Escherichia coli K1 induces IL-8 expression in human brain microvascular endothelial cells

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ABSTRACT. Microbial penetration of the blood-brain barrier (BBB) into the central nervous system is essential for the development of meningitis. Considerable progress has been achieved in understanding the pathophysiology of meningitis, however, relatively little is known about the early inflammatory events occurring at the time of bacterial crossing of the BBB. We investigated, using real-time quantitative PCR, the expression of the neutrophil chemoattractants alpha-chemokines CXCL1 (Groα) and CXCL8 (IL-8), and of the monocyte chemoattractant beta-chemokine CCL2 (MCP-1) by human brain microvascular endothelial cells (HBMEC) in response to the meningitis-causing *E. coli* K1 strain RS218 or its isogenic mutants lacking the ability to bind to and invade HBMEC. A nonpathogenic, laboratory *E. coli* strain HB101 was used as a negative control. CXCL8 was shown to be significantly expressed in HBMEC 4 hours after infection with *E. coli* K1, while no significant alterations were noted for CXCL1 and CCL2 expression. This upregulation of CXCL8 was induced by *E. coli* K1 strain RS218 and its derivatives lacking the ability to bind and invade HBMEC, but was not induced by the laboratory strain HB101. In contrast, no upregulation of CXCL8 was observed in human umbilical vein endothelial cells (HUVEC) after stimulation with *E. coli* RS218. These findings indicate that the CXCL8 expression is the result of the specific response of HBMEC to meningitis-causing *E. coli* K1.

Keywords: blood-brain barrier, *E. coli*, interleukin-8, meningitis, human brain microvascular endothelial cell

Bacterial meningitis remains a "top-ten" infectious cause of death worldwide, and neurological sequelae affect up to half of survivors [1-3]. The study of bacterial passage across the blood-brain barrier (BBB) has become feasible because of the availability of both *in vitro* and *in vivo* BBB models [1-3]. A well-characterized *in vitro* model comprised of human brain microvascular endothelial cells (HBMEC) has been used for the study of translocation of the BBB by bacteria, fungi, and parasites [1-7]. At present, *E. coli*-HBMEC interactions represent the most characterized system of how circulating bacteria cross the BBB [1-3]. *E. coli* is the most common cause of neonatal gram-negative meningitis. Binding to and invasion of HBMEC are a prerequisite for *E. coli* penetration of the BBB *in vivo* [1-3]. Several *E. coli* K1 determinants contribute to HBMEC adherence and invasion, such as type 1 fimbriae and outer membrane protein A (OmpA), and cytotoxic necrotizing factor 1 (CNF1) [1-3, 8].

Following bacterial penetration, a series of inflammatory mediators has been found to be increased in cerebrospinal fluid (CSF). Among them, chemokines play key roles in leukocyte migration across the BBB [9-11]. Very little is known about the release of chemokines from HBMEC [4, 6, 7]. The aim of this study was to investigate the contribution of the BBB endothelial cells to the early chemokine RNA expression, as a response to stimulation by *E. coli* strains. This expression in HBMEC was compared to that in human umbilical vein endothelial cells (HUVEC) [6, 7, 12-14].

Abbreviations:

BBB	blood-brain barrier
HBMEC	human brain microvascular endothelial cells
CNF1	cytotoxic necrotizing factor 1
CNS	central nervous system
CSF	cerebrospinal fluid
HUVEC	human umbilical vein endothelial cells
MOI	multiplicity of infection
OmpA	outer membrane protein A

MATERIALS AND METHODS

Materials

RPMI medium 1640 was obtained from Cambrex Bio Science (Walkersville, MD, USA). Phosphate-buffered saline (PBS), Hanks balanced salt solution (HBSS), trypsin-EDTA and penicillin/streptomycin were obtained from Invitrogen Corporation (Grand Island, NY, USA). L-glutamine was purchased from Irvine Scientific (Santa Ana, CA, USA). Minimum essential medium (MEM)-

doi: 10.1684/ecn.2006.0042

vitamins, MEM-non essential amino acids, and sodium pyruvate were from Cellgro Mediatech (Washington, DC, USA). Fetal bovine serum was obtained from Omega Scientific (Tarzana, CA, USA) and NuSerum and rat tail collagen from BD Biosciences (Bedford, MA, USA). HUVEC were purchased from Cambrex (Walkersville, MD, USA).

Bacterial strains and growth conditions

Five *E. coli* strains were used in this study. The wild type *E. coli* K1 strain RS218 serotype 018:K1:H7 has been isolated from the cerebrospinal fluid of a neonate with meningitis and found to bind to and invade HBMEC [1-3, 15, 16]. *E. coli* K1 RS218 depleted of FimH adhesin on type 1 fimbriae (RS218*ΔfimH*), *E. coli* K1 RS218 depleted of OmpA (RS218*ΔompA*), and *E. coli* K1 RS218 depleted of CNF1 (RS218*Δcnf1*) strains have been shown to be respectively defective in binding to and invasion of HBMEC [1-3, 8, 17-19]. The nonpathogenic laboratory *E. coli* K12 strain HB101 has been shown to be non-adherent and non-invasive [1-3] and was used as a negative control in the present study. All *E. coli* strains were grown in brain-heart infusion (BHI) broth (Difco Lab, Detroit, MI, USA) to mid-log phase ($\approx 10^8$ CFU/mL), at which point the cells were harvested and used for the experiments.

Cultures of HBMEC and HUVEC

The HBMEC line, which has been isolated, immortalized and characterized as described previously [5, 15, 20] was used for this study. The frozen stock of HBMEC between passages 13 and 18 was thawed, cultured and maintained in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum (FBS), 10% NuSerum, L-glutamine (2 mM), sodium pyruvate (1 mM), 1% MEM nonessential amino acids, 1% vitamins, 100 U/mL penicillin, and 100 µg/mL streptomycin in 75cm^2 flasks. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2–95% air. Culture media were changed every other day. After reaching confluency, the cells were detached with 0.05% trypsin–0.5 mM EDTA solution, resuspended in fresh culture medium and split in a ratio of 1:3. HBMEC at a density of 1 x 10^5 cells/well were seeded into 24-well tissue culture plates precoated with rat tail collagen. At this cell density, confluent monolayers were formed within 24h and used for the assays. HUVEC were cultured according to the supplier (Cambrex, Walkersville, MD, USA) in endothelial cell growth medium EGM-2 supplemented with bullet kit containing 2% FBS, hydrocortisone, heparin, rhFGF-B, R^3-IGF-1, rhEGF, VEGF, ascorbic acid, gentamicin sulfate and amphotericin B. HUVEC cultures were maintained in an identical fashion to HBMEC until becoming confluent. First passaged HUVEC were used for all experiments.

In vitro association/invasion assays

Association and invasion assays were performed as previously described [5, 16, 21-23]. Approximately 10^7 bacteria in 500 µL of experimental medium (Ham's F12: Medium 199, 5% heat inactivated bovine serum, 1 mM sodium pyruvate, 2 mM glutamine) were added to HBMEC monolayers at a multiplicity of infection (MOI) of 100 bacteria per cell. Plates were centrifuged at 700 x g for 5 min, and then incubated at 37°C for 90 minutes. For the association assay, the monolayers were washed to remove the unbound bacteria and lysed with sterile water. The adherent bacteria were inoculated on sheep blood agar plates. Results were expressed as percentage association: [(number of bacteria recovered)/(number of bacteria inoculated)] x 100. For the invasion assay, the monolayers were incubated with *E. coli* as described above and then incubated with experimental medium containing gentamicin (100 µg/mL) for 1 hour to kill extracellular bacteria. The monolayers were washed again, lysed with sterile water and inoculated on sheep blood agar plates. Results were expressed as percentage invasion: [(number of intracellular bacteria recovered)/(number of bacteria inoculated)] x 100. All association and invasion experiments were conducted in triplicate. For the chemokine expression assay, a MOI of 10 was used. Plates were centrifuged at 700 *g* for 5 min, and then incubated at 37°C for 0.5, 1, 2, and 4 hours. At these time points, the supernatant was removed from the monolayers and HBMEC monolayers were stabilized using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA). Samples from control HBMEC wells without *E. coli* were collected at all time points. The experimental medium for HUVEC consisted of EGM-2 containing 2% FBS.

RNA extraction and reverse transcription

Total RNA was extracted using RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) applying the optional on-column Dnase treatment according to the manufacturer's instructions. The amount and the quality of the RNA were verified by measuring the absorbance at 260 and 280 nm. Oligo (dT) primed reverse transcription of RNA was performed using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) using 0.5 µg of RNA for each reaction; reactions without reverse transcriptase were concurrently prepared for each RNA sample to verify the absence of genomic DNA contamination in PCR.

Real-time quantitative PCR analysis of chemokine expression in HBMEC

Real-time PCR analysis was performed in a LightCycler® System using the LightCycler FastStart DNA Master^{PLUS} SYBR Gren I Kit (Roche Applied Science, Indianapolis, IN, USA). Primers were designed using Primo 3.4 online software (Chang Bioscience; www.changbioscience.com/primo/) for CXCL8 (TTGAGGCCAAGGGCCAAGAG, position f781; CTAGCAGACTAGGGTTGCCAG, position r881), MCP-1 (CCAAGCAGAAGTGGGTTCAGG, position f303; GTGTCTGGGGAAAGCTAGGG, position r434) and GROα/GROβ (AGACCCTGCAGGGAATTCACC, position f218; AGGGCCTCCTTCAGGAACAG, position r452). The sequence for GROα also matched for GROβ. QuantumRNA β-actin Internal Standards (Ambion, Austin, TX, USA) were used as reference primers. The PCR program consisted of one preincubation at 95° C for 10 min and 40 cycles at 95° C for 10 seconds, 60° C for 5 seconds and 72° C for 10 seconds, followed by the melting curve analysis program described

by the manufacturer. Efficiency-corrected calibrator-normalized relative quantification was performed according to Roche Applied Science Technical Note No. LC 13/2001. Relative Expression Software Tool (REST) [24] was used to analyze the statistical significance of the data.

Statistical analysis

Association and invasion assays were analyzed using the Mann Whitney rank sums test, with a p value < 0.05 considered as significant. Statistical analysis of the PCR results was performed as described previously [24].

RESULTS

The association frequency (mean ± SD) for the *E. coli* K1 strain RS218 was 27.1% ± 2.86% and for the laboratory *E. coli* strain HB101, 2.3% ± 0.60%. The invasion frequencies (mean ± SD) were 0.325% ± 0.050% and 0.018 ± 0.002%, respectively, for *E. coli* K1 strain RS218 and laboratory strain HB101. Association and invasion rates for all five *E. coli* strains are presented in *figure 1*.
E. coli K1 derivatives lacking the ability to bind (*i.e.*, RS218Δ*ompA* and RS218Δ*fimH*) exhibited, as expected, significantly decreased association and invasion frequencies compared to the parent strain RS218 ($p < 0.05$). The decreased invasion frequencies of these strains were secondary to their decreased binding. In contrast, the *E. coli* strain lacking the ability to invade (RS 218Δ*cnf1*) exhibited a similar rate of binding, but exhibited a significantly decreased invasion frequency as compared to the parent *E. coli* K1 strain ($p < 0.05$).
Chemokine expression by HBMEC infected with *E. coli* K1 strain RS218 at time points 30 min, 1 hour, 2 hours and 4 hours, as compared to the expression at time 0 and to the expression by non-infected HBMEC, revealed that changes for CXCL1 (Groα) and CCL2 (MCP-1) did not differ significantly and were less than twofold *(figure 2)*. In contrast, *E. coli* K1 RS218-infected HBMEC exhibited a more than a 3-fold increase in CXCL8 (IL-8) expression at the 4-hour time point.

The CXCL8 expression by HBMEC stimulated with the *E. coli* K1 strain RS218 and its derivatives lacking the abilities to bind and invade HBMEC is further presented in *figure 3*. In contrast to *E. coli* HB101, which did not trigger any expression by infected HBMEC, all four *E. coli* K1 strains showed the capability of inducing upregulation of CXCL8 ($p < 0.001$ [24]), as compared to the expression by uninfected HBMEC. In contrast to HBMEC, no increased CXCL8 expression was noted in HUVEC in response to *E. coli* K1 RS218 at 4 hours incubation (data not shown).

DISCUSSION

Penetration of the BBB by bacteria is followed by a complex interaction among immune, vascular, and central nervous system (CNS) cells. The secretion of adhesion molecules, selectins, integrins, cytokines, chemokines, proteolytic enzymes, and oxidants forms the inflammatory cascade, which leads not only to pleocytosis and increased permeability of the BBB, but also to brain tissue damage and neurological sequelae [25, 26]. Adjunctive measures focusing on the prevention of the detrimental effects of the host immune response may thus have the potential of reducing sequelae following meningitis [25, 27-30].
Chemokines are key players in regulating the recruitment, adhesion and diapedesis of leukocytes to the sites of inflammation [6, 7, 14]. In the CSF of patients with bacterial meningitis, significantly higher levels of several alpha- and beta-chemokines, including CXCL1, CXCL8, CCL2, CCL3, CCL4 have been demonstrated [6, 26, 31-34]. However, CSF specimens are usually obtained several days after the onset of meningeal infection. On the other hand, chemokines can be released in the CSF by different cell populations, such as microglial and endothelial cells as well as migrating leukocytes [6], and their cellular source appears to differ, depending on the stage of disease [13]. Recent studies show that the BBB endothelial cells can produce chemokines after stimulation with cytokines or microbial pathogens, and can express chemokine receptors [4, 6, 9, 12, 26]. However, knowledge of the contribution of brain endothelial cells to chemokine production is limited

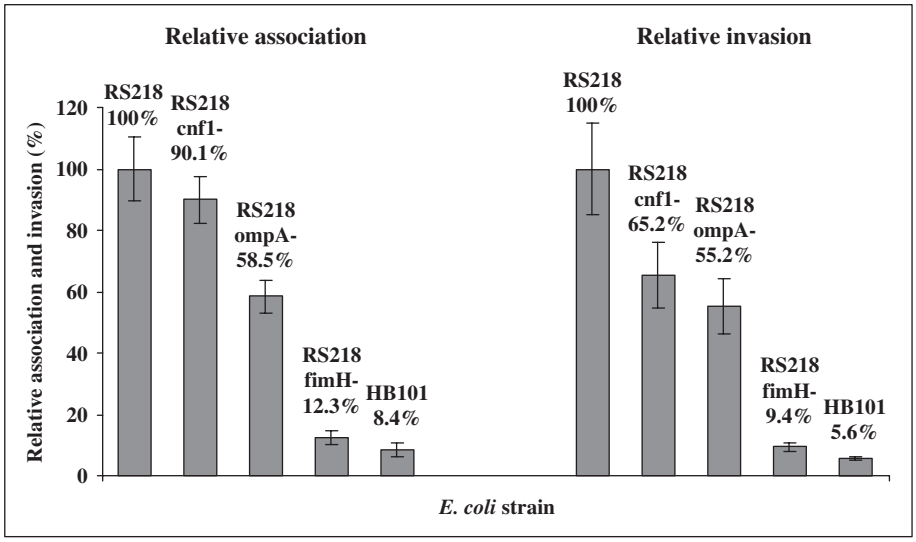

Figure 1
Association and invasion rates to HBMEC shown by 5 different *E. coli* strains (% normalized to the association and invasion by the parent strain RS218).

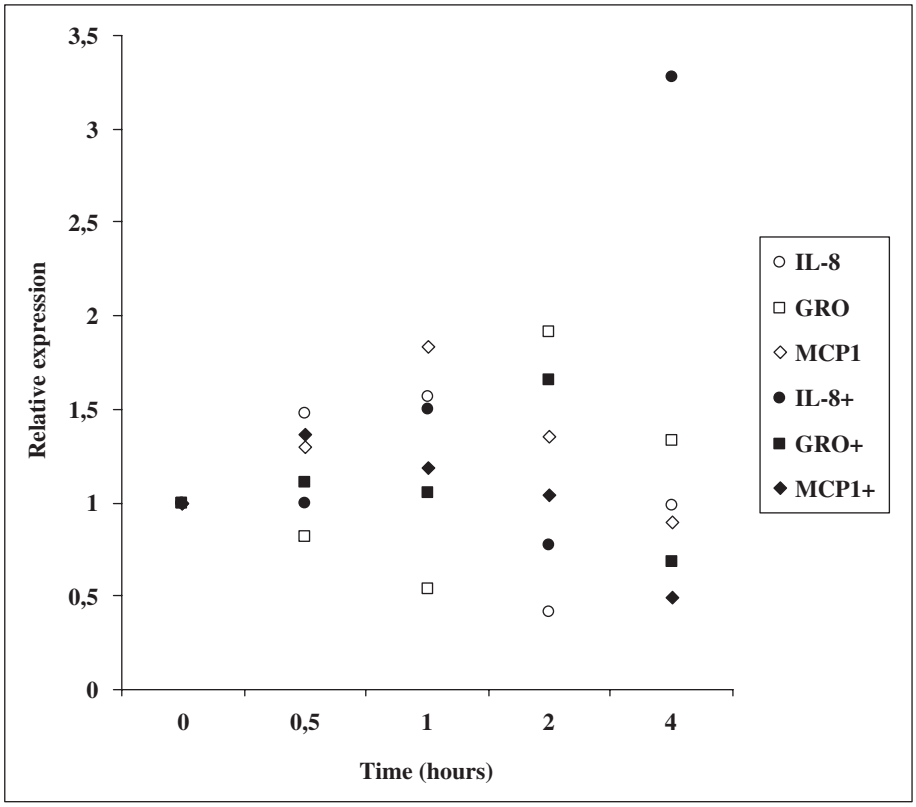

Figure 2
Alpha- and beta-chemokine expression by HBMEC after stimulation by *E. coli* RS218. The expression by HBMEC stimulated by *E. coli* is marked as IL-8+, CXCL1+, and CCL2+, as opposed to the expression by cells not stimulated by *E. coli*, which is marked as IL-8, CXCL1, and CCL2, respectively. RNA expressions were determined at various time points by using real-time quantitative PCR.

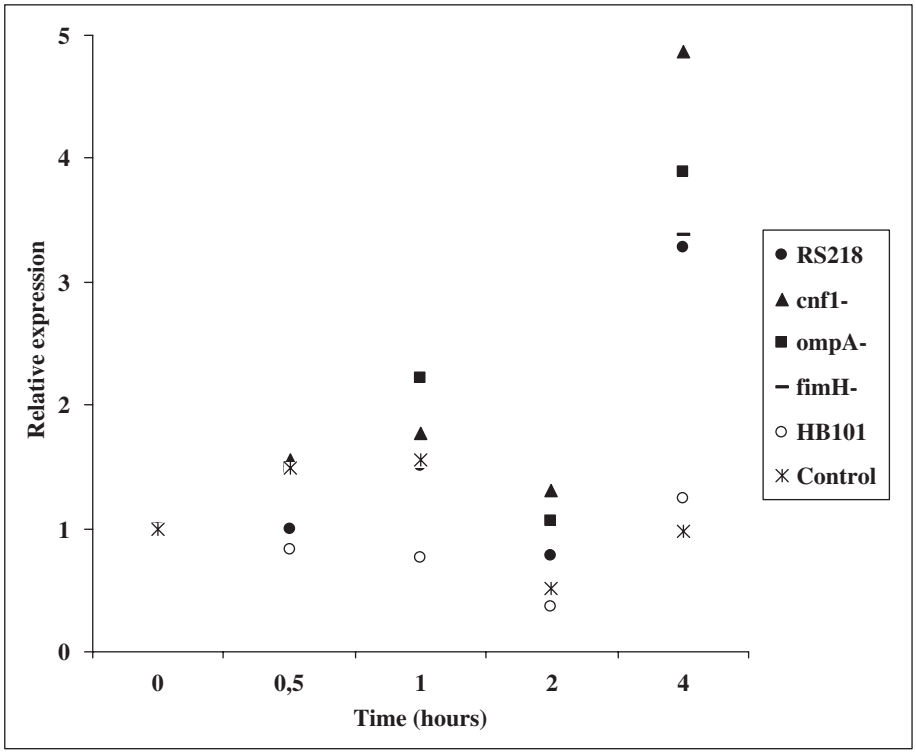

Figure 3
Time-dependent CXCL8 (IL-8) expression in HBMEC stimulated by *E. coli* strains of variable pathogenic capacity. RNA expressions were determined at various time points by using real-time quantitative PCR. *E. coli* HB101 did not trigger any CXCL8 expression by infected HBMEC, but all four *E. coli* K1 strains induced CXCL8 upregulation ($p < 0.001$, 24) as compared to the expression by uninfected HBMEC.

[11]. HBMEC, when cultivated on a collagen-coated surface, form a continuous lining of endothelial cells and demonstrate the unique properties of the BBB [1-3, 5, 30].

Among the chemokines investigated in this study, CXCL8 (IL-8) was the first to be expressed by HBMEC 4 hours after infection with a meningitis-causing *E. coli* K1 strain. The expression of CXCL8 by HBMEC four hours after infection with another meningitis-causing organism, group B *Streptococcus* has also been reported [4]. CXCL8 belongs to the alpha-chemokine family, acting mainly on cells of neutrophil lineage, in which it stimulates adhesion and degranulation [4]. CXCL8 has been found significantly elevated in the CSF of patients with bacterial meningitis and CXCL8 inhibition on the blood side of the BBB effectively reduces CSF pleocytosis [28, 29]. An interesting finding of this study was that the *E. coli* K1 strain RS218 and its derivatives induced the same CXCL8 response by HBMEC, although RS218 isogenic mutants have been shown to be significantly less able than the parent strain to penetrate the CNS [1-3, 18, 19, 22]. In other epithelial cells such as uroepithelial cells, a relationship between P fimbriae or type 1 fimbriae expressing *E. coli* strains and CXCL8 expression has been documented, and FimH negative strains clearly stimulated less CXCL8 expression than their FimH positive parent strains [35, 36]. This type 1 fimbriae-mediated activation was not demonstrated for HBMEC, as both FimH+ and FimH- *E. coli* strains induced CXCL8 expression.

In our study no increased CXCL8 expression was noted in HUVEC in response to *E. coli* K1 RS218 after 4 hours of incubation. Previous reports have shown that, contrary to microvascular endothelial cells, prolonged stimulation of HUVECs with inflammatory mediators such as human IL-1beta is required for CXCL8 release [37, 38]. We have previously shown that *E. coli* K1 binding to and invasion of endothelial cells are specific to HBMEC, and are not observed in non-brain endothelial cells, such as HUVEC [2, 3]. In this study, *E. coli* K1 was able to upregulate the expression of CXCL8 in HBMEC, but not in HUVEC, indicating that CXCL8 expression is the result of specific interaction of meningitis-causing *E. coli* K1 with HBMEC. The specificity of CXCL8 expression in HBMEC and not in HUVEC has also been demonstrated with *Streptococcus suis* serotype b [6]. Additional studies with other, non-brain microvascular endothelial cells may elucidate whether our finding is related to microvessel-, not macrovessel-derived endothelial cells.

CXCL1 and CCL2 were not found to be upregulated in the early stage of BBB infection with *E. coli* K1, and it is of interest that *E. coli* K1 strain RS218 appeared to induce a decrease rather than an increase in CXCL1 and CCL2 mRNA expression in the first four hours of stimulation. These chemokines can be expressed by HBMEC and an upregulation of expression and secretion following treatment with pro-inflammatory cytokines has been described [13]. Elevated CXCL1 has been demonstrated in the CSF of patients with bacterial meningitis [26, 33], and the mRNA levels of CXCL1 have been found to be increased in HBMEC 4 hours after infection with group B *Streptococcus*, although to a lesser degree than CXCL8 [4]. CCL2 is a potent chemokine for monocytes, T cells and microglia [13] and has been found to be increased mainly in the CSF of patients with viral meningitis. HBMEC have been shown to produce CCL2 after stimulation by heat-killed *Streptococcus suis* serotype 2, but only after 48 hours of incubation [6], a finding which is compatible with the absence of early CCL2 expression in our study.

In this study, we did not measure the LPS content in the *E. coli*-stimulated endothelial cell cultures, therefore we could not conclude on the potential impact of LPS on CXCL8 production. LPS is known to induce chemokine production in HBMEC [11]. Furthermore, our study focused on the early HBMEC response and did not assess the kinetics after the first 4 hours of stimulation. The primer sequence for CXCL1 also matches for CXCL2, hence CXCL1 expression could not be specifically demonstrated. Additionally, the study focused on chemokine expression rather than secretion, and quantification of the amount of proteins secreted was not performed. Despite these limitations, the preliminary detection of CXCL8 at an early stage of CNS infection is suggestive of its important role in the initial recruitment of inflammatory cells across the BBB. This upregulation of CXCL8 was independent of the ability of *E. coli* K1 to bind and invade HBMEC, and failed to occur with the laboratory *E. coli* strain HB101. *E. coli* K1 also failed to induce XCCL8 upregulation in HUVEC. These findings indicate that the BBB endothelium possesses unique properties in reacting to meningitis-causing pathogens and that the CXCL8 (IL-8) expression is the result of specific interaction of meningitis-causing *E. coli* K1 with HBMEC.

Acknowledgements. *This work was supported by NIH grants NS 26310 and AI 47225. Dr. E. Galanakis was on a Sabbatical from the University of Crete, Greece.*

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