

Escherichia coli Binding to and Invasion of Brain Microvascular Endothelial Cells Derived from Humans and Rats of Different Ages

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***Escherichia coli* meningitis commonly occurs in the neonatal period, but the basis of this age dependency is unclear. We have previously identified two types of *E. coli*-brain microvascular endothelial cell (BMEC) interactions contributing to *E. coli* traversal of the blood-brain barrier (i.e., binding and invasion). The present study examined whether the age dependency of *E. coli* meningitis stemmed from differences in the capacities of neonatal and adult BMECs to interact with *E. coli*. BMECs were isolated from rats of different ages (10 days, 20 days and 3 months) as well as from humans of different ages (fetuses, 4- to 7-year-old children, and a 35-year-old adult, and 60- to 85-year-old geriatrics). The bindings of *E. coli* to young and old rat BMECs were similar. Also, the abilities of *E. coli* to invade BMECs were similar for BMECs derived from young and old rats and from human fetuses, children, adults, and geriatrics. These findings suggest that the predominance of *E. coli* meningitis in neonates is not likely due to greater binding and invasion capacities of newborn compared to adult BMECs.**

The mortality and morbidity associated with neonatal gram-negative bacillary meningitis have remained significant despite advances in antimicrobial chemotherapy and supportive care (3, 18). Both clinical and experimental data indicate the limited efficacy of antimicrobial chemotherapy alone (5, 7). Inadequate knowledge of the pathogenesis and pathophysiology of the disorder has contributed to this limited reduction in mortality and morbidity. For example, *Escherichia coli* meningitis is common during the neonatal period, but the basis of this age dependency is unclear.

We have previously shown that *E. coli* S fimbriae mediate binding to brain microvascular endothelial cells (BMEC) and that OmpA contributes to the invasion of BMEC (12, 14, 17). Parkkinen et al. (11) reported that the binding of S fimbriae to rat brain is decreased after the neonatal period, suggesting that the density of bacterial receptors on host tissue may be responsible for the age dependency of *E. coli* meningitis. The present study therefore examined and compared the capacities of *E. coli* to bind to and invade BMEC from young (10- or 20-day-old) and adult (3-month-old) rats as well as from human fetuses, children, an adult, and geriatrics.

Rat brain capillaries were isolated by a modification of the method previously described (17). Briefly, fresh rat brains were obtained from 10- or 20-day-old pups or 3-month-old rats, and the cerebellum, brain stem, choroid plexus, and meninges were carefully removed. Cortices were homogenized in Dulbecco's modified Eagle medium containing 2% fetal bovine serum (FBS) (DMEM-S) by using a Dounce homogenizer with a loose fitting. The homogenate was centrifuged in 15% Dextran in DMEM-S for 10 min at 10,000 × g. The pellet containing crude microvessels was further digested in a solution containing 1 mg of collagenase-dispase per ml in DMEM-S for 1 h at

37°C. Microvascular capillaries were isolated by adsorption to a column of glass beads, and the capillaries were then washed off the beads and recovered in growth medium. Human brain capillaries were isolated and cultured as described previously (16). Briefly, fetal brain specimens were obtained from Advanced Bioscience Resources, Inc. (Alameda, Calif.) via Milan Fiala of the UCLA School of Medicine. These specimens were derived from fetuses aborted at 19 weeks of gestation. Small fragments of cerebral cortex were obtained from surgical resections of 4- to 7-year-old children with seizure disorders at Children's Hospital Los Angeles. Brain tissue was also obtained from a 35-year-old patient after cortical resection for epilepsy. Postmortem brain samples were obtained from the Alzheimer's Disease Research Center of the University of Southern California via Carol Miller (the age of the patients at time of death varied from 60 to 85 years). The cerebral cortex specimens from non-Alzheimer patients were used for this study. Visible large blood vessels, if present, were carefully removed from brain specimens. Brain tissue specimens were subsequently processed for isolation of microvascular capillaries as described above, and the resulting endothelial cells were designated as fetal, child, adult, or geriatric BMEC.

The rat brain microvessels were plated on rat-tail-collagen-coated dishes or glass coverslips and were cultured in minimal essential medium containing D-valine (to inhibit growth of nonendothelial cells) (2, 17), 20% FBS, endothelial cell growth supplement (30 µg/ml), heparin (120 U/ml), L-glutamine (2 mM), sodium pyruvate (2 mM), nonessential amino acids, vitamins, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cultures were incubated at 37°C in a humid atmosphere of 5% CO₂. For binding and invasion experiments, rat brain endothelial cells of passage 1 or 2 were used at confluence. The human brain microvessels were plated on rat-tail-collagen-coated supports and cultured in RPMI 1640-based medium with 10% FBS, 10% NuSerum, endothelial cell growth supplement (30 µg/ml), heparin (5 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids, vitamins, penicillin, and streptomycin. Cultures were incubated at 37°C

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in a humid atmosphere of 5% CO₂. For invasion experiments, human BMEC of passage 6 or 8 were used.

Morphologic and immunocytochemical analyses of BMEC monolayers were performed with cells grown to confluency on collagen-coated coverslips and were examined with an Olympus IX 70 microscope with phase contrast. Specific marker studies were carried out as previously described (16, 17). Briefly, cells were grown on collagen-coated glass coverslips, washed with Hanks balanced salt solution (HBSS), fixed in cold acetone-methanol (1:1, vol/vol) for 15 min, and were air dried, sealed, and stored at -20°C. Cells were rehydrated, washed with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.01% Tween, preincubated with 10% normal goat serum for 15 min, and incubated with the appropriate antibody for 1 h at room temperature. After incubation with the primary antibody, the monolayers were washed with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.01% Tween, incubated with peroxidase-labeled secondary antibody for 30 min, and mounted on slides with glycerol or aquamount. Specimens were viewed in a Nikon Diaphot fluorescence microscope equipped with a standard fluorescein isothiocyanate filter combination. Endothelial cells possessing factor VIII-Rag were identified by using rabbit anti-human factor VIII (dilution, 1:200), pericytes were identified by using mouse anti-smooth muscle actin (1:400), and glial cells were identified by using rabbit anti-cow glial fibrillary acidic protein (GFAP) antibodies (1:200). Antibodies against carbonic anhydrase IV were used at a dilution of 1:500. Primary and/or secondary antibodies were omitted for controls, resulting in the absence of staining.

Endothelial cells were also identified by their ability to take up acetylated low density lipoprotein (AcLDL) as previously described (16, 17). Cultures were tested for the presence of gamma glutamyl transpeptidase (GGTP) specific for brain endothelial cells (8, 10, 16, 17, 19). Monolayers of acetone-methanol-fixed cells were rehydrated, and GGTP activity was histochemically assessed as described previously (16, 17).

Transformants of *E. coli* HB101 with or without S fimbrial plasmids were previously described (12, 17). Strain HB101 was shown not to invade BMEC and not to possess the structures contributing to BMEC invasion (e.g., Ibe A protein) (4). Strain HB101/13 carrying pANN801-13 produced the wild-type S fimbria adhesin complex, and strain HB101/322 carried the vector pBR322. *E. coli* strains E69 and E91 were derived from strain RS218 (O18:K1:H7) by P1 transduction as described previously (14). Strain RS218 was shown to invade BMEC and to possess the structures contributing to BMEC invasion (e.g., Ibe A protein) (4). Strain E69 (OmpA⁺) contained the entire *ompA* gene, and strain E91 (OmpA⁻) lacked the *ompA* gene. The presence or absence of *ompA* was verified by Southern hybridization using ³²P-labeled cloned *ompA* gene from pRD87, and OmpA expression was verified by Western blotting using anti-OmpA antibody (14). All *E. coli* strains were grown in brain heart infusion broth containing appropriate antibiotics. Bacteria were metabolically labeled with [³H]adenine (25 mCi/mmol) during logarithmic growth until a concentration of approximately of 10⁹ bacteria/ml was reached. These bacteria were harvested by centrifugation and washed four times at 10,000 × g for 10 min, were resuspended in HBSS containing 20% glycerol, and were stored at -20°C.

Assays for *E. coli* binding to BMEC were performed by the method previously described (17). Briefly, BMEC were subcultured into 24-well tissue culture plates and grown to confluence. Monolayers were washed three times with HBSS and incubated at 4°C for 2 h with ³H-labeled bacteria (multiplicity of infection of approximately 10) in M199-Ham F-12 medium

containing 5% heat-inactivated FBS. Thereafter, the monolayers were washed four times, and the BMEC plus adherent bacteria were dissolved in 0.2 M NaOH. Subsequently, ice-cold 10% trichloroacetic acid was added to each well. The resulting precipitates were collected on glass microfiber filters and assayed by liquid scintillation counting. Binding was expressed as percentage of the total added trichloroacetic acid precipitable counts.

Invasion assays were performed as described previously (4, 14). Approximately 10⁷ bacteria were added to the confluent BMEC monolayer (multiplicity of infection of 100) which was then incubated at 37°C for 1.5 h. The monolayers were washed four times and then incubated for 1 h at 37°C with gentamicin (100 µg/ml) to kill extracellular bacteria. The monolayers were again washed four times and lysed with 0.5% Triton X-100 (bacterial viability was not affected by 0.5% Triton X-100 treatment), and released intracellular bacteria were enumerated by plating on blood agar. Percent invasion was calculated by dividing the number of surviving intracellular bacteria by the number of bacteria inoculated and multiplying by 100. Each assay was run in triplicate and repeated at least two times. The condition of the cells was morphologically assessed after each binding and invasion experiment. Cells were more than 95% viable as estimated by trypan blue exclusion, and no detachment of cells was observed.

Freshly isolated microvessels displayed a viability of more than 95% as judged by trypan blue exclusion. After seeding on collagen-coated flasks, the isolated microvessels readily attached, and cells migrated away from the initial attachment sites. The rat cells were spindle shaped, took up acLDL, and stained positive for factor VIII-Rag and carbonic anhydrase IV, typical for endothelial cells. The cells also stained positive for GGTP, indicative of their brain origin, although the intensity of the staining varied. Contamination of other cell types varied from less than 1 to 5% for GFAP-positive cells and from less than 1 to 20% for smooth-muscle-actin-positive pericytes. With increasing passage number the morphology of the cells changed to a more flat appearance, and staining for factor VIII-Rag, carbonic anhydrase IV, and GGTP decreased. Our binding and invasion experiments used only early passage cells (passage 1 or 2) which had positive staining for factor VIII, carbonic anhydrase IV, and GGTP as well as contaminating nonendothelial cells (<5%).

The BMEC from children displayed a somewhat spindly morphology as previously described (16) while the fetal, adult, and geriatric human BMEC showed a more cobblestone-like morphology. Human BMEC from different ages stained positive for factor VIII-Rag, took up acLDL, and were positive for GGTP. No GFAP-positive cells were present, and very few cells stained positive for smooth muscle actin, indicating that the cultures were more than 95% pure (16).

Table 1 summarizes the degrees to which *E. coli* bound to and invaded BMEC derived from rats of different ages. As shown, binding to BMEC was significantly greater (*P* < 0.01) for S-fimbriated *E. coli* HB101/13 than for nonfimbriated HB101/322. We have previously shown that both E69 and E91 strains possess S fimbriae and that the number of BMEC-associated bacteria did not differ between strains E69 and E91 (14). But as shown in Table 1, binding to rat BMEC was statistically different for strains E69 and E91 (*P* < 0.05). The basis of the differences in binding to BMEC observed for strains E69 versus E91 in the present study is not clear. Of interest, the magnitude of binding was significantly less with strain E69 than with strain HB101/13. In contrast, only OmpA⁺ strain E69 exhibited invasive capacity, while HB101 transformants and strain E91 failed to invade BMEC. More

TABLE 1. Comparison of the degrees to which *E. coli* bound to and invaded BMEC derived from rats of different ages

<i>E. coli</i> strain	Results of assays of rats aged ^a :					
	10 Days		20 Days		3 Months	
	Binding	Invasion	Binding	Invasion	Binding	Invasion
HB101/13	1.2 ± 0.08 ^b	<0.0001	1.3 ± 0.1 ^b	<0.0001	2.1 ± 0.02 ^b	<0.0001
HB101/322	0.33 ± 0.01	<0.0001	0.4 ± 0.15	<0.0001	0.8 ± 0.03	<0.0001
E69	0.88 ± 0.05 ^c	0.02 ± 0.01 ^d	0.9 ± 0.1 ^c	0.02 ± 0.01 ^d	1.13 ± 0.02 ^c	0.02 ± 0.01 ^d
E91	0.55 ± 0.11	<0.0001	0.4 ± 0.12	<0.0001	0.10 ± 0.02	<0.0001

^a Expressed as mean percentages ± standard deviations.

^b $P < 0.01$, compared to HB101/322 by two-tailed *t* test.

^c $P < 0.05$, compared to E91 by two-tailed *t* test.

^d $P < 0.01$, compared to HB101 and E91.

importantly, *E. coli* binding and invasion characteristics did not differ between neonatal and adult rat BMEC.

We have shown that invasion of BMEC is required for *E. coli* K1 to cross the blood-brain barrier in vivo (4, 14). We therefore examined whether or not the invasion capacities of *E. coli* K1 strains E69 and E91 differed among human BMEC derived from fetuses, children, an adult, and geriatrics. As shown in Table 2, OmpA⁺ strain E69 invaded approximately 4- to 15-fold more efficiently than OmpA⁻ strain E91 in all human BMEC examined. These results are consistent with those of our previous study illustrating that OmpA⁺ *E. coli* invaded bovine BMEC significantly more than OmpA⁻ *E. coli* (14). Similar to the results obtained from rat BMEC, *E. coli* invasion did not differ between BMEC from fetuses, children, adults, and geriatrics.

E. coli is the most common gram-negative organism causing neonatal meningitis, but it is unclear why *E. coli* meningitis is predominant in the neonatal period. Most cases of *E. coli* meningitis occur as a result of hematogenous spread (1). We have shown that a high degree of bacteremia is a primary determinant of meningeal invasion by *E. coli* K1 (6), suggesting that one possible explanation for the prevalence of *E. coli* meningitis in the neonatal period is the high relative susceptibility of neonates to bacteremia compared to adults. Another possibility is that *E. coli* may be able to interact with neonatal BMEC to a greater extent than with adult BMEC.

At present, it is unclear how circulating *E. coli* crosses the blood-brain barrier. Our previous studies have identified two types of *E. coli*-BMEC interactions contributing to *E. coli* traversal of the blood-brain barrier, i.e., binding and invasion (4, 12, 14, 17). We have shown that *E. coli* binds BMEC via S fimbriae, but binding via S fimbriae was not accompanied by invasion (14, 17). We, therefore, speculate that *E. coli* binding to BMEC is an important attribute for the initial attachment of circulating *E. coli* to BMEC to withstand blood flow in vivo. In contrast, we have shown that *E. coli* invasion of BMEC is a prerequisite for *E. coli* crossing the blood-brain barrier in vivo and that several bacterial determinants contribute to this invasion phenotype (i.e., OmpA and Ibe) (4, 14). We have also

shown that *E. coli* binding to and invading endothelial cells pertaining to the pathogenesis of meningitis is specific to endothelial cells of brain origin and that such characteristics are not observed in the endothelial cells of systemic vessels such as human umbilical vein endothelial cells and human aortic arterial endothelial cells (13, 17).

The present study, therefore, examined whether the basis of the age dependency of *E. coli* meningitis is due to differences in the capabilities of host tissues to interact with *E. coli*. This study was feasible because of our ability to successfully isolate and cultivate human BMEC derived from children and adults. As shown previously (9, 15, 16), these human BMEC were found to be pure (>99%) and exhibited tightness, as demonstrated by the limited permeability of inulin and transendothelial electrical resistance of at least 100 Ω per cm², a property unique to BMEC monolayers among systemic vascular endothelia. The present study extended those BMEC to include human BMEC derived from fetuses and geriatrics. The morphologic characteristics of these human BMEC derived from fetuses, children, adults, and geriatrics were similar in exhibiting factor VIII-Rag, carbonic anhydrase IV, and GGTP and by uptake of AcLDL, and contaminating pericytes and glial cells were rarely observed. As shown in the present study, the abilities of *E. coli* to invade human BMEC derived from fetuses, children, an adult, and geriatrics were similar, suggesting that the age dependency of *E. coli* meningitis is not due to differences in host tissue interaction with *E. coli*.

We have previously shown in the rat model of experimental hematogenous meningitis that the prevalence of meningitis (defined as positive cerebrospinal fluid cultures) is similar between newborn and adult rats who developed bacteremia of greater than 10⁴ CFU/ml of blood (45 versus 65%) (6). We, therefore, isolated and cultivated BMEC derived from newborn (10- and 20-day-old) and adult rats and examined their ability to interact with *E. coli*. Similar to the findings with BMEC derived from humans of different ages, BMEC from newborn and adult rats also exhibited similar characteristics in their interactions with *E. coli* (i.e., binding and invasion). These findings are concordant with those of Parkkinen et al.

TABLE 2. Comparison of *E. coli* invasion of BMEC derived from humans of different ages

<i>E. coli</i> strain	Human BMEC source ^a			
	Fetus	Child	Adult	Geriatric
E69	0.025 ± 0.007 ^b	0.020 ± 0.001 ^b	0.075 ± 0.005 ^b	0.025 ± 0.006 ^b
E91	0.006 ± 0.001	0.002 ± 0.001	0.005 ± 0.003	0.006 ± 0.003

^a Expressed as mean percentages ± standard deviations.

^b $P < 0.05$, compared to E91 by two-tailed *t* test.

(11) who have shown that the binding of S fimbriae to the luminal surfaces of the brain vascular endothelium is similar between 3- and 30-day-old rats. However, these investigators have shown that the binding of S fimbriae to the choroid plexuses and ventricular epithelium is less prevalent in 3-day-old rats than in 30-day-old rats, suggesting that this age-dependent difference in bacterial receptor densities in choroid plexuses may contribute to the age-specific predisposition to *E. coli* meningitis. We have previously shown in the experimental hematogenous meningitis model that the entry of *E. coli* K1 into the central nervous system occurs initially in the cerebral vasculature, not in choroid plexus (6). Thus, it remains unclear whether the above-mentioned differences in binding to choroid plexuses contribute to the increased susceptibility of newborn rats to *E. coli* meningitis. Taken together, our in vitro and in vivo findings indicate that the age dependency of *E. coli* meningitis is not likely due to greater binding and invasion capabilities of newborn compared to adult BMEC.

Of interest, we have previously shown that the induction of the high degree of bacteremia necessary for the development of meningitis requires different bacterial inocula for newborn and adult rats, i.e., an approximately 10^6 -fold greater inoculation of *E. coli* K1/gm of body weight is required in adult compared to newborn rats to induce similarly high levels of bacteremia (e.g., $>10^4$ CFU/ml of blood) (6). Taken together, these findings indicate that one of the reasons for the close association of newborns with *E. coli* meningitis is the relative ease with which *E. coli* K1 evades the neonatal host defenses to achieve the threshold level of bacteremia necessary for meningeal invasion. It is, therefore, prudent to suggest that the prevention of bacterial multiplication in the blood necessary for bacterial entry into the central nervous system would be one potential approach to the prevention of neonatal *E. coli* meningitis.

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