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Human Meningitis-Associated *Escherichia coli*

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Abstract

E. coli is the most common Gram-negative bacillary organism causing meningitis and *E. coli* meningitis continues to be an important cause of mortality and morbidity throughout the world. Our incomplete knowledge of its pathogenesis contributes to such mortality and morbidity. Recent reports of *E. coli* strains producing CTX-M-type or TEM-type extended-spectrum β-lactamases create a challenge. Studies using *in vitro* and *in vivo* models of the blood-brain barrier have shown that *E. coli* meningitis follows a high-degree of bacteremia and invasion of the blood-brain barrier. *E. coli* invasion of the blood-brain barrier, the essentials step in the development of *E. coli* meningitis, requires specific microbial and host factors as well as microbe- and host-specific signaling molecules. Blockade of such microbial and host factors contributing to *E. coli* invasion of the blood-brain barrier is shown to be efficient in preventing *E. coli* penetration into the brain. The basis for requiring a high-degree of bacteremia for *E. coli* penetration of the blood-brain barrier, however, remains unclear. Continued investigation on the microbial and host factors contributing to a high-degree of bacteremia and *E. coli* invasion of the blood-brain barrier is likely to identify new targets for prevention and therapy of *E. coli* meningitis.

INTRODUCTION

Gram-negative bacillary meningitis continues to be an important cause of mortality and morbidity throughout the world despite advances in antimicrobial chemotherapy and supportive care. Case fatality rates have ranged between 15 and 40%, and approximately 50% of the survivors sustain neurological sequelae (1-10). Both clinical and experimental data indicate limited efficacy with antimicrobial chemotherapy alone (11, 12). A major contributing factor to such mortality and morbidity is our incomplete understanding of the pathogenesis of this disease.

E. coli is the most common Gram-negative bacillary organism that causes meningitis, in particular during the neonatal period. Most cases of *E. coli* meningitis develop as a result of hematogenous spread (13, 14), but it is incompletely understood how circulating *E. coli* traverses the blood-brain barrier. Given the plethora of *E. coli* serotypes, it is striking that *E. coli* strains possessing the K1 capsular polysaccharide are predominant (approximately 80%) among isolates from neonatal *E. coli* meningitis (15-17), and most of these K1 isolates are associated with a limited number of O serotypes (e.g., O18, O7, O16, O1, O45) (14,

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18-20). The basis of this association of K1 and certain O antigens with *E. coli* meningitis remains unclear.

The development of both *in vitro* and *in vivo* models of the blood-brain barrier has facilitated the current understanding of the microbial penetration of the blood-brain barrier, a key step for the development of *E. coli* meningitis. Meningitis-causing pathogens cross the blood-brain barrier transcellularly, paracellularly, and/or by means of infected phagocytic cells ("Trojan horse" mechanism) (21-25), and this review describes the microbial and host factors contributing to *E. coli* penetration of the blood-brain barrier.

THE BLOOD-BRAIN BARRIER

The blood-brain barrier is a structural and functional barrier that is formed by brain microvascular endothelial cells (BMECs), astrocytes and pericytes. It regulates the passage of molecules into and out of the brain to maintain the neural microenvironment. BMECs possess distinct features such as tight junctions between them and low rates of pinocytosis (25, 26). The blood-brain barrier protects the brain from microbes and toxins circulating in the blood, and astrocytes and pericytes help maintain the barrier property of BMEC. Recent studies, however, have shown that meningitis-causing *E. coli* traverses the blood-brain barrier as live organisms and cause intracranial inflammation, resulting in meningitis (7, 21-28). The contributions of astrocytes and pericytes to *E. coli* penetration of the blood-brain barrier are shown to be minimal. The *in vitro* blood-brain barrier model has been developed with human brain microvascular endothelial cells (HBMECs). Upon cultivation on collagen-coated Transwell inserts these HBMECs exhibit morphologic and functional properties of tight junction formation as well as a polar monolayer. These are shown by the demonstrations of tight junction proteins (such as claudin 5 and ZO-1) and adherens junction proteins (such as VE-cadherin and β-catenin) and their spatial separation, limited transendothelial permeability to inulin (molecular weight, 4,000) and dextran (molecular weight, 70,000), and development of high transendothelial electrical resistance (29-32). Studies with transmission electron microscopy demonstrate that meningitis-causing *E. coli* invades HBMECs and internalized bacteria are found within membrane-bound vacuoles of HBMECs (Fig. 1) and transmigrate through HBMECs via an enclosed vacuole without intracellular multiplication and without any change in the integrity of HBMEC monolayers (20, 30, 33). No free bacteria are found in the cytoplasm of HBMECs or between adjacent HBMECs.

Experimental hematogenous meningitis models have been developed in infant rats and mice for assessing the role of the blood-brain barrier in microbial penetration into the brain *in vivo*. In this animal model, *E. coli* is injected via intravenous, intracardiac or subcutaneous administration, resulting in bacteremia and subsequent entry into the brain (14, 34-41), which mimics the pathogenesis of *E. coli* meningitis in humans. Studies in these hematogenous meningitis models indicate that the primary site of entry into the brain for circulating *E. coli* is the cerebral microvessels, not the choroid plexus (14).

As indicated above, meningitis-causing pathogens exhibit the ability to penetrate the blood–brain barrier, the essential step in the development of central nervous system infection, and

their penetration of the blood–brain barrier occurs transcellularly, paracellularly, or by means of infected phagocytes (so-called Trojan-horse mechanism) (21-25). Transcellular traversal refers to microbial penetration through barrier cells without any demonstration of organisms between the cells or any evidence of intercellular tight junction disruption. Paracellular traversal is defined as microbial penetration between barrier cells with and/or without evidence of tight junction disruption. The Trojan-horse mechanism involves microbial penetration of the barrier cells via transmigration within infected phagocytes. *E. coli* penetration into the brain was documented without accompanying host inflammatory cells (e.g., polymorphonuclear leukocytes, macrophages) (14), and *E. coli* entry into the brain was shown to occur without any change in the blood-brain barrier permeability (42). In addition, *E. coli* transmigrate through HBMECs via an enclosed vacuole without any change in the integrity of HBMEC monolayers (21, 30, 33), and no free bacteria are found in the cytoplasm of HBMECs or between adjacent HBMECs. These findings indicate that initial entry of *E. coli* into the brain is less likely to involve paracellular penetration of the blood-brain barrier and/or the Trojan horse mechanism via transmigration of *E. coli*-infected phagocytic cells. Taken together, meningitis-causing *E. coli* traverses the blood-brain barrier using a transcellular mechanism without altering the blood-brain barrier permeability.

E. COLI PENETRATION OF THE BLOOD-BRAIN BARRIER

Studies using the above-mentioned *in vitro* and *in vivo* models of the blood-brain barrier have revealed that successful crossing of the blood-brain barrier by circulating *E. coli* requires (a) a high degree of bacteremia, (b) *E. coli* binding to and invasion of HBMEC, and (c) traversal of the blood-brain barrier as live bacteria (21-25, 27, 43) (Table 1).

A HIGH DEGREE OF BACTEREMIA REQUIRED FOR *E. COLI* PENETRATION INTO THE BRAIN

Several studies of *E. coli* meningitis in humans and experimental animals point to a relationship between the magnitude of bacteremia and the development of meningitis. For example, a significantly higher incidence of *E. coli* meningitis was noted in neonates who had bacterial counts in blood higher than 10^3 colony forming units (CFUs)/ml (6 of 11 or 55%), compared to those with blood bacterial counts lower than 10^3 CFUs/ml (1 of 19 or 5%) (13). A high degree of bacteremia was also shown to be a primary determinant for penetration into the brain by circulating *E. coli* in neonatal and adult animals with experimental hematogenous *E. coli* meningitis (14, 34-38), but an approximately 10^6-fold greater inoculum of *E. coli* is required to induce a similar high-level bacteremia in adult animals compared to neonatal animals (14).

These findings suggest that the age dependency of *E. coli* meningitis is most likely due to the relative resistance of adults to high-level bacteremia, which precedes the development of meningitis, and less likely due to greater invasion of meningitis-causing *E. coli* in HBMECs derived from neonates compared to those from adults. This concept is supported by the demonstration that the abilities of meningitis-causing *E. coli* to bind and invade BMEC are similar between BMEC derived from young and old rats as well as HBMEC derived from different ages (44). Thus, one of the reasons for the close association of meningitis-causing

E. coli strains with neonatal meningitis is their ability to escape from host defenses and then to achieve a threshold level of bacteremia necessary for invasion of the blood-brain barrier. Taken together, these findings indicate (a) that a high-degree of bacteremia is required for meningitis-causing *E. coli* penetration of the blood-brain barrier, and (b) that the prevention of bacterial multiplication in the blood that is required for penetration into the brain would be one potential approach for prevention of *E. coli* meningitis. The basis for requiring a high-degree of bacteremia for penetration into the brain, however, remains unclear

Previous studies have identified that the expression of K1 capsular polysaccharide and O-lipopolysaccharide (LPS) are shown to be critical for induction of a high degree of bacteremia (14, 45, 46), but the feasibility of using the K1 capsule and O-LPS for the prevention of *E. coli* bacteremia has been shown to be limited (19, 47-49). Recent functional *E. coli* genomic studies identified several *E. coli* factors that are shown to contribute to bacteremia (21, 43, 50, 51). For example, NlpI, named after new lipoprotein I, has been shown to contribute to a high-level *E. coli* bacteremeia (52). NlpI's evasion of serum-mediated killing is through regulation of the complement regulator C4bp deposition on the bacterial surface (53). Studies are in progress to determine the broadly conserved antigens or a multi-epitope subunit vaccine for the prevention of *E. coli* bacteremia and subsequent meningitis (50, 51, 54).

E. COLI BINDING TO AND INVASION OF HBMECs

Subsequent studies have shown that a high degree of bacteremia is necessary, but not sufficient for *E. coli* penetration of the blood-brain barrier *in vivo*, and that *E. coli* binding to and invasion of HBMEC is a prerequisite for penetration into the brain (34-38, 55). This was shown by the demonstration in infant rats with experimental hematogenous meningitis that isogenic mutants of meningitis-causing *E. coli* deleted of determinants contributing to HBMEC binding and invasion were significantly less able to induce meningitis than the parent strain despite having similar levels of bacteremia (Table 2). These findings indicate that those *E. coli* determinants contributing to HBMEC binding and invasion are necessary for penetration of the blood-brain barrier *in vivo*.

E. COLI STRUCTURES AFFECTING HBMEC BINDING

Infections caused by pathogenic *E. coli* are often initiated by the binding of the bacteria to the host cell surface, and this concept is likely to be important for circulating *E. coli* to withstand the blood flow and cross the blood-brain barrier *in vivo*. Several *E. coli* determinants have been identified to be involved in HBMEC binding and subsequent invasion into HBMECs (52, 56-59). The roles of those *E. coli* structures in HBMEC binding have been verified by deletion and complementation experiments, as shown by the demonstration that isogenic deletion mutants were significantly less able to bind HBMECs and their binding abilities were restored to the levels of parent strain by complementation with wild type genes.

Fimbriae—Pathogenic *E. coli* express several types of fimbrial adhesins, which can be divided into different groups by their affinity to specific receptor structures such as α-d-mannosides (type 1 fimbrial adhesins), α-d-Gal-(1-4)-β-d-Gal (P fimbrial adhesins), and

NeuAc α2,3-galactose (S fimbrial adhesins) (60). A study using *E. coli* DNA microarray examined the gene expression patterns of HBMEC-associated *E. coli*, which revealed that type 1 fimbriae play an important role in *E. coli* binding to HBMEC (59). The HBMEC-associated *E. coli* showed significantly higher expression levels of the *fim* cluster genes than the non-associated bacteria. Expression of type 1 fimbriae in wild-type *E. coli* is regulated by phase variation in which each bacterium can alternate between fimbriated and non-fimbriated states, so-called phase-ON and phase-OFF, respectively. *E. coli* associated with HBMECs are found to be predominantly type 1 fimbria phase-ON bacteria. To determine the role of type 1 fimbriae in *E. coli* binding to HBMEC without phase variation, the type 1 fimbria locked-ON and locked-OFF mutants of meningitis-causing *E. coli* were constructed, whose *fim* promoters are fixed in the ON and OFF orientation, respectively. The binding to HBMECs is found to be significantly greater with the locked-ON mutant than the wild-type strain, while it is significantly less with the locked-OFF mutant (59). Decreased binding as the result of the *fimH* deletion or the locked-OFF mutant resulted in decreased invasion into HBMECs.

From *E. coli* DNA microarray experiments, a novel site-specific recombinase, HbiF, was identified which inverted the molecular switch *fimS* independent of the two known recombinases, FimB and FimE, that invert *fimS* and control the expression of the downstream *fim* operon (61). Discovery of HbiF-mediated *fimS* switching provides a new opportunity for investigating the regulation of type 1 fimbriae expression, which will help in developing a novel strategy for the prevention and therapy of *E. coli* bacteremia and meningitis. FimH is shown to interact with a glycosylphosphatidylinositol-anchored receptor, CD48 on the surface of HBMEC, and FimH-CD48 interaction contributes to *E. coli* binding to HBMEC and increases in intracellular Ca^{2+} ($[Ca^{2+}]i$) in HBMEC (56, 62). This concept is shown by the demonstration that CD48 antibody blocks FimH-mediated binding to HBMEC and FimH-induced $[Ca^{2+}]i$ changes in HBMEC.

S fimbriae, which bind to terminal NeuAc α2,3-galactose sequences present on glycoproteins and glycolipids containing terminal Gal(3SO4)β-1 residues, have been implicated in *E. coli* binding to HBMECs. This concept was shown by the demonstration that purified S fimbriae or a recombinant *E. coli* strain HB101 expressing S fimbriae was shown to bind to the luminal surfaces of the brain vascular endothelium in neonatal rat brain tissues (63). A previous study using S fimbriated transformants of *E. coli* strain HB101 also showed that S fimbriae allowed this laboratory *E. coli* strain to bind to HBMECs (64, 65), suggesting that S fimbriae play an important role in *E. coli* binding to HBMECs. However, in-frame deletion of the S fimbria operon in meningitis-causing *E. coli* did not significantly affect *E. coli* binding to and invasion of HBMECs and also did not affect *E. coli* penetration into the brain in the experimental hematogenous meningitis animal model (39). These findings indicate that S fimbriae are not critical in meningitis-causing *E. coli* binding to HBMECs in vitro and traversal of the blood-brain barrier in vivo.

Flagella—A study comparing the gene expression patterns of HBMEC-associated versus non-associated *E. coli* with *E. coli* DNA microarray demonstrated that flagella play an important role in *E. coli* binding to HBMECs (66, 67). This concept is supported by the demonstration (a) that the mutant deleted of *fliC* was significantly defective in binding to

and invasion of HBMECs, and this defect was restored by complementation with wild type *fliC*, (b) that recombinant flagellin (FliC) binds directly to the surface of HBMECs and (c) that exogenous recombinant flagellin inhibits *E. coli* binding to HBMECs (66).

Outer membrane proteins—OmpA is one of the major outer membrane proteins in *E. coli* and its N-terminal domain crosses the outer membrane eight times in antiparallel β-strands with four hydrophilic surface-exposed loops and short periplasmic turns. Several studies have shown that the N-terminal portion of OmpA and its surface-exposed loops contribute to binding to HBMEC (57, 58, 68), and that OmpA interacts with HBMEC through N-acetylglucosamine (GlcNAc) residues of gp 96 (69, 70). The chitooligomers (GlcNAc β1, 4-GlcNAc oligomers) and chitohexose block meningitis-causing *E. coli* invasion of HBMEC and traversal of the blood–brain barrier in the infant rat model of experimental hematogenous meningitis (68, 70). A recent study comparing the *ompA* deletion mutant with its parent *E. coli* strain RS218 using an *E. coli* DNA microarray, however, revealed that the *ompA* deletion mutant exhibited significantly lower expression of the *fim* cluster genes, and lower expression of type 1 fimbriae on the bacterial surface (71). These findings suggest that decreased binding of the *ompA* deletion mutant may be related to its lower expression of type 1 fimbriae. The *ompA* deletion mutant was significantly less efficient in its penetration into the brain in vivo compared to the parent *E. coli* strain (38). Additional studies are needed to determine whether these *in vitro* and *in vivo* defects of the *ompA* deletion mutant are in part related to its decreased expression of type 1 fimbriae and also understand how the deletion of *ompA* affects type 1 fimbria expression.

Lipoproteins—NlpI is shown to be an important factor of Crohn's disease-associated *E. coli* strain LF82 (083:H1) to interact with intestinal epithelial cells (72). Deletion of *nlpI* in *E. coli* strain LF82 decreased expression of type 1 fimbriae and flagella (72). NlpI is found to be an outer membrane-anchored protein and contributes to meningitis-causing *E. coli* binding to and invasion of HBMEC (52). Unlike strain LF82, deletion of *nlpI* in meningitis-causing *E. coli,* however, did not affect the expression of type 1 fimbriae, flagella and OmpA, indicating that the contribution of NlpI to HBMEC binding and invasion is independent of those bacterial factors in meningitis-causing *E. coli*. This concept is shown by the demonstration that mutants deleted of type 1 fimbriae, OmpA and NlpI exhibited significantly decreased HBMEC binding and invasion compared to mutants deleted of individual factors or a combination of the two factors (52). These findings suggest that type 1 fimbriae, OmpA and NlpI are likely to contribute to HBMEC binding and invasion independent of each other. It remains, however, incompletely understood how and why several bacterial factors of meningitis-causing *E. coli* are involved in HBMEC binding.

E. COLI STRUCTURES CONTRIBUTING TO INVASION OF HBMECs

Previous studies using Tn*phoA* mutagenesis, signature-tagged mutagenesis, and differential fluorescence induction with screening of a *gfp* fusion library identified several *E. coli* determinants contributing to invasion of HBMECs, which include Ibe (named after invasion of brain endothelial cell) proteins and cytotoxic necrotizing factor 1 (CNF1) (34-36, 55, 73, 74). Mutants deleted of the above-mentioned invasion factors were significantly less invasive in HBMECs and less able to traverse the blood-brain barrier in vivo (Table 2), and their

invasive abilities were restored to the levels of parent strain by complementation with wild type genes. Recombinant Ibe proteins inhibit *E. coli* invasion of HBMECs (34), suggesting that Ibe proteins contribute to HBMEC invasion by a ligand-receptor interaction. This concept was supported by the demonstration of a HBMEC surface protein interactive with IbeA, and a polyclonal antibody raised against this receptor protein inhibited *E. coli* invasion of HBMECs (25).

CNF1 is a bacterial virulence factor associated with pathogenic *E. coli* strains causing urinary tract infection and meningitis (75). CNF1 is an AB-type toxin, composed of the N-terminal cell binding domain and the C-terminal catalytic domain possessing a deaminase activity through the site-specific deamination of a Gln residue to Glu (76, 77). CNF1 has been shown to activate Rho GTPases and induce uptake of latex beads, bacteria, and apoptotic bodies into nonprofessional phagocytes such as epithelial and endothelial cells by macropinocytosis (78). CNF1 contributes to *E. coli* invasion of HBMECs *in vitro* and penetration into the brain *in vivo*, and these *in vitro* and *in vivo* effects of CNF1 depend on RhoA activation (55). This concept was shown by (a) decreased invasion and RhoA activation with the *cnf1* deletion mutant in HBMEC and (b) restoration of the *cnf1* mutant's invasion frequency to the level of the parent strain in HBMECs expressing constitutively active RhoA. CNF1 has been suggested to be internalized via receptor-mediated endocytosis upon binding to a cell surface receptor (75), but it is unclear how CNF1 enters the HBMEC and activates Rho GTPases. A yeast two-hybrid screening of the HBMEC cDNA library using the N-terminal cell binding domain of CNF1 as bait identified the HBMEC receptor for CNF1 (79). This receptor, 37-kDa laminin receptor precursor (LRP), interacted with the N-terminal CNF1 and full-length CNF1 but not with the C-terminal CNF1. CNF1-mediated RhoA activation and bacterial uptake were inhibited by exogenous LRP or LRP antisense oligodeoxynucleotides, whereas they were increased in LRP-overexpressing cells, demonstrating correlation between effects of CNF1 and levels of LRP expression in HBMEC (79). These findings indicate that CNF1 interaction with its receptor, 37-kDa LRP, is the initial step required for CNF1-mediated RhoA activation and bacterial uptake in eukaryotic cells. The 37-kDa LRP is a ribosome-associated cytoplasmic protein and shown to be a precursor of 67-kDa laminin receptor (LR). It is unclear how 67-kDa LR is matured and synthesized from the 37-kDa LRP, but mature 67-kDa LR is shown to be present on the cell surface and functions as a membrane receptor for the adhesive basement membrane protein laminin (80). CNF1-expressing *E. coli* has been shown to up-regulate 67-kDa LR expression on the surface of HBMEC and recruit 67-kDa LR to the site of invading *E. coli* in a CNF1-dependent manner (81). Increased expression of 67-kDa LR has been shown to be associated with invasive and metastatic properties of a variety of tumors (82), and it remains speculative whether CNF1-expressing *E. coli* has any role in malignant transformation of certain cancers. Although CNF1 is shown to interact with 37-kDa LRP/67-kDa LR on the cell surface of HBMEC, resulting in RhoA activation and increased internalization of CNF1-expressing *E. coli*, CNF1 is a bacterial cytoplasmic protein (83, 84), and it remains unclear how it is secreted into the outer membrane and interacts with 37-kDa LRP/67-kDa LR on the blood-brain barrier. Taken together, these findings indicate that meningitis-causing *E. coli* invades HBMECs through ligand-receptor interactions. Of interest, 37-kDa LRP/67-kDa LR has been shown to be a cellular target for various CNS-infecting microorganisms,

including *S pneumoniae*, *N meningitidis*, *H. influenzae* type b, dengue virus, adeno-associated virus, Venezuelan equine encephalitis virus, and prion protein (7). The mechanism by which the same receptor is involved in CNS penetration by different organisms remains to be established.

E. COLI TRAVERSAL OF THE BLOOD-BRAIN BARRIER AS LIVE BACTERIA

The ability of meningitis-causing pathogens to cross the blood-brain barrier as live bacteria is a critical factor for the development of meningitis. Meningitis-causing *E. coli* has been shown to traverse the blood-brain barrier without altering the integrity of the HBMEC monolayer and without affecting the blood-brain barrier permeability (30, 42). HBMECs have been shown to exhibit the complete trafficking machinery required to deliver the microbe-containing vacuoles to cathepsin D-containing components (i.e., lysosomes) (85). Vacuoles containing the *E. coli* K1 capsule deletion mutant interact sequentially with early endosomal marker proteins (e.g., early endosomal auto-antigen 1 and transferrin receptor) and late endosome and late endosome/lysosomal markers (e.g., Rab7 and lysosome-associated membrane proteins, respectively) and allow lysosomal fusion, with subsequent degradation of bacteria inside vacuoles. In contrast, vacuoles containing *E. coli* K1+ (*E. coli* with the K1 capsule) obtained early and late endosomes without fusion with lysosomes (85), thereby allowing *E. coli* K1 to cross the blood-brain barrier as live bacteria, indicating that *E. coli* K1 modulates intracellular trafficking to avoid lysosomal fusion in HBMECs. *E. coli* K1 capsule is well recognized for its serum resistance and antiphagocytic properties (14, 45, 46), which are the essence of inducing a high degree of bacteremia. Another novel property of the K1 capsule is to modulate the maturation process of *E. coli* K1+-containing vacuoles and prevent their fusion with lysosomes, which is an event necessary for traversal of the blood-brain barrier as live bacteria. Additional studies are needed to elucidate how the K1 capsule is able to modulate intracellular trafficking of *E. coli* K1+-containing vacuoles to avoid fusion with lysosomes in HBMECs and whether similar events occur with other meningitis-causing microbes.

THE PATHOGNESIS OF *E. COLI* MENINGITIS: CELLULAR MICROBIOLOGY APPROACHES

Pathogenic microbes internalize into nonprofessional phagocytes such as epithelial and endothelial cells via exploiting various strategies affecting host cell actin cytoskeleton rearrangements (21, 25). Electron microscopy studies have shown that meningitis-causing *E. coli* invasion of HBMECs is associated with microvillus-like protrusions at the entry site on the surface of HBMECs (24, 33) (Fig. 1), suggesting the involvement of host cell actin cytoskeleton rearrangement in *E. coli* invasion of HBMECs. This concept is supported by the demonstrations that the F-actin condensation occurs with invading bacteria and blockade of actin condensation with microfilament-disrupting agents such as cytochalasin D inhibits *E. coli* invasion of HBMECs (33).

Several host cell signal transduction pathways have been shown to be involved in meningitis-causing *E. coli* invasion of HBMECs, most likely through their effects on host cell actin cytoskeleton rearrangements. These include focal adhesion kinase (FAK), paxillin,

phosphatidylinositol 3-kinase (PI3K), Src kinase, signal transducers and activators of transcription 3 (STAT3), Rho GTPases (RhoA and Rac1), cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$), 5-lipoxygenase and cysteinyl leukotrienes, epidermal growth factor receptor (EGFR) tyrosine kinase, vascular endothelial growth factor (VEGF) receptor-1, ezrin, radixin and moesin (ERM), calmodulin-dependent myosin light-chain kinase, and protein kinase C (PKC) (7, 21, 40, 41, 52, 55, 68, 86-89) (Figure 2). The host–microbial factors exploiting such host cell signaling molecules for *E. coli* invasion of the blood-brain barrier, however, remain incompletely elucidated.

It is important to note that the above-mentioned host cell signaling molecules are shown to be activated in response to specific microbial factors of meningitis-causing *E. coli* and their interactions with HBMEC factors, and that participation of the same bacterial or host factors does not necessarily lead to activation of the same host cell signaling molecules. For example, FimH of meningitis-causing *E. coli* has been shown to induce RhoA activation, not FAK activation in HBMECs (56). In contrast, FimH of uropathogenic *E. coli* induces FAK activation in bladder epithelial cells (90). A similar concept is shown with host factors, e.g., gp96 functions as the receptor for *E. coli* OmpA and *L. monocytogenes* Vip. The OmpA–gp96 interaction resulted in FAK activation in HBMECs, but no FAK activation occurred with the Vip–gp96 interaction in mouse fibroblasts (21).

Elucidation of the mechanisms involved in *E. coli* penetration of the blood-brain barrier has been facilitated by determination of the above-mentioned host cell signaling molecules contributing to *E. coli* invasion of HBMECs, as exemplified below, (a) identification of the *E. coli* factors contributing to activation of specific host cell signaling molecules and (b) examination of the interrelationship of the host cell signaling molecules for elucidating how different *E. coli* factors contribute to HBMEC invasion.

OmpA and IbeA proteins of meningitis-causing *E. coli* are shown to be involved in FAK and PI3K activations as well as in STAT3 and Rac1 activations, while FimH and CNF1 in RhoA activation, OmpA and NlpI in $cPLA_2\alpha$ and PKCα activations, and CNF1 in ERM activation. This information has been useful for elucidating how several bacterial factors contribute to *E. coli* binding to and invasion of HBMEC. For example, Rac1 activation occurs in response to OmpA or IbeA, and RhoA activation occurs in response to CNF1 or FimH, while $cPLA_2\alpha$ activation occurs in response to OmpA or NlpI (25, 52, 56, 69, 88). *E. coli* mutants deleted of OmpA and CNF1, OmpA and FimH, or FimH and NlpI exhibit significantly greater defects in invasion of HBMEC compared to individual deletion mutants. In contrast, mutants deleted of OmpA and IbeA are not shown to exhibit significantly greater defects in HBMEC invasion compared to individual deletion mutants. Thus, the reasons for the additive (non-redundant) versus indifferent (redundant) effects of different bacterial factors in *E. coli* binding to and invasion of HBMEC can be in part explained by their underlying host cell signaling mechanisms (e.g., involving different or same host cell signaling molecules, respectively).

In addition, meningitis-causing *E. coli* strains exploit FAK and PI3K for invasion of HBMEC, as shown by significantly decreased invasion in HBMEC expressing dominant-negative FAK and PI3K and in HBMEC treated with pharmacologic inhibitors of FAK and

PI3K, but FAK is upstream of PI3K in *E. coli* invasion of HBMEC. This is shown by the demonstration that PI3K activation was abolished in HBMEC expressing dominant-negative FAK (86). Similarly, both STAT3 and Rac1 are involved in meningitis-causing *E. coli* invasion of HBMEC, but STAT3 is upstream of Rac1, as shown by blockade of Rac1 activation in HBMEC expressing dominant-negative STAT3 (88). Also, cPLA$_2\alpha$ and PKCα are involved in meningitis-causing *E. coli* invasion of HBMEC, but cPLA$_2\alpha$ is upstream of PKCα, as shown by the demonstration that inhibition of cPLA$_2\alpha$ prevents PKCα activation in response to meningitis-causing *E. coli* in HBMEC (40).

As indicated before, despite the comprehensive information on host cell signaling molecules contributing to *E. coli* invasion of HBMECs, it remains incompletely understood why and how several microbial factors are involved in HBMEC binding and invasion. It also remains to be determined whether complete abolition of HBMEC binding and invasion requires deletion of all the non-redundant bacterial factors contributing to HBMEC binding and invasion.

THE PATHOGENESIS OF *E. COLI* MENINGITIS: FUNCTIONAL GENOMIC APPROACHES

Genome sequencing information of meningitis-causing microbes is likely to provide a new tool for elucidating the pathogenesis of meningitis, but its utilization so far has been limited to meningitis-causing *E. coli*. Comparative genome analysis of the prototypic meningitis-causing *E. coli* strain RS218 (O18:K1) versus laboratory *E. coli* strain MG1655 identified 22 RS218-derived islands that are larger than 10 kb and are absent in strain MG1655 (51). These RS218-derived islands are termed RDIs. The total length of these RDIs is approximately 793 kb, which replaced approximately 80 kb of MG1655-specific sequences. The actual chromosomal size difference between RS218 and MG1655 was approximately 450 kb, which is slightly smaller than the previously estimated genome size difference between RS218 and MG1655 (91). Previous studies using comparative macrorestriction mapping and subtractive hybridization of the chromosomes of meningitis-causing *E. coli* (e.g., O18:K1 strains RS218 and C5) compared with nonpathogenic *E. coli* have identified 500 kb spread over at least 12 chromosome loci specific to meningitis-causing *E. coli* (92, 93). Mapping studies reveal that those *E. coli* loci are located at different regions of *E. coli* chromosome. Twenty-two RDIs have been shown to be located at different regions of *E. coli* RS218 chromosome (51).

By use of RDI deletion mutants, eight RDIs have been shown to be involved in the pathogenesis of *E. coli* meningitis (i.e., induction of a high degree of bacteremia and HBMEC binding/invasion) (51). The size and characteristics of these eight RDIs are summarized in Table 3. Two RDIs include a P4-family integrase and are directly adjacent to tRNAs (RDI 4-*serX* and RDI 21-*leuX*), and four RDIs (RDI 7, RDI 16, RDI 21, and RDI 22) have markedly lower GC percentages compared with the whole RS218 genome, suggesting that those RDIs are likely to be acquired through horizontal gene transfer. Further identification and characterization of microbial determinants from those RDIs that are

involved in the pathogenesis of *E. coli* meningitis should help in elucidating the microbial-host interactions that are involved in meningitis.

At present, a few virulence factors identified from prototypic meningitis-causing O18:K1 *E. coli* strains (e.g., strains RS218 and C5) have been used to understand the pathogenesis of meningitis (21-24, 51, 94-97), but it is unclear whether the information derived from these *E. coli* K1 strains is comprehensive and relevant to other *E. coli* meningitis isolates. For example, some of the identified *E. coli* factors are shown to be uncommon in CSF isolates (e.g., CNF1) (98).

A comparative genomic hybridization (CGH) with an *E. coli* DNA microarray was carried out to examine the basis of meningitis caused by representative *E. coli* strains isolated from blood and CSF (97). These strains include RS218 (O18:K1), C5 (18:K1), IHE3034 (O18:K1), EC10 (O7:K1), A90 (O1:K1), RS168 (O1:K1), RS167 (O16:K1), E253 (O12:K1), E334 (O12:K1), S88 (O45:K1), and S95 (O45:K1). A hierarchical clustering revealed that these strains can be categorized into two groups. Group 1 includes strains RS218, C5, IHE3034, A90, RS167, E334, S88, and S95, while strains EC10, RS168, and E334 belong to group 2. All group 1 strains belong to the phylogenetic group B2, which is predominant in meningitis isolates, and group 2 strains belong to less common phylogenetic groups A and D (97). All group 2 strains have been shown to harbor some genes from *E. coli* type III secretion system 2 (ETT2), but none of group 1 strains harbor ETT2 (97). The existence of a degenerate ETT2 gene cluster has been shown in septicemic *E. coli* O78 strains (99). Sequence analysis of the ETT2 genes showed premature stop codons in *eprI* and *eprJ* encoding the needle structure and deletion of the *invG* gene, which encodes a conserved component of the outer membrane ring. This ETT2 lacks the gene (*eivC*) for the cytoplasmic ATPase that energizes secretion and some other conserved components of type III secretion system (e.g., *epaS*). However, a deletion mutant of genes coding for the putative inner membrane ring of the secretion complex showed significantly reduced virulence in a 1-day-old chick model, even though the mutation does not seem to affect the secreted proteome (99). Meningitis-causing *E. coli* strain EC10 from group 2 was found to harbor all the genes needed to encode type III secretion apparatus proteins compared with the aforementioned septicemic *E. coli* O78 strain 789 (97, 100). The type III secretion system has been shown to be involved in EC10's invasion and intracellular survival in HBMECs (101), and additional studies are needed to elucidate the role of type III secretion system in the pathogenesis of *E. coli* meningitis.

The CGH was also utilized to examine the distribution of the eight RDIs that are relevant to the pathogenesis of *E. coli* meningitis among representative meningitis-causing *E. coli* isolates (67). RDI 16 harbors the K1 capsule biosynthesis gene cluster and, as expected, is present in all of the meningitis isolates. The other pathogenic RDIs are found to exist in strains belonging to the above-mentioned group1 and phylogenetic group B2. For example, RDI 1, 7, 13, 20, and 22 are widely distributed among this group of *E. coli* strains. Previous studies using PCR, dot blot, and Southern blot suggest that PAI III_{536}-like, PAI II_{J96}-like, and GimA-like ectochromosomal DNA domains (ECDNAs) are prevalent among O18:K1 strains, the most common serogroup in meningitis-causing *E. coli* (94). Based on their virulence signatures, those ECDNAs correspond to RDI 4, 21, and 22, respectively. The

distribution of these three RDIs among O18:K1 strains based on CGH is consistent with previous findings (51, 94). The CGH analysis also revealed that type VI secretion system (T6SS)-like gene clusters, including the *icmF*-like component, *clpV*, *dotU*, and *hcp2*, are present in the RDI 1 (51, 102). Of interest, the T6SS clusters have two *hcp*-like genes located next to each other in the chromosome of strain RS218, and the two Hcp family proteins have been shown to exhibit different roles in meningitis-causing *E. coli* infection and coordinately contribute to the pathogenesis of *E. coli* interaction with HBMEC, e. g., *E. coli* binding to and invasion of HBMEC as well as release of IL-6 from HBMEC (102).

In addition, microbial DNA microarrays offer new opportunities for exploring microbial gene expression profiles during microbe-host interactions. For example, using *E. coli* DNA microarray analysis with microarray-grade bacterial RNA isolated from *E. coli* interacting with HBMECs, the expression of the type 1 fimbria genes is shown to be significantly higher for *E. coli* associated with HBMEC than for *E. coli* not associated with HBMECs (59). Subsequently, type 1 fimbriae are shown to play an important role in *E. coli* binding to and invasion of HBMECs (59), indicating that microbial DNA microarray analysis has a potential for elucidating microbial-host interactions that are relevant to the pathogenesis of meningitis.

Some meningitis-causing *E. coli* strains (e. g., strains S88 and RS218) harbor large plasmids (103, 104). The plasmids from strains S88 (045:K1) and RS218 (018:K1) have been shown to contribute to a high-degree of bacteremia, suggesting that they are involved in the development of *E. coli* meningitis, but the underlying mechanisms remain incompletely understood.

PREVENTION OF BACTERIAL PENETRATION INTO THE BRAIN BY TARGETING THE MICROBIAL-HOST FACTORS CONTRIBUTING TO *E. COLI* INVASION OF THE BLOOD-BRAIN BARRIER

Meningitis-causing *E. coli* penetration into the brain requires *E. coli* binding to and invasion of HBMEC, involving specific microbial and host factors, and host cell signaling molecules (7, 21-25, 28). The information on host cell receptors and host cell signal transduction pathways in the microbial invasion of the blood–brain barrier is likely to provide a new paradigm for prevention and therapy of meningitis by targeting such host cell receptors or signaling molecules (7, 21-25, 28, 40, 41, 68, 79, 88, 105). A proof-of-concept study has shown that down-modulation of the HBMEC receptor for CNF1 (37LRP) and blockade or inhibition of host cell signaling molecules involved in *E coli* invasion of HBMECs (e.g., $cPLA_2\alpha$) were efficient in preventing *E coli* penetration into the brain (7, 21, 25, 28, 41, 41, 79). In addition, pharmacological inhibition and gene deletion of host cell signaling molecules (e. g., $cPLA_2\alpha$) involved in *E. coli* invasion of HBMECs was efficient in preventing *E. coli* penetration into the brain (40, 41).

Determination of the host cell receptors that interact with *E. coli* factors and host cell signaling molecules contributing to *E. coli* invasion of HBMEC also provides a novel strategy for elucidating the pathogenesis of *E. coli* meningitis. The feasibility of this novel

strategy was shown by the demonstration that pharmacological inhibition of the host cell signaling molecules involved in *E. coli* invasion of HBMEC (e. g., cPLA$_2\alpha$) was beneficial in elucidating the novel mechanisms involved in *E. coli* penetration into the brain (e.g., cPLA$_2\alpha$-cysteinyl leukotrienes) (7, 21-25, 28, 40, 41). Additional studies are needed to elucidate the microbial–host factors that contribute to *E. coli* invasion of HBMEC and also can serve as a novel target for prevention and therapy of *E. coli* meningitis.

An additional novel strategy for prevention and therapy of *E. coli* meningitis is to modulate the expression and/or secretion of the microbial factors contributing to HBMEC binding and invasion. For example, CNF1 is a key factor contributing to *E. coli* invasion of HBMEC and penetration into the brain via the interaction with its receptor (37LRP) on HBMEC (55, 79). CNF1, however, is a cytoplasmic protein and execution of its contribution to *E. coli* invasion of the blood–brain barrier requires its secretion from the bacterial cytoplasm. No signal peptide is found in the CNF1 sequence. CNF1 secretion is, therefore, a strategy utilized by meningitis-causing *E. coli* to invade the blood–brain barrier. It, however, remains unclear how CNF1 secretion occurs across the bacterial inner membrane and outer membranes. Elucidation of the mechanisms involved in CNF1 secretion is, therefore, likely to enhance our knowledge on the pathogenesis of *E. coli* meningitis and also help in developing a novel strategy targeting CNF1 secretion in prevention and therapy of *E. coli* meningitis (83, 84). Taken together, these findings suggest that modulation of bacterial secretion systems (CNF1 secretion, type II secretion, type III secretion, type VI secretion) represents a novel target for prevention and therapy of *E. coli* meningitis.

THE MECHANISMS INVOLVED IN CNS INFLAMMATION FOLLOWING *E. COLI* PENETRATION INTO THE BRAIN

Bacterial meningitis is characterized by inflammation of the meninges that occurs in response to bacteria and bacterial products, resulting in release of cytokines and chemokines as well as pathophysiological alterations such as infiltration of leukocytes and blood–brain barrier dysfunction (7, 21, 24, 25, 28). Several studies have shown that the mechanisms involved in microbial invasion of the blood–brain barrier differ from those involved in the release of cytokines and chemokines in response to meningitis-causing pathogens (7, 21, 24, 25, 28). For example, interleukin-8 (IL-8) secretion in response to *E coli* strain happens in HBMEC, but not in non-brain endothelial cells (e.g., human umbilical vein endothelial cells). However, *E coli* factors involved in HBMEC binding and invasion did not affect the release of IL-8 from HBMEC (106). Similar findings were demonstrated for *N. meningitidis* (107). These findings suggest that targets for prevention of bacterial penetration across the blood-brain barrier are likely to differ from those involved in CNS inflammation associated with bacterial meningitis.

CONCLUSION

A major limitation to advances in prevention and therapy of *E. coli* meningitis is our incomplete understanding of the pathogenesis of this disease. Successful *E. coli* penetration of the blood-brain barrier requires a high-degree of bacteremia as well as *E. coli* binding to and invasion of HBMECs, but the underlying mechanisms remain incompletely understood.

Studies with the *in vitro* and *in vivo* blood-brain barrier models have shed light on the mechanisms of microbial translocation of the blood-brain barrier, a key step for the development of meningitis. At present, the basis for requiring a high-degree of bacteremia for *E. coli* penetration of the blood-brain barrier, however, remains unknown. Meningitis-causing *E. coli* penetrates the blood-brain barrier transcellularly without altering the integrity of the HBMEC monolayer and without affecting blood-brain barrier permeability. Meningitis-causing *E. coli* penetration of the blood-brain barrier exploits specific microbial and host factors as well as specific host cell signal transduction pathways. Complete understanding of the microbial-host interactions that are involved in *E. coli* penetration of the blood-brain barrier as well as blood-brain barrier penetration-induced intracranial inflammation should help in developing a new strategy for prevention and therapy of *E. coli* meningitis.

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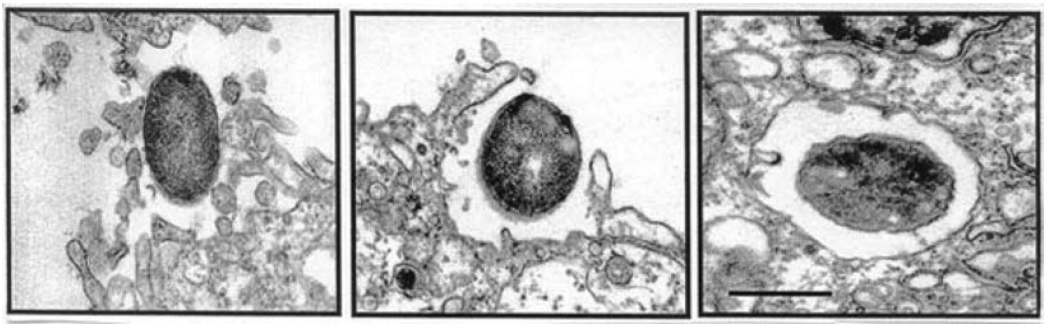

Figure 1.

Transmission electron micrographs of human brain microvascular endothelial cell monolayers infected with meningitis-causing *E. coli* strain RS218 (O18:K1). Scale bar = 1 μm.

Modified with permission from (25).

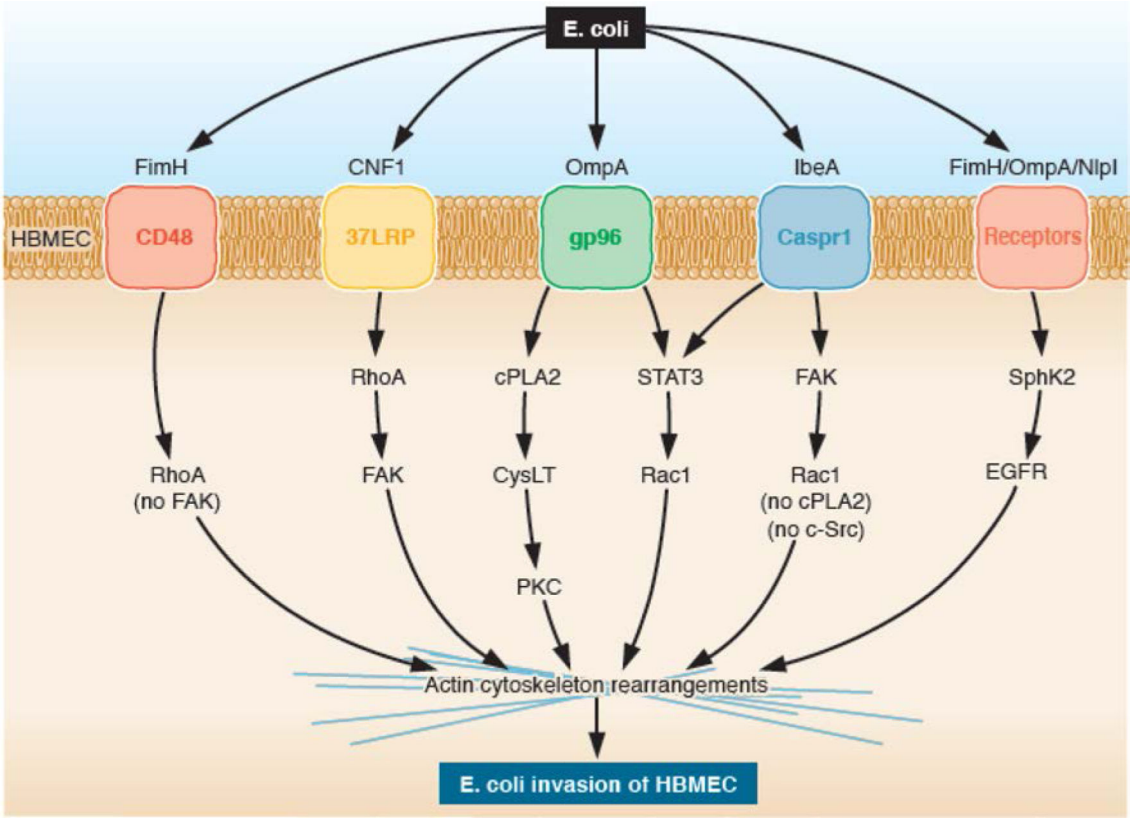

Figure 2.

Host cell signaling molecules exploited by specific microbial-host interactions involved with meningitis-causing *E. coli* for invasion of the blood-brain barrier.

Modified with permission from (25).

Table 1

Mechanisms involved in *E. coli* penetration of the blood-brain barrier and *E. coli* factors contributing to translocation of the blood-brain barrier

Mechanism	***E. coli* factors**
1. A high-degree of bacteremia	K1, O-LPS, NlpI, AVF, SslE
2. *E. coli* binding to HBMECs	FimH, HbiC, FliC, OmpA, NlpI
3. *E. coli* invasion of HBMECs	IbeA, IbeB, IbeC, AslA, CNF1
4. *E. coli* traversal of the blood-brain barrier as live bacteria	K1

Table 2

Development of bacteremia and meningitis (defined as positive CSF cultures) in newborn rats receiving meningitis-causing *E. coli* strain RS 218 or its isogenic mutants

E. coli strain	No. of animals	Bacteremia (log_{10} CFU/ml blood)	No. of animals with meningitis (%)
RS 218	19	7.18 ± 0.63	12 (63)
Δ *ompA*	22	7.05 ± 0.49	6 (27)[a]
RS 218	24	7.51 ± 1.25	16 (67)
Δ *ibe A*	25	6.97 ± 1.21	4 (16)[a]
RS 218	27	7.01 ± 1.17	15 (56)
Δ *ibe B*	25	7.06 ± 1.29	4 (16)[a]
RS 218	24	7.53 ± 0.40	18 (75)
Δ *ibeC*	24	7.80 ± 0.67	10 (42)[a]
RS 218	17	7.50 ± 0.32	14 (82)
Δ *aslA*	22	7.60 ± 0.49	7 (32)[a]
RS 218	26	7.64 ± 1.00	20 (77)
Δ *cnf1*	27	7.24 ± 1.60	12 (44)[a]

Modified from (22)

[a]Significantly less than RS 218

Table 3

Size and characteristics of eight RDIs derived from meningitis-causing *E. coli* strain RS218 that are involved in the pathogenesis of *E. coli* meningitis

RDI	Size (kb)	Anchor tRNA	%GC[a]	Defects in bacteremia[b]	Defects in HBMEC binding/invasion[b]
1	28.7	aspV	51.52	-	+
4	61.7	serX	48.07	+	+
7	14.9		41.91	+	+
13	12.1		50.49	-	+
16	27.7	pheW	45.68	+	-
20	10.9		48.86	-	+
21	116.5	leuX	46.22	+	-
22	20.3		46.11	-	+

Data from (51)

[a]The average %GC in the *E. coli* strain RS218 genome is 50.63.

[b]+ indicates RDI deletion mutants exhibiting defects in inducing a high-degree of bacteremia and/or HBMEC binding/invasion.