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Binding Characteristics of S Fimbriated *Escherichia coli* to Isolated Brain Microvascular Endothelial Cells

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To assess the role of S fimbriae in the pathogenesis of* Escherichia coli *meningitis, transformants of* E. coli *strains with or without S fimbriae plasmid were compared for their binding to microvessel endothelial cells isolated from bovine brain cortices (BMEC). The BMEC's displayed a cobblestone appearance, were positive for factor VIII, carbonic anhydrase IV, took up fluorescent-labeled acetylated low density lipoprotein, and exhibited gamma glutamyl transpeptidase activity. Binding of S fimbriated* E. coli *to BMEC was approximately threefold greater than nonfimbriated* E. coli *Similarly S fimbriated* E. coli *bound to human brain endothelial cells approximately threefold greater than nonfimbriated* E. coli *Binding was reduced approximately 60% by isolated S fimbriae and about 80% by anti-S adhesin antibody. Mutating the S adhesin gene resulted in a complete loss of the binding, whereas mutagenesis of the major S fimbriae subunit gene sfaA did not significantly affect binding. Pretreatment of BMEC with neuraminidase or prior incubation of S fimbriated* E. coli *with NeuAcα2,3-sialyl lactose completely abolished binding. These findings indicate that S fimbriated* E. coli *bind to NeuAcα2,3-galactose containing glycoproteins on brain endothelial cells via a lectin-like activity of SfaS adhesin. This might be an important early step in the penetration of bacteria across the blood-brain barrier in the development of* E. coli *meningitis. (Am J Pathol 1994, 145:1228–1236)

Neonatal bacterial meningitis still results in a high mortality and morbidity, despite advances in antimicrobial chemotherapy and supportive care.[1] A contributing factor may be the incomplete understanding of pathogenesis and pathophysiology associated with bacterial meningitis. *Escherichia coli* is the most common gram-negative bacteria that cause meningitis during the neonatal period.[1–5] However, the mechanism(s) of *E. coli* entry into the central nervous system are not clear.

Adhesion of bacteria to mammalian cells is mediated by filamentous surface proteins, called fimbriae. *E. coli* expresses many types of fimbriae, which are characterized by their carbohydrate binding specificity.[2] Sialyl galactosides recognizing fimbriae (S fimbriae) are common in cerebrospinal fluid isolates from neonates with *E. coli* meningitis, therefore, S fimbriae have been implicated as one of the factors responsible for neonatal *E. coli* meningitis.[2,6–8] In addition, histochemical studies have revealed that isolated S fimbriae bind to luminal surfaces of vascular endothelium in neonatal rat brain tissues.[9] S fimbriae adhesin, a functional protein complex, consists of four proteins, the major subunit SfaA (16 kd) and minor subunits SfaG (17 kd), SfaS (14 kd), and SfaH (29 kd).

To further investigate the role of S fimbriae in the pathogenesis of *E. coli* meningitis *in vitro*, we studied the binding of different *E. coli* strains with intact or mutated S fimbriae or mutated S fimbriae subunits to monolayers of brain endothelial cells. We show that the presence of S fimbriae is important in the binding of *E. coli* to endothelial cells isolated from both bovine and human brains and that S adhesin is the major determinant.

Materials and Methods

Bovine calf serum was obtained from HyClone (Logan, UT). Dextran, molecular weight 87,000, bovine serum albumin (BSA), endothelial cell growth supple-

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ment (ECGS), fibronectin, glass beads (0.25 to 0.3 mm), mouse antismooth muscle actin IgG2a, FITC-labeled goat anti-mouse IgG, neuraminidase from *Vibrio cholera*, NeuAcα2-3-galactose from bovine colostrum, neuraminic acid, mannose, and sucrose were purchased from Sigma (St. Louis, MO). Collagenase/dispase was from Boehringer Mannheim (Indianapolis, IN). Dulbecco's modified Eagle medium (DMEM), Ham's F-12, medium 199 with D-valine, and Hanks' balanced salt solution (HBSS) were purchased from Irvine Scientific (Irvine, CA). Heparin was from Elkins Sinn Inc. (Cherry Hill, NJ). The 1,1′-dioctadecyl-1-3,3,3′3′-tetramethyl-indocarbocyanine perchlorate-labeled acetylated low density lipoprotein (DiI-AcLDL) was from Biomedical Technologies Inc. (Stoughton, MA). FITC-labeled swine anti-rabbit IgG (SWAR-F), rabbit IgG's against human F-VIII, and cow glial fibrillary acidic protein (GFAP) were from Dako (Glostrup, Denmark). Peroxidase-labeled goat anti-rabbit and goat anti-mouse IgG and IgM were from Bio-Rad (Richmond, CA). Glass microfiber filters (1.2 µ) were obtained from Whatman (Maidstone, UK). Brain heart infusion (BHI) broth was from Difco Laboratories (Detroit, MI). [^{3}H]adenine was from ICN Biomedical Inc. (Costa Mesa, CA).

Isolation of Brain Microvessels

Bovine brain capillaries were isolated by a modification of the method described by Bowman et al.[10] Briefly, fresh brains were kept on ice unless otherwise indicated and meninges were removed. Cortices were dissected and homogenized in DMEM containing 5% bovine calf serum (DMEM-S) using a Dounce homogenizer with a loose fitting. The homogenate was centrifuged in 25% BSA in DMEM for 10 minutes at 1000 *g*. The pellet containing crude microvessels was further digested in a solution containing 0.5 to 1 mg/ml collagenase/dispase in DMEM-S for 1 hour at 37 C. Microvascular capillaries were isolated by absorption to a column of glass beads then washed off the beads and recovered in growth medium.

Culture of Brain Endothelial Cells

The bovine brain microvessels were plated on rat tail collagen fibronectin-coated dishes or glass coverslips and cultured in MEM containing D-valine (to inhibit growth of nonendothelial cells),[11] 10% heat-inactivated fetal calf serum (FCS), ECGS (30 µg/ml), heparin (120 U/ml), L-glutamine (2 mmol/L), sodium pyruvate (11 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). After passage 5, bovine microvessel endothelial cells were cultured in M199-HAMs F12 1:1 (v/v) containing 10% FCS, 12 U/ml heparin, 11 µg/ml sodium pyruvate, 2 mmol/L L-glutamine, and antibiotics. Cultures were incubated at 37 C in a humid atmosphere of 5% CO_2. For binding experiments bovine brain endothelial cells were used at confluence between passages 5 through 13. Human brain endothelial cells were provided by W. Carley (Miles Laboratories Inc., West Haven, CT). Human umbilical vein endothelial cells were purchased from Clonetics (San Diego, CA).

Immunocytochemical Analysis of Brain Endothelial Cell Monolayers

For specific marker studies, cells were grown on collagen-coated glass coverslips, washed with HBSS, fixed in cold acetone:methanol (1:1, v/v) for 15 minutes, air-dried, sealed, and stored at −20 C. Cells were rehydrated, washed with phosphate-buffered saline (PBS) containing 0.1% BSA (PBS-B), preincubated with 5% normal goat serum for 15 minutes, and incubated with the appropriate antibody for 1 hour at room temperature. After incubation with the primary antibody the monolayers were washed with PBS-B, incubated with peroxidase- or FITC-labeled secondary antibody for 30 minutes, and mounted on slides with glycerol or aquamount. Specimens were viewed in a Nikon Diaphot fluorescence microscope equipped with standard FITC filter combination and photographed on Kodak Ektachrome or T max film. Endothelial cells possessing factor VIII antigen[12] were identified using rabbit anti-human F-VIII (1:40), pericytes by using mouse antismooth muscle actin (1:400) and glial cells with rabbit anti-cow GFAP (1:100). Antibodies against carbonic anhydrase IV[13] were used in a dilution 1:500.

Uptake of Acetylated Low Density Lipoprotein (AcLDL)

To identify endothelial cells by their ability to take up AcLDL uptake,[14] monolayers of brain endothelial cells were incubated with DiI-labeled AcLDL (10 µg/ml) in DMEM containing 3% BSA for 4 hours at 37 C. The monolayers were washed with HBSS, fixed in 4% paraformaldehyde in PBS for a minimum of 30 minutes at 4 C, and mounted on slides with glycerol. Specimens were viewed in a Nikon Diaphot fluorescence microscope equipped with standard rhodamine filter combination and photographed on Kodak T max film.

Assay for Gamma Glutamyl Transpeptidase (GGTP)

Cultures were tested for the presence of GGTP.[15–19] Monolayers of acetone-methanol-fixed cells were rehydrated and GGTP activity was histochemically assessed using the method of DeBault and Cancilla.[20] GGTP was also biochemically assessed using Wahlefeld's modified method of Sasz.[21] Briefly, bovine brain cortices (BMECs) were washed with cold PBS then detached with 0.1% trypsin/EDTA in PBS and centrifuged. The pellet was resuspended in 0.1% Triton X-100 and sonicated. The enzymatic GGTP activity was determined spectrophotometrically by reading optical density at 405 nm over 3 to 4 minutes of 3-carboxyl-4-nitroaniline released from L-gamma-glutamyl-3-carboxyl-4-nitroanilide by GGTP in the presence of glycylglycine. GGTP activity was expressed as unit per gram of protein. One unit was defined as the amount of activity that will liberate 1 μmol of 3-carboxy-4-nitroaniline per minute at room temperature. Protein was measured using the bicinchoninic acid method.[22]

Bacterial Strains

E. coli strains HB101 (K12), E412 (K1), and E412M (K1-negative mutant of E412) were used for transformation, as described previously.[23–26,27] HB101/13 carrying the recombinant plasmid pANN 801-13 produced the wild-type S fimbriae adhesin (sfa) complex. Non fimbriated *E. coli* HB101/322 was mock transfected and carried the vector pBR 322.[23–26] *E. coli* strains HB101/1 (pANN 801-1) and HB101/1321 (pANN 801-1321) contained the plasmid pANN 801-13, lacking major (sfaA) or minor subunit genes (sfaS), respectively.[24,25]

The strains HB101/116 (pANN 801-116), HB101/118 (pANN 801-118), and HB101/122 (pANN 801-122) were derived by site-directed mutagenesis of single amino acids encoded by the sfaS gene.[23] An exchange of lysine for threonine at position 116 (HB101/116) and an exchange of arginine for serine at position 118 (HB101/118) resulted in the loss of SfaS-mediated hemagglutination, whereas exchange of lysine for threonine at position 122 (HB101/122) had no effect on hemagglutination.[23]

E. coli strains E412/13 and E412M/13 contained plasmid pANN 801-13. *E. coli* strains E412/1321 and E412M/1321 contained plasmid pANN 801-1321 lacking the minor subunit adhesin, sfaS. *E. coli* strains E412/322 and E412M/322 contained only pBR 322.

All recombinant *E. coli* strains of HB101, E412, and E412M were grown in BHI broth containing appropriate antibiotics (Table 1). Bacteria were metabolically labeled with [^{3}H]adenine (25 mCi/mmol) during logarithmic growth, until a concentration of approximately 10^9 bacteria/ml was reached. They were harvested by centrifugation and washed four times at 10,000 × *g* for 10 minutes, resuspended in HBSS containing 20% glycerol, and stored at −20 C.

Binding of Bacterial Strains to Brain Endothelial Cells

Brain endothelial cells were subcultured into 24-well tissue culture plates and grown to confluence. Monolayers were washed three times with HBSS and in-

Table 1. *Phenotypic and Genotypic Characteristics of E. coli Strains*

E. coli Strain	Recombinant DNA*	S Fimbriae Phenotype†	S Fimbriae Genotype
HB101/13	pANN 801–13 (Ap^r)	Mrh+ Fim+	sfaA, sfaG, sfaS, sfaH
HB101/322	PBR 322 (Ap^rTc^r)	Mrh− Fim−	−, −, −, −
HB101/1	pANN 801–1 (Tc^r)	Mrh+ Fim−	−, sfaG, sfaS, sfaH
HB101/1321	pANN 801–1321 (Ap^r)	Mrh− Fim+	sfaA, sfaG, −, sfaH
HB101/116	pANN 801–116 (Ap^r) SfaS (Lys→116 Thr)	Mrh− Fim+	sfaA, sfaG, sfaS, sfaH
HB101/118	pANN 801–118 (Ap^r) SfaS (Arg→118 Ser)	Mrh− Fim+	sfaA, sfaG, sfaS, sfaH
HB101/122	pANN 801–122 (Ap^r) SfaS (Arg→118 Ser)	Mrh+ Fim+	sfaA, sfaG, sfaS, sfaH
E412/13	pANN 801–13 (Ap^r)	Mrh+ Fim+	sfaA, sfaG, sfaS, sfaH
E412/322	PBR 322 (Ap^rTc^r)	Mrh− Fim−	−, −, −, −
E412/1321	pANN 801–1321 (Ap^r)	Mrh− Fim+	sfaA, sfaG, −, sfaH
E412M/13	pANN 801–13 (Ap^r)	Mrh+ Fim+	sfaA, sfaG, sfaS, sfaH
E412M/322	PBR 322 (Ap^rTc^r)	Mrh− Fim−	−, −, −, −
E412M/1321	pANN 801–1321 (Ap^r)	Mrh− Fim+	sfaA, sfaG, −, sfaH

* A_p^r, resistant to ampicillin; Tc^r, resistant to tetracycline; Km^r, resistant to kanamycin.
† Mrh, mannose-resistant hemagglutination; Fim, fimbriation by electron microscopy.

cubated at 4 C with ^{3}H-labeled bacteria in M199/Ham F-12 containing 5% heat-inactivated calf serum. The dishes were gently shaken throughout the incubation. Thereafter, the monolayers were washed four times and the brain endothelial cells plus adherent bacteria were dissolved in 500 µl 0.2 N NaOH. Subsequently, 500 µl 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.

To characterize the specificity of the binding at 4 C, bacteria were incubated with antibodies or trisaccharides or monosaccharides before addition to endothelial cell monolayers. Neuraminidase treatment of the BMEC was used with the *Vibrio cholera* enzyme at 0.1 U/ml in PBS containing 2 mmol/L $CaCl_2$ at 37 C for 1 hour, followed by three washings in PBS for 5 minutes each.

The condition of the cells was morphologically assessed after each experiment. Cells were >95% viable, as estimated by trypan blue exclusion, and no detachment of cells was observed.

To examine the effect of temperature on binding of bacteria to endothelial cells, binding was also performed at 37 C. To prevent internalization, cells were gently fixed with 0.2% glutaraldehyde in PBS for 5 minutes then extensively washed and further processed as described above for binding.

Antibodies for Blocking Bacterial Interaction

Monoclonal antibodies A1 (IgG1) and F1 (IgG2) were directed against S adhesin and the major fimbrial subunit SfaA, respectively.[27,28] Monoclonal antibody 9A10 (IgG1) is directed against the core oligosaccharide of *E. coli* HB101 and monoclonal antibody 2-2-B (IgM) is specific for capsular polysaccharides of *E. coli* KI.[3]

Visualization of Binding of E. coli *to Brain Endothelial Cells*

Brain endothelial cells were grown to confluence on coverslips in 24-well plates and fixed with 0.2% glutaraldehyde in PBS for 10 minutes followed by extensive washing before adding unlabeled bacteria. Incubations were performed as described for binding assays. Subsequently, the wells were washed and stained for bacteria and cells according to Wright[29] and photographed.

Statistics

All data are presented as mean ± SD. A two-tailed unpaired *t*-test was used to compare means. P values ≤ 0.05 were considered significant.

Results

Characterization of Isolated and Cultured Brain Microvessel Cells

Cells of isolated bovine brain microvessels attached to the matrix and migrated away from initial sites. The individual cells displayed both a somewhat spindly and cobblestone-like morphology. After passage 5 these cells showed a typical cobblestone-like pattern at confluence.

The bovine endothelial cells were tested up to passage 14 and were positive for factor VIII and DiI-AcLDL uptake, thereby establishing their endothelial origin.[12,14] Specific features of brain endothelial cells were studied by assaying for GGTP[15–19] and carbonic anhydrase IV. Carbonic anhydrase IV, a specific marker for rat brain endothelial cells,[13] is now shown in primary bovine endothelial cells and present in culture up to approximately passage 6. The GGTP activity of bovine brain endothelial cells was histochemically detected until approximately passage 6, but GGTP activity could be measured biochemically up to passage 14. The GGTP activity was measured biochemically at several points up to 28 days in culture. At 6 days in culture the GGTP activity was 14.08 ± 3.00 U/g protein, at 13 days in culture (postconfluent) the enzyme activity was reduced by 45% to 7.86 ± 1.08 U/g protein. Culturing up to 28 days decreased GGTP activity to 2.35 ± 0.24 U/g protein.

A few contaminating glial cells were found only at early passages. After passage 5 no contaminating nonendothelial cells were detected in the cultures. Also, no pericytes could be detected using anti smooth muscle cell actin antibody. D-valine-containing medium is known to reduce fibroblast contamination,[11] therefore, the microvessel cells were grown in this medium. Morphological examination at confluence showed no fibroblast-like cells in the microvessel cultures.

Based on the presence of factor VIII, carbonic anhydrase IV, GGTP, and uptake of AcLDL by these cells, we conclude that we isolated relatively pure BMEC and specific brain endothelial cell characteristics are present up to at least passage 14. Human endothelial cells also took up DiI-AcLDL and were

positive for GGTP. Human umbilical vein endothelial cells were positive for factor VIII and took up DiI-AcLDL.

Binding of S Fimbriated and Nonfimbriated E. coli *to BMEC*

Binding of S fimbriated (HB101/13 and E412/13) and nonfimbriated *E. coli* (HB101/322 and E412/422) to BMEC was assessed and the results are expressed as the percentage of inoculum size. Optimal binding of bacteria to BMEC was observed with an inoculum of 2×10^6 CFU for both HB101/13 and E412/13, which is equivalent to a bacteria/endothelial cell ratio of 10:1. At this ratio, binding of strains HB101/13 and E412/13 to the BMEC was significantly greater ($P < 0.05$) than that of strains HB101/322 and E412/322, respectively. Figure 1 shows that binding of HB101/13 and HB101/322 increased with time and reached a maximum at 2 hours of incubation. At this time, binding of HB101/13 was 4.2% and binding of HB101/322 was 1.1%, a more than threefold difference ($P < 0.02$). Similar observations were made for strain E412 and its KI-negative mutants. Bindings were maximal after 2 hours of incubation (data not shown) and at this time binding of E412/13 to BMEC was 3.3% *versus* 1.5% for E412/322 ($P < 0.05$). For strain E412M/13 and E412M/322 5.5% *versus* 2.0% ($P < 0.02$) bound to BMEC. These binding characteristics of S fimbriated *E. coli* to BMEC were similar between passage 5 through passage 13. We also examined binding of HB101/13 and HB101/322 to human brain endothelial cells. Again, binding of HB101/13 was approximately threefold greater than that of HB101/322, 3% *versus* 1% ($P < 0.01$). In contrast, binding to human umbilical vein endothelial cells did not differ between HB101/13 and HB101/322 (0.3% *versus* 0.27%). Binding of bacteria at 37 C to gently fixed BMEC gave comparable results to those at 4 C; 3.35% for HB101/13 *versus* 0.65% for HB101/322. These results indicate that S fimbriae are important in the binding of *E. coli* to brain endothelial cells of both bovine and human origin.

Characterization of Binding Domains of S Fimbriated E. coli *to BMEC's*

The specificity of the binding of S fimbriated *E. coli* to BMEC was assessed by the addition of isolated proteins to BMEC monolayers before addition of the bacteria or by preincubating the bacteria with monoclonal antibodies. Figure 2 shows that addition of isolated S fimbriae protein to BMEC reduced binding of HB101/13 by approximately 60%. Preincubation of anti-S adhesin antibody A1 with the bacteria resulted in an approximately 80% decrease of the binding of HB101/13 to BMEC. Anti-SfaA antibody F1 also decreased binding of HB101/13 by about 65%. In contrast, antibody 9A10, directed against core oligosaccharide, did not inhibit binding of HB101/13. Comparable results were obtained with the E412 strain (Figure 3). Anti-S adhesin antibody A1 reduced

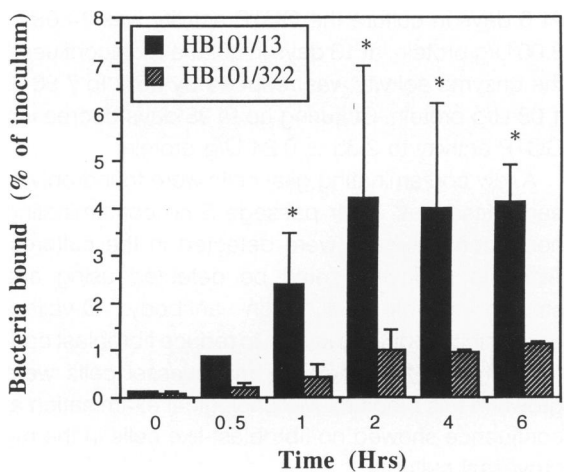

Figure 1. *Time-dependent binding of HB101* E. coli *strains to bovine brain endothelial cells. Monolayers were incubated at 4 C with [³H]S fimbriated* E. coli *(HB101/13) and [³H]nonfimbriated* E. coli *(HB101/322) for the indicated time. The cells were washed, solubilized, and trichloroacetic acid precipitable counts, indicating cell-associated bacteria, were determined. Data are expressed as percentage of the total added bacteria (10^7 CFU/well), bars indicate standard deviation from means of triplicate determinations ($P < 0.05$)*.*

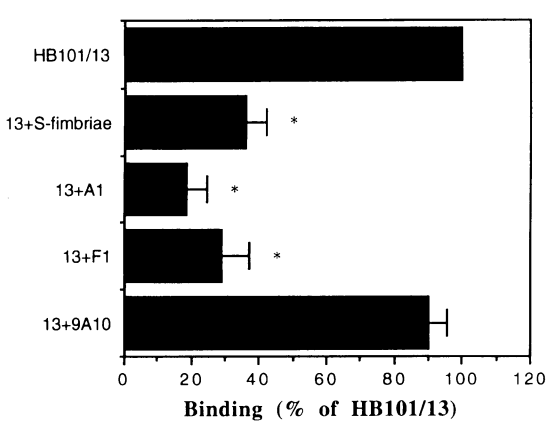

Figure 2. *Effect of antibodies and isolated S fimbriae on the binding of S fimbriated* E. coli *to bovine brain endothelial cells.* E. coli *strain HB101/13 was preincubated for 1 hour on ice with anti-S adhesin (A1), anti-SfaA fimbriae (F1), or anticore oligosaccharide (9A10) antibodies before addition to BMEC. BMEC monolayers were preincubated with S fimbriae (sfa) (10 μg/ml). Binding was determined as trichloroacetic acid precipitable counts; values are expressed as percentage of binding of HB101/13 (100% is 2×10^7 CFU/well). Bars indicate standard deviation from means of triplicate determinations ($P < 0.02$)*.*

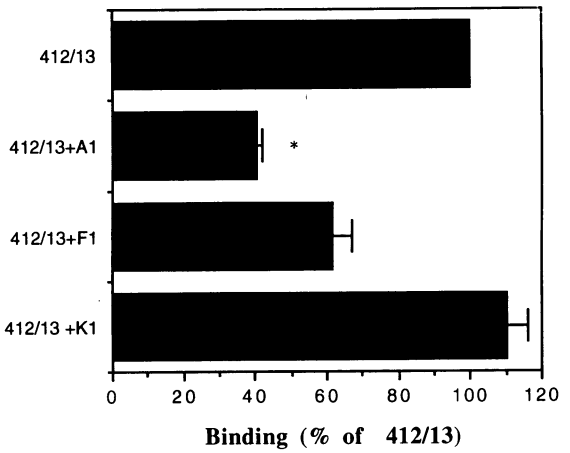

Figure 3. *Effect of antibodies on the binding of S fimbriated E412 strain to bovine brain endothelial cells.* E. coli *strain E412/13 was preincubated for 1 hour on ice with anti-S adhesin (A1), anti-SfaA fimbriae (F1), or anticore oligosaccharide (9A10) antibodies before addition to BMEC. Binding of E412/13 to BMEC was determined as trichloroacetic acid precipitable counts; values are expressed as percentage of binding of E412/13 (100% is 2.8 × 10^7 CFU/well). Bars indicate standard deviation from means of triplicate determinations ($P < 0.05$).**

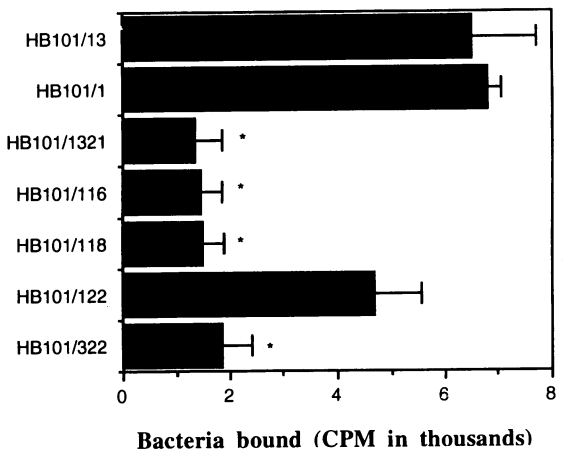

Figure 4. *Binding of transformants of HB101 to bovine brain endothelial cells. The phenotypic and genotypic characteristics of* E. coli *transformants are indicated in Table 1. Endothelial cell monolayers were incubated with the indicated* E. coli *strains for 2 hours at 4 C, and trichloroacetic acid precipitable counts associated with the cells were determined. Per well 200,000 cpm or 1.5 × 10^7 CFU were added. Bars indicate standard deviation from the means of triplicate determinations ($P < 0.01$)**.

the binding of E412/13 by approximately 60% and anti-SfaA antibody F1 resulted in approximately 35% inhibition. In contrast, anti-K1 capsular polysaccharide antibody was unable to affect binding. These data suggest that the binding of S fimbriated *E. coli* to BMEC may be mediated via SfaS and SfaA proteins of the Sfa complex.

Effect of Mutation in E. coli *S Fimbriae on Binding to BMEC*

To further examine which part of S fimbriae was responsible for the binding to brain endothelial cells, genes encoding for subunits of S fimbriae were altered by deletion or site-specific mutagenesis (Figure 4). Only a mutation in the minor subunit gene for S adhesin sfaS (HB101/1321) significantly decreased the binding compared with HB101/13. Similarly, a significant decrease in binding was found for HB101/116 and HB101/118 but not for HB101/122. Deletion of the major S fimbriae subunit gene sfaA (HB101/1) did not alter binding. This is in contrast with the previously observed decrease in binding by the anti-SfaA antibody F1, suggesting that the blocking induced by antibody F1 might be due to a steric hindrance effect. Similar results were found for strain 412 and its derivatives. These findings indicate that the binding of S fimbriae to brain endothelial cells occurs via S adhesin.

Specificity of Binding of S Fimbriae to Glycoproteins on BMEC Surface

S fimbriae have been shown to bind to terminal NeuAcα2,3-galactose units of cell surface glycoproteins.[1] The role of this lectin-like activity of S adhesin in binding to BMEC was examined by treating monolayers with neuraminidase and by preincubating S fimbriated *E. coli* with NeuAcα2,3-sialyl lactose (Figure 5). Both treatments completely abolished the binding of S fimbriated *E. coli* to BMEC. In contrast,

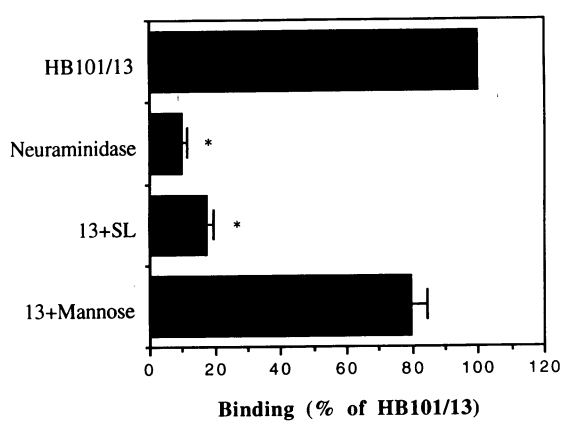

Figure 5. *Effect of simple sugars and neuraminidase treatment on the binding of S fimbriated* E. coli *to bovine brain endothelial cells. Bacteria were preincubated with NeuAcα2,3-sialyl lactose (SL) (50 μ), mannose (50 μ), or BMEC were pretreated with neuraminidase. Binding of bacteria was determined as trichloroacetic acid as precipitable counts associated with the cells; values are expressed as percentage of binding of HB101/13 (100% is 1.8 × 10^7 CFU/well). Bars indicate standard deviation from means of triplicate determinations* (P < *0.05*)*.

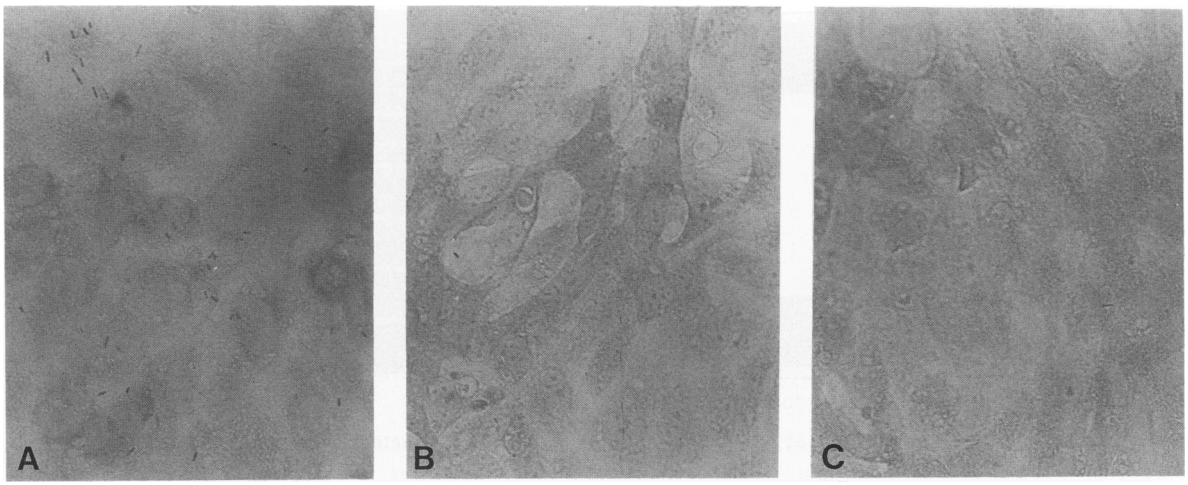

Figure 6. *Microscopic evaluation of binding of HB101 strains to bovine brain endothelial cells. BMEC monolayers were incubated for 2 hours at 4 C with bacteria (1 × 10^7 CFU/well) HB101/13* (**A**), *HB101/322* (**B**), *and HB101/13 preincubated with anti-SfaS antibody A1* (**C**). *Monolayers were then washed and stained for bacteria according to Wright (magnification ×150).*

preincubating bacteria with simple sugars like mannose, sialic acid, or sucrose (data not shown) resulted in minimal or no inhibition on the binding of S fimbriated *E. coli* to BMEC. This indicates a role for sialyl lactose moieties on brain endothelial glycoproteins in the binding of *E. coli* S fimbriae.

Visualization of Binding to S Fimbriated E. coli *to BMEC*

To show that intact bacteria bind to BMEC monolayers and that binding does not involve adherence of bacterial cell wall debris, we visualized binding of bacteria to monolayers.

Binding patterns of S fimbriated *E. coli* HB101/13 and HB101/322 are shown in Figure 6. S fimbriated HB101/13 bound to the surface of BMEC. Adhesion mostly occurs over the apical endothelial cell surface and not at cell borders (Figure 6A). In contrast, the binding of nonfimbriated HB101/322 was hardly discernable (Figure 6B). Anti-S adhesin antibody A1 almost completely blocked binding of S fimbriated HB101/13 (Figure 6C).

Discussion

Inadequate knowledge of the pathogenesis and pathophysiology of neonatal bacterial meningitis has contributed to the significant mortality and morbidity associated with this disease. Most cases of bacterial meningitis in newborns and children develop as a result of hematogenous spread, however, it is not apparent how circulating *E. coli* cross the blood-brain barrier. The adhesion of *E. coli* to the blood-brain barrier is likely to be an important first step in the pathogenesis of meningitis. However, it is not clear whether specific microbial and host structures are required for this bacterial-host interaction. In this study, we characterized the interaction of S fimbriated *E. coli* with bovine brain endothelial cells. Our findings support the role of S fimbriae in promoting bacterial binding to brain endothelial cells. We demonstrated that regardless of the capsular serotypes (K12 for HB101 or K1 for E412) binding of *E. coli* to brain endothelial cells was approximately two- to threefold greater with S fimbriated than with nonfimbriated strains. Interestingly, human brain endothelial cells also gave a comparable difference in binding of S fimbriated *versus* nonfimbriated strains. Because of the limited availability of human brain endothelial cells, this finding validates the use of bovine brain endothelial cells to study the pathogenesis of human meningitis, specifically interactions between S fimbriated *E. coli* and brain endothelial cells.

Binding of S fimbriated bacteria to BMEC could be inhibited by addition of purified S fimbriae or antibodies against S fimbriae. Moreover, anti-S adhesin antibody decreased binding and transformants lacking S adhesin (HB101/1321, HB101/116, HB101/118, E412/1321, and E412M/1321) showed binding at control (nonfimbriated) level. These findings indicate that the SfaS adhesin is a critical determinant in the binding of S fimbriated *E. coli* to brain endothelial cells. Anti-SfaA antibody, F1 exhibited the inhibitory activity on the binding of S fimbriated *E. coli*. Binding of HB101/1 lacking SfaA, however, was similar to that

of HB101/13 and this binding was inhibited by anti-S adhesin antibody but not by anti-SfaA antibody, indicating that SfaA is not involved in the binding. Thus, the inhibition by anti-SfaA antibody appears to be due to steric hindrance.

In this study, the binding of S fimbriated *E. coli* to brain endothelial cells was completely inhibited after pretreatment of endothelial cells with neuraminidase or by prior incubation of *E. coli* with NeuAcα2,3-sialyl lactose. In contrast, simple sugars lacking sialyl lactose residues failed to reduce binding, indicating the importance of sialyl lactose moieties as receptors on membrane glycoproteins. These findings, in accordance with previous findings,[9] suggest that glycoprotein receptors are present on both bovine and human brain endothelial cells with a specificity for S adhesin of S fimbriated *E. coli*. In rat tissues terminal NeuAcα2,3-galactose units of cell surface glycoproteins on vascular endothelium are involved in the binding of S fimbriae/SfaS adhesin.[9] We previously determined that sulfated glycolipids on bovine brain endothelial cells can serve as receptors for the major fimbrillin protein SfaA.[27] Thus, it appears that at least two distinct binding sites on brain endothelial cells are involved in the binding of S fimbriated *E. coli*; sialoglycoproteins for the SfaS adhesin and sulfated glycolipids for the sfaA protein. The SfaS adhesin is preferentially located at the tips of the S fimbriae.[28] We speculate that initial attachment of bacteria to endothelial cells occurs via sialic acid-containing receptor(s) and mediated by the SfaS adhesin. This event is followed by an association of the bacteria to sulfated glycolipids on the cell surface, possibly for a more intimate contact of the organism to satisfy nutritional requirements, which might result in a subsequent crossing of the blood-brain barrier. Further characterization of the roles of these binding sites in the pathogenesis of *E. coli* meningitis may provide novel ways to prevent this devastating disease.

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