdorferi* across the blood-brain barrier (BBB) and other vascular barriers. MMPs induced by TNF-α subsequent to systemic or local inflammatory responses are known to play a role in BBB integrity by compromising or reorganizing tight junctions (18, 21, 33, 38). Occludin, a tight junction protein that contains a putative MMP cleavage site (8), serves as substrate for both MMP-3 and MMP-9 (18, 21). Furthermore, MMP-3 can also degrade tight junction claudins and most extracellular matrix proteins (21, 33). Aside from proteolytic effects on endothelial cell tight junctions, MMPs also modulate inflammation by either activating or inactivating cytokines and other inflammatory factors. These results reveal clear evidence that the presence of both bacteria enhances inflammatory cytokine/chemokine production and strengthen the hypothesis that *A. phagocytophilum* enhances the degranulation of MMPs from neutrophils.

A critical question that remains is whether these MMPs enhance *B. burgdorferi*, *A. phagocytophilum*, or coinfection pathogenesis. MMPs (except membrane-type MMPs) are secreted in proenzyme forms and require proteolytic cleavage at the N terminus for activation. The activation cascade for MMPs in the healthy host is closely tied to the fibrinolytic pathway, and activated MMP-3 is believed to be the major physiological activator of most MMPs, including MMP-9 (27, 33). While regulation of MMP production in normal cells is tightly controlled and occurs at many levels, the dysregulation of MMPs often associated with disease (27) could be an important consequence of coinfection. This hypothesis could also explain the worsening of arthritis in the mouse model of coinfection with *A. phagocytophilum* and *B. burgdorferi* (47).

Although the most direct explanation for the observation that BMEC monolayer integrity is compromised not only in *B. burgdorferi* infection but also, to a greater degree, with *A. phagocytophilum* coinfection is that the compromised integrity is due to the actions of MMPs on tight junction proteins, another possibility is that *A. phagocytophilum*-infected neutro-

phils are markedly activated for the production of chemokines (IL-8) and cytokines (IL-6) (15, 26), biologically active compounds with multiple effects, including enhanced changes in vascular permeability related to alterations in the endothelial cell cytoskeleton. For example, IL-8 and TNF-α induce permeability changes in cerebral vascular endothelial cells (9) and nonbrain microvascular cells (7, 9) by altering actin rearrangements (F-actin polymerization/stress fiber formation) through Rho and Rac GTPase-mediated signaling (7, 39, 45). IL-6 can also influence the physiologic function of the BBB and contributes to parenchymal CNS injury (10), whereas both IL-6 and IL-10 could act as compensatory neuroprotective factors (42, 49). In keeping with this hypothesis, we showed enhanced secretion of chemokines (IL-8 and MIP-1α) and cytokines (IL-6, IL-10, and TNF-α) during coinfection that could directly contribute to the transient breakdown in human BMEC monolayer integrity, allowing more spirochete transmission with coinfection than with *B. burgdorferi* alone. Furthermore, the biological consequences of enhanced chemokine/cytokine secretion might be further amplified by the direct action of an MMP whose expression was also enhanced. That such effects might occur was recently suggested by Tester et al., who show that MMP-8 cleavage at Arg5-Ser6 can activate IL-8 (46).

Additionally, *A. phagocytophilum* infection of neutrophils impairs phagocytosis, and this could result in an increased availability of *B. burgdorferi* to transmigrate (17). Regardless, the combined effects of enhanced MMP, cytokine, and chemokine release and impaired neutrophil phagocytosis with coinfection could collectively lead to enhanced entry of *B. burgdorferi* into the CNS and other tissues, potentially worsening clinical manifestations of Lyme disease. A precedent for *A. phagocytophilum*-enhanced clinical disease in the CNS exists in sheep coinfected with louping ill virus, a tick-transmitted flavivirus of the tick-borne encephalitis group (11, 37), although the mechanism is not understood.

In summary, these data show that *B. burgdorferi-A. phagocytophilum* coinfection results in higher levels of MMP, cytokine, and/or chemokine production, as well as more-extensively compromised endothelial barrier function, than *B. burgdorferi* or *A. phagocytophilum* infection alone. Together, these factors could play a role in the observed enhancement of *B. burgdorferi* transmigration across the BBB in the human model. Further investigation will be required to prove the hypothesis that increased transmigration results from MMP/cytokine/chemokine-enhanced tight junction degradation and/or signal-mediated alterations of the host cell cytoskeleton. Importantly, these data provide a plausible alternate explanation for the enhanced tissue dissemination of Lyme disease spirochetes with *A. phagocytophilum* coinfection in animal models and set the stage for further work if concurrent HGA proves to exacerbate and facilitate spirochete dissemination in human Lyme disease.

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