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Outer Membrane Protein A of *Escherichia coli* Contributes to Invasion of Brain Microvascular Endothelial Cells

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***Escherichia coli* is the most common gram-negative bacteria causing meningitis during the neonatal period, but it is unclear what microbial factors mediate traversal of *E. coli* across the blood-brain barrier. Outer membrane protein A (OmpA), a highly conserved 35-kDa protein, was examined for its role in *E. coli* K1 invasion of brain microvascular endothelial cells (BMEC). The invasive capability of the OmpA$^+$ strains was 25- to 50-fold greater than that of OmpA$^-$ strains, and the invasive capability of OmpA$^-$ strains was restored to the level of the OmpA$^+$ strain by complementation with the *ompA* gene. Purified OmpA proteins and polyclonal anti-OmpA antibodies inhibited the invasion of OmpA$^+$ *E. coli* into BMEC. Two short synthetic peptides (a hexamer, Asn-27–Glu-32, and a pentamer, Gly-65–Asn-69) generated from the N-terminal amino acid sequence of OmpA exhibited significant inhibition of OmpA$^+$ *E. coli* invasion, suggesting that these two sequences represent the OmpA domains involved in *E. coli* invasion of BMEC. These findings suggest that OmpA is the first microbial structure identified to enhance *E. coli* invasion of BMEC, an important event in the pathogenesis of *E. coli* meningitis.**

Escherichia coli is the most common gram-negative bacterium that causes meningitis during the neonatal period (36). Most cases of *E. coli* meningitis develop as a result of hematogenous spread, but it is not clear how circulating bacteria cross the blood-brain barrier. The invasion process, preceded by binding to host cells, is thought to involve specific interaction of bacterial surface determinants with the host cell and subsequent triggering of an event that resembles phagocytosis (8). We have previously shown that S-fimbriae are important in binding of *E. coli* to brain microvascular endothelial cells (BMEC), but their role in invasion appears to be insignificant (27).

There is growing evidence that outer membrane proteins contribute to the invasion of several gram-negative organisms into cultured mammalian cells. For example, the invasive potential of enteropathogenic *E. coli* into epithelial cells is mediated by a 94-kDa membrane protein encoded by the *eae* gene (14, 15). Another well-characterized invasive property of *Yersinia* spp. is dependent on the *inv* gene product, a 986-amino-acid outer membrane protein (13). The prominent outer membrane proteins of *Neisseria gonorrhoeae*, Opa, have been implicated in the invasion of epithelial cells (22, 38) and interaction with human neutrophils (1), whereas expression of another surface protein, PIII, increases the resistance of bacteria to complement-mediate killing (30). *E. coli* outer membrane protein A (OmpA) has been shown to have sequence homology with these two surface antigens of *N. gonorrhoeae*; the membrane-associated portion of OmpA (amino acids 41 to 171) is homologous to Opa, and the periplasmic portion of OmpA (amino acids 176 to 316) is homologous to PIII (11). The structural similarities of *E. coli* OmpA to *Neisseria* Opa and PIII suggest that OmpA may have a role similar to that of Opa (e.g., in invasion) in the pathogenesis of *E. coli* infection.

OmpA is one of the major proteins in the outer membrane of *E. coli*. This heat-modifiable 35-kDa protein is highly conserved among gram-negative bacteria, and its N-terminal domain encompassing amino acid residues 1 to 177 is thought to cross the membrane eight times in antiparallel β-strands (17). The C-terminal portion is thought to be located in an aqueous environment of periplasm (17). This protein is multifunctional: in addition to its nonphysiological function as a phage and colicin receptor, it serves as a mediator in F-factor-dependent conjugation (31), and in combination with the outer membrane lipoprotein (2), it is required for the structural integrity of this membrane and the generation of normal cell shape (32). The OmpA protein also forms small nonspecific diffusion channels (24). Weiser and Gotschlich have reported that OmpA contributes to pathogenicity and serum resistance of *E. coli* K1 (39). An OmpA$^-$ mutant was significantly less virulent in both embryonic chicken and neonatal rat models and exhibited increased sensitivity to serum bactericidal activity compared with that of its parent OmpA$^+$ strain. These investigators showed that the growth characteristics of both the OmpA$^-$ mutant and its parent OmpA$^+$ were similar, indicating that the observed decreased virulence of the OmpA$^-$ mutant was unlikely to be the result of its diminished growth.

In the present study, we investigated the role of OmpA in *E. coli* K1 invasion of BMEC, using isogenic OmpA$^+$ and OmpA$^-$ strains. We showed that the invasive capability of OmpA$^+$ strains is 25- to 50-fold greater than that of OmpA$^-$ strains and that the invasive capability of the OmpA$^-$ mutant is restored by complementation with the *ompA* gene. OmpA$^+$ *E. coli* invasion of BMEC was inhibited by purified OmpA proteins and anti-OmpA antibodies. We further determined that the functional domains of OmpA reside in the N-terminal amino acid sequence.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and chemicals. All strains used in this study were derived from a cerebrospinal fluid isolate of *E. coli* K1 strain RS218

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TABLE 1. Invasion frequencies of *E. coli* K-1 OmpA$^+$ and OmpA$^-$ strains into BMEC

E. coli strain	Relevant characteristic(s)	Mean CFU (± SD)/well		% Invasion (mean ± SD)[b]
		Cell associated	Intracellular[a]	
E44	RS218 spontaneous Rif^r mutant	$(3.2 \pm 0.3) \times 10^5$	$(7.5 \pm 0.2) \times 10^3$	0.07 ± 0.02[c]
E58	E44 OmpA::Tn*phoA′*	$(3.9 \pm 0.3) \times 10^5$	$(5.1 \pm 0.5) \times 10^3$	0.04 ± 0.02[c]
E69	E58 OmpA$^+$ Tet^s Kan^r	$(4.2 \pm 1.0) \times 10^5$	$(1.1 \pm 0.3) \times 10^4$	0.11 ± 0.03[c]
E91	E44 OmpA$^-$ Tet^r	$(3.0 \pm 1.1) \times 10^5$	$(2.1 \pm 0.5) \times 10^2$	0.002 ± 0.002
E92	E44 OmpA$^-$ Tet^r	$(3.5 \pm 0.7) \times 10^5$	$(3.1 \pm 0.5) \times 10^2$	0.005 ± 0.002
E105	E91 OmpA$^+$ Amp^r	$(3.7 \pm 0.9) \times 10^5$	$(1.2 \pm 0.5) \times 10^4$	0.10 ± 0.02[c]
E109	E91 truncated OmpA$^+$ Amp^r	$(3.6 \pm 0.6) \times 10^5$	$(6.3 \pm 0.2) \times 10^3$	0.05 ± 0.02[c]
E111	E91 OmpA$^-$ Amp^r	$(3.1 \pm 0.4) \times 10^5$	$(2.8 \pm 0.5) \times 10^2$	0.005 ± 0.005

[a] The bacteria survived after gentamicin treatment.
[b] % invasion, percent of input bacteria recovered after Triton lysis; the values are the means for at least three separate experiments.
[c] $P < 0.001$ by two-tailed, unpaired *t* test.

(O18:K1:H7) as described previously (39) and are shown in Table 1. Briefly, Tn*phoA* mutagenesis of *E. coli* K1 was carried out by using strain E44, a spontaneous rifampin-resistant mutant of RS218. The OmpA$^-$ strain (E58) was identified by screening the kanamycin- and rifampin-resistant colonies by colony immunoblotting with anti-OmpA antibody. A corrected mutant E69 strain (OmpA$^+$) was generated by P1 transduction of the *E. coli* K-12 *ompA* gene into strain E58. A mutant lacking the entire *ompA* gene (strain E91) was generated from strain E44 in two steps. First, a tetracycline resistance marker was mobilized from strain DME 558, which contains the marker *neo-ompA*, by P1 transduction to *E. coli* K-12 strain BRE51 in which *ompA* had been deleted (4, 39). Then, a P1 lysate was used to transduce *E. coli* K1 strain E44. The tetracycline-resistant colonies were screened for lack of OmpA expression by Western blot (immunoblot) analysis to identify mutant E91. E92 was the same phenotype as E91 but was generated in a separate experiment. E105 was obtained by transformation of E91 with pRD87, which contains the *ompA* gene on pUC9 and expresses the same physiological characteristics as K-12 OmpA (10). E111 was obtained by transformation of E91 with pUC9. pRD87 was digested with *Bam*HI and *Bgl*II and religated to delete the C-terminal 53 amino acids of OmpA. pRD87 containing this deletion was transformed into E91 to yield E109. All the strains were found to possess the K1 capsule by the antiserum agar technique and agglutination with an anti-K1 monoclonal antibody as described previously (19). Expression of O18 lipopolysaccharide and S-fimbriae was verified by colony blotting of intact bacteria with anti-O18 lipopolysaccharide (6) and anti-S-fimbriae antibodies (33), respectively. Bacteria were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) with appropriate antibiotics at the following concentrations, unless otherwise stated; ampicillin, 100 μg/ml; tetracycline, 12.5 μg/ml; rifampin, 100 μg/ml; and kanamycin, 40 μg/ml. All chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified.

Isolation and culture of BMEC. Bovine brain capillaries were isolated as described previously (33). Briefly, the meninges of fresh bovine brains were removed, and the cortices were dissected and homogenized in Dulbecco's modified Eagle's medium (DMEM) containing 5% bovine fetal calf serum (DMEM-S). The homogenate was centrifuged in 25% bovine serum albumin (BSA) in DMEM for 10 min at 1,000 × *g*. The pellet containing crude microvessels was further digested in a solution containing 0.5 to 1 mg of collagenase-dispase per ml in DMEM-S for 1 h at 37°C. Microvascular capillaries were isolated by absorption to a column of glass beads and then washed off the beads in growth medium. The microvessels were plated on rat tail collagen-fibronectin-coated dishes or glass coverslips and cultured in minimal essential medium (MEM) containing D-valine (to inhibit the growth of nonendothelial cells), 10% heat-inactivated fetal calf serum, endothelial cell growth supplement (30 μg/ml), heparin (120 U/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). After passage 5, BMEC were cultured in M199-Ham F-12 (1:1) containing 10% fetal calf serum, 12 U of heparin per ml, 11 μg of sodium pyruvate per ml, 2 mM L-glutamine, and antibiotics. Cultures were incubated at 37°C in a humid atmosphere of 5% CO_2. The isolated BMEC were positive for factor VIII; fluorescently labeled, acetylated, low-density lipoprotein; carbonic anhydrase IV; and gamma glutamyl transpeptidase, demonstrating their brain endothelial cell characteristics. For invasion experiments, BMEC were used at confluence between passages 5 through 12.

Invasion assay. BMEC were cultured in T75 flasks (Costar, Cambridge, Mass.) in complete growth medium, and after reaching confluence, the cells were subcultured in collagen-coated 24-well tissue culture plates and incubated at 37°C in a humid atmosphere of 5% CO_2–room air for approximately 5 days. The invasion assays were performed by a modification of the method of Tang et al. (35). Approximately 10^7 bacteria were added to confluent monolayers of BMEC (approximate BMEC and bacterium ratio of 1:100) in experimental medium (500 μl) containing M199-Ham F-12 (1:1 [vol/vol]) with 5% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. The plates were incubated for 1.5 h at 37°C in 5% CO_2 without shaking. After the incubation period, the monolayers were washed four times with prewarmed M199 medium, and the number of cell-associated bacteria was determined after the BMEC were lysed with 0.5% Triton X-100 in distilled water for 10 min. The number of intracellular bacteria was determined after extracellular bacteria were eliminated by incubation of the monolayers with experimental medium containing gentamicin (100 μg/ml) for 1 h at 37°C. The monolayers were again washed four times with M199 and lysed with 0.5% Triton X-100. The released intracellular bacteria were enumerated by plating on sheep blood agar. Bacterial viability was not affected by 0.5% Triton X-100 treatment for all strains tested. The percent invasion was calculated as (the number of surviving bacteria divided by the number of bacteria inoculated) × 100. Each assay was run in triplicate and also repeated at least three times. BMEC monolayers were also treated with 5 mM sodium *meta*-periodate in phosphate-buffered saline (PBS) for 10 min in the dark, washed five times with PBS, and used for the invasion assays as described above. For desialylation of BMEC, the monolayers were treated with either *Vibrio cholerae* or *Arthrobacter ureafaciens* neuraminidase (0.1 U/ml) in 50 mM sodium acetate buffer (pH 5.5) containing 5 mM $CaCl_2$ and 0.1% (wt/vol) BSA for 1 h at 37°C.

For inhibition studies, methyl mannoside or sialyl lactose (NeuAcα2,3-galβ1,4-glc), monoclonal antibody A1 directed against SfaS adhesin (23) or anti-group B streptococcus (GBS) antibody directed against type III GBS polysaccharide (7), or polyclonal anti-OmpA antibody was incubated with OmpA$^+$ or OmpA$^-$ *E. coli* strains for 1 h on ice before being added to the BMEC monolayers. With purified OmpA protein, the BMEC monolayers were incubated with different concentrations of protein for 1 h at 37°C in humid incubator, and then either the OmpA$^+$ or OmpA$^-$ *E. coli* strain was added to continue the invasion assays. The viability of bacteria was assessed by exposing the bacteria to these compounds for different times up to 5 h in experimental medium and by determining the number of CFU on agar with appropriate antibiotics. The viability of BMEC was tested by trypan blue exclusion assay (5) after the BMEC had been exposed to various inhibitors.

Isolation of *E. coli* OmpA proteins. *E. coli* E69 (OmpA$^+$) was grown overnight in brain heart infusion broth containing 100 μg of rifampin per ml and 40 μg of kanamycin per ml. The bacteria were collected by centrifugation, washed three times with 50 mM PBS (pH 7.4), and resuspended in PBS containing 0.5% Triton X-100 and the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and aprotinin. The bacterial suspension was briefly sonicated, frozen at −70°C, thawed again, and sonicated for 30 s. Freezing and thawing were repeated three times to obtain good yields of membrane proteins. The suspension was centrifuged at 8,000 × *g* to remove debris, and the supernatant was subjected to ultracentrifugation at 120,000 × *g* for 1 h at 4°C. The pellet was extracted with 3% lauroylsarcosine, and the mixture was centrifuged at 100,000 × *g* for 45 min at 4°C. The pellet was suspended in 0.3% lithium dodecyl sulfate (LDS) containing 5 mM EDTA in 10 mM Tri-HCl (pH 7.5) to give a final protein concentration of about 2 mg/ml. After 30 min at 0°C, the suspension was centrifuged at 100,000 × *g* for 1 h. The pellet was then extracted with the same volume of 3% LDS–5 mM EDTA–10 mM Tris-HCl (pH 7.5) for 30 min at 0°C and centrifuged at 100,000 × *g* for 1 h. The supernatant was loaded onto a Sephacryl S-300-HR column (1.5 by 70 cm) equilibrated with 0.1% LDS–0.4 M LiCl–10 mM Tris-HCl (pH 7.5), and the proteins were eluted with the same buffer. OmpA-containing fractions (2 ml) were concentrated by using Centricon tubes and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The OmpA preparation did not contain any major contaminant.

Preparation of proteoliposomes for inhibition studies. Proteoliposomes were prepared as described previously (25). Briefly, a mixture of 2.5 μmol of acetone-washed (24) egg phosphatidylcholine (type XVI-E; Sigma) and 0.2 μmol of dicetylphosphate was dried under nitrogen. The lipids were dissolved in anhydrous benzene, dried to remove traces of water, dissolved in ethyl ether, and then dried to produce a thin, even film. The drying process was completed by leaving the tubes for at least 2 h in an evacuated desiccator over silica gel. The lipid film was then resuspended in 0.2 ml of water or water containing the purified OmpA protein. Most detergent from the sample was removed, before its use in reconstitution, by passing the sample through an Extracti Gel-D (Pierce) column. The

lipid-protein mixture was dried in a desiccator under vacuum. The dried protein-lipid film was resuspended in 5 mM Tris-HCl buffer (pH 8.0), containing 15% of Dextran-40. Proteoliposomes were used after having been left at room temperature for 1 h. Liposomes containing 10 to 30 μg of OmpA protein were incubated with BMEC for 1 h before the bacteria were added, and the invasion assays were carried out as described above. Liposomes without OmpA protein and liposomes containing 3% LDS-soluble OmpA$^-$ strain E91 proteins were used as controls.

Preparation of polyclonal antibodies to OmpA and Fab fragments. Purified OmpA protein (100 μg) in 200 μl of PBS was mixed with an equal volume of complete Freund's adjuvant and injected subcutaneously into rabbits. On day 14, another batch of OmpA protein in incomplete Freund's adjuvant was injected subcutaneously. On day 31, the rabbits were test bled, the sera were heat inactivated at 56°C for 30 min, and the reactivity of serum against OmpA was tested by Western blot analysis. In addition, the anti-N-terminal OmpA antibodies (raised against the N-terminal fragment of OmpA [16]), provided by U. Henning, were also used for Western blot analysis. Fab fragments of OmpA antibodies were isolated according to the manufacturer's instructions (Pierce). Briefly, OmpA antibodies purified by using Protein A-Sepharose columns were dialyzed against 20 mM phosphate–10 mM EDTA buffer (pH 7.0) for 24 h at 4°C and lyophilized. The lyophilized immunoglobulin G was dissolved in 1 ml of digestion buffer containing cysteine hydrochloride in phosphate buffer and incubated with 0.5 ml of immobilized papain (prewashed three times with digestion buffer) at 37°C in a shaker water bath overnight at high speed. The solubilized Fab and Fc fragments were separated from the immobilized papain and passed through an immobilized Protein A column. The eluant containing Fab fragments were collected, dialyzed against PBS, and checked for complete cleavage of antibodies by SDS-PAGE.

SDS-PAGE and Western blotting with anti-OmpA antibodies. *E. coli* whole membrane proteins or 3% LDS-soluble purified outer membrane proteins were subjected to SDS–10 or 12% PAGE. Samples were mixed with an equal or greater volume of sample buffer (0.06 M Tris, 4% SDS, 10% glycerol, 0.003% bromophenol blue), boiled for 3 min in a boiling-water bath, loaded onto the gels and electrophoresed at a 10- to 20-mA constant current (Bio-Rad mini-gel apparatus). For Western blots, the proteins were transferred onto a polyvinylidene difluoride nitrocellulose (Millipore) sheet with a Millipore semi-dry transblot apparatus by using a three-buffer system according to the manufacturer's instructions. After transfer, the blots were washed with PBS two times and blocked with either 5% nonfat dry milk in PBS for anti-OmpA antibodies or 1% milk for anti-N-terminal OmpA antibodies for 1 h at room temperature. Then, the blots were incubated with anti-OmpA antibody (1:3,000) for 2 h, washed four times with PBS, and incubated with peroxidase-labeled goat anti-rabbit immunoglobulin G at a dilution of 1:2,000 in milk for 2 h at room temperature. The blots were washed with PBS containing 0.05% Tween 20 for 15 min with five changes of buffer, and the color was developed with diaminobenzidine and hydrogen peroxide as the substrates. Control blotting was carried out by omitting anti-OmpA antibodies.

Preparation of synthetic peptides. Peptide amides were synthesized on a Biosearch model 9600 peptide synthesizer with a standard base-catalyzed 9-fluorenylmethoxycarbonyl (FMOC) protection and deprotection chemistry with benzotiazolyloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) as the activating agent. The synthesis of each of the three peptides was initiated by coupling the activated C-terminal amino acid to a polystyrene FMOC support (PAL resin). The completed peptides were cleaved from the resin and deblocked with a trifluoroacetic acid-thioanisole-ethanedithiol-anisole mixture (90:5:3:2). Peptides were precipitated and washed in anhydrous diethyl ether. The dried peptides were used in invasion assays.

RESULTS

Expression of OmpA in *E. coli* strains. To determine the expression of OmpA at the protein level and also to verify whether any other proteins are affected by either insertional mutagenesis or deletion of the *ompA* gene, we examined the whole membrane proteins and Sarcosyl-insoluble and 3% LDS-soluble outer membrane proteins of all the strains by SDS-PAGE and Western blotting with anti-OmpA antibody. As shown in Fig. 1, 3% LDS-soluble outer membrane proteins of all the strains revealed similar patterns except that strains E44, E69, and E105 showed a 35-kDa protein which reacted with anti-OmpA antibody (OmpA$^+$ strains), whereas E91, E92, and E111 strains did not show the 35-kDa proteins by either SDS-PAGE or Western blotting (OmpA$^-$ strains). Similar findings were observed for whole membrane proteins (data not shown). Of interest, strain E58, which was generated by Tn*phoA* mutagenesis of strain E44, no longer showed a 35-kDa OmpA protein by SDS-PAGE, but it showed a protein with an apparent molecular size of 20 kDa which reacted with anti-OmpA antibody on Western blots. Strain E58 was previously shown to contain no proteins reactive with the anti-OmpA antibody (39). It was, however, noted that the polyclonal anti-OmpA antibody used was directed primarily against the carboxy-terminal portion of OmpA (unpublished data), thus making it possible that the Tn*phoA* insertion in E58 may still allow expression of truncated OmpA, which was not detected by the anti-C-terminal OmpA antibody. To verify this possibility, we used anti-OmpA antibody raised against the N-terminal fragment containing 1 to 177 amino acids of OmpA (obtained from U. Henning [16]) on Western blots. The antibody reacted with a 20-kDa protein, in agreement with the SDS-PAGE results. Similarly, strain E109, which contained a truncated *ompA* gene, did not show 35-kDa protein by SDS-PAGE but showed a protein with approximate size of 27 kDa which reacted with the anti-N-terminal OmpA antibody. Other phenotypic characteristics such as expression of S-fimbriae and LPS were unaffected as examined by colony blot assays using monoclonal antibodies against S-fimbriae and the anti-O18 LPS antibody, respectively.

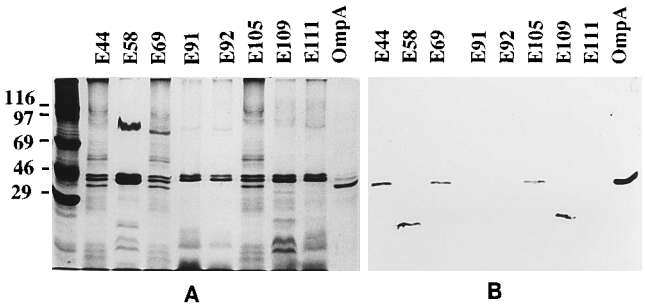

FIG. 1. SDS-PAGE (A) and anti-OmpA antibody Western blot (B) of outer membrane proteins of OmpA$^+$ and OmpA$^-$ strains. The 3% LDS-soluble membrane proteins of different bacteria were separated on SDS–10% polyacrylamide gels and stained with Coomassie blue. A duplicate gel was transferred to a nitrocellulose sheet and immunoblotted with anti-OmpA antibody as described in the text. The molecular mass markers (in kilodaltons) are indicated on the left.

Inoculum size and time-dependent invasion of OmpA$^+$ and OmpA$^-$ *E. coli* K1 strains. The characteristics of OmpA$^+$ (E69) and OmpA$^-$ (E91) invasion into BMEC were examined and compared to optimize the experimental conditions. *E. coli* HB101 was used as a negative control. Initially, the effect of increasing inoculum size on cellular invasion by these two strains was studied. Overnight cultures of both OmpA$^+$ and OmpA$^-$ strains were added to BMEC monolayers at increasing concentrations (Fig. 2A). The number of intracellular bacteria increased for both strains with increases in inoculum size. Nonetheless, a clear distinction between these two strains was observed. The number of internalized E69 bacteria increased as the inoculum size was increased up to 10^7 CFU per well. The number of internalized OmpA$^-$ strain E91 bacteria appeared to increase, but the increase was considerably less than that of strain E69. *E. coli* HB101 showed invasion responses more or less similar to those of E91 at all inoculum sizes (data not shown). All three strains were equally susceptible to gentamicin (MIC, 5 μg/ml), and the inoculation of wells without BMEC monolayers did not reveal any viable organisms for E69, E91, and HB101 strains. The growth rates of OmpA$^+$, OmpA$^-$, and HB101 strains in experimental media during the invasion assays were similar. Hereafter, 10^7 CFU (each) of OmpA$^+$, OmpA$^-$, and HB101 cells per well was added to the BMEC monolayers for invasion assays unless otherwise stated. We also examined whether the lack of invasion by OmpA$^-$ strains was related to a failure of the bacteria to become

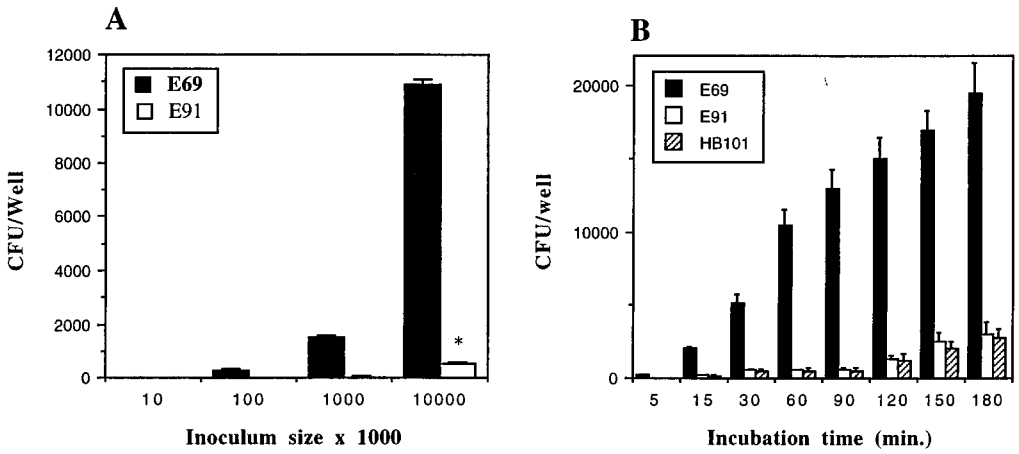

FIG. 2. Inoculum size and time-dependent invasion of OmpA$^+$ E69, OmpA$^-$ E91, and HB101 strains into BMEC. Overnight cultures of bacteria were incubated with confluent monolayers of BMEC, and the invasion assays were carried out as described in the text. The results presented are the means for at least three different experiments; error bars indicate standard deviations. *, $P < 0.01$ by two-tailed, unpaired *t* test.

associated with BMEC. As shown in Table 1, no significant difference was observed in the number of bacteria associated with BMEC between OmpA$^+$ ($4.2 \times 10^5 \pm 1.0 \times 10^5$) and OmpA$^-$ ($3.0 \times 10^5 \pm 1.1 \times 10^5$) *E. coli* at an inoculum size of 10^7 CFU.

Next, the time courses of invasion for OmpA$^+$ and OmpA$^-$ strains were compared (Fig. 2B). BMEC monolayers were infected with approximately 10^7 CFU and incubated for increasing periods of time before gentamicin was added. The number of OmpA$^+$ E69 bacteria invading BMEC increased up to 180 min after infection. The invasive capability of OmpA$^+$ strain E69 was approximately 25- to 50-fold greater than that of OmpA$^-$ strain E91 up to 120 min; however, the difference in invasion frequency was reduced to 3- to 4-fold after 120 min of infection. The latter finding with prolonged incubation might be due to a decrease in internalized OmpA$^+$ bacteria, which were subsequently killed by gentamicin, and/or increased nonspecific interaction of OmpA$^-$ bacteria with BMEC. The differences in invasion by OmpA$^+$ and OmpA$^-$ strains (3 to 4 fold) up to 4 h of incubation were similar to those described above for shorter incubation periods (data not shown). However, prolonged incubation with these bacteria caused the monolayer to detach from the wells, precluding experiments with longer time periods. As shown in Fig. 2B, HB101 behaved in a manner similar to E91. Because the invasive capability of the OmpA$^+$ strain into BMEC was significantly greater than that of the OmpA$^-$ strain and could be reproducibly documented at 90 min, a 90-min incubation time was used for additional experiments.

Invasion of OmpA$^+$ and OmpA$^-$ strains into BMEC. The abilities of *E. coli* K1 OmpA$^+$ and OmpA$^-$ strains to invade BMEC were compared (Table 1). The 35-kDa OmpA-expressing bacteria E44, E69, and E105 invaded BMEC in numbers approximately 25- to 50-fold higher than those of the OmpA$^-$ strains E91, E92, and E111 (approximately 0.1% invasion versus 0.002 to 0.005%, respectively; $P < 0.01$). Strain E58, in which the *ompA* gene was inactivated by a Tn*phoA* insertion, still expressed the truncated N-terminal OmpA protein and showed 50 to 70% invasion compared with the parent strain E44. Similarly, strain E109, which contains OmpA with a deletion of the C-terminal 53 amino acids, also showed approximately 50 to 70% invasion of BMEC. We (33) and other investigators (26) have previously shown that S-fimbriae mediate the binding of *E. coli* to BMEC. All *E. coli* strains listed in Table 1 were found to possess S-fimbriae by colony blot assay with the anti-S fimbria monoclonal antibody. Thus, it is unlikely that the difference in invasion frequencies between OmpA$^+$ and OmpA$^-$ strains reflect the difference in S-fimbria-mediated binding of these *E. coli* strains to BMEC. These results indicate that OmpA contributed to the invasion of BMEC by *E. coli* K1, which is independent of binding.

Inhibition of E69 invasion of BMEC by OmpA proteins and by anti-OmpA antibodies. To demonstrate that OmpA$^+$ *E. coli* invasion of BMEC is specific to OmpA, we examined the effect of purified OmpA on the E69 invasion of BMEC. The membrane proteins of E69 were extracted with 0.3% LDS containing 5 mM EDTA in 10 mM Tris-HCl (pH 7.5) to remove most of the non-OmpA proteins. Subsequent extraction of the pellet with 3% LDS in the same buffer produced a preparation enriched in OmpA. The 3% LDS extract was subjected to Sephacryl S-300-HR gel filtration in the presence of 0.1% LDS containing 0.4 M LiCl in Tris buffer. OmpA was eluted as a sharp band at a position similar to that of bovine serum albumin (apparent molecular size of 67 kDa) (Fig. 3A). This was significantly earlier than the position expected for the OmpA monomer because an equal amount of LDS bound to OmpA under these conditions shifted the apparent molecular size of OmpA to 70 kDa (34). The purified OmpA protein showed a single 35-kDa protein by SDS-PAGE and reacted with the anti-OmpA antibody on Western blots (Fig. 3B, lane 4). Since the OmpA fraction obtained from the Sephacryl S-300-HR column contained a considerable amount of LDS, which was not suitable for blocking experiments on BMEC monolayers, it was passed through an Extractgel-D column to remove excess LDS. The OmpA protein solution did not show any precipitation after Extractgel-D column extraction. Purified OmpA was reconstituted into proteoliposomes and used in E69 invasion assays. The OmpA proteoliposomes showed significant inhibition of E69 invasion into BMEC whereas liposomes containing OmpA$^-$ strain E91 proteins did not show any inhibitory activity (Fig. 4). Approximately 80% inhibition was achieved with 30 μg of OmpA protein per well. Similar results were also obtained with the OmpA proteins provided by H. Nikaido. Under these conditions the bacterial viability was unaffected by the OmpA proteins. These results suggest that the 35-kDa OmpA protein mediates the *E. coli* K1 invasion of BMEC.

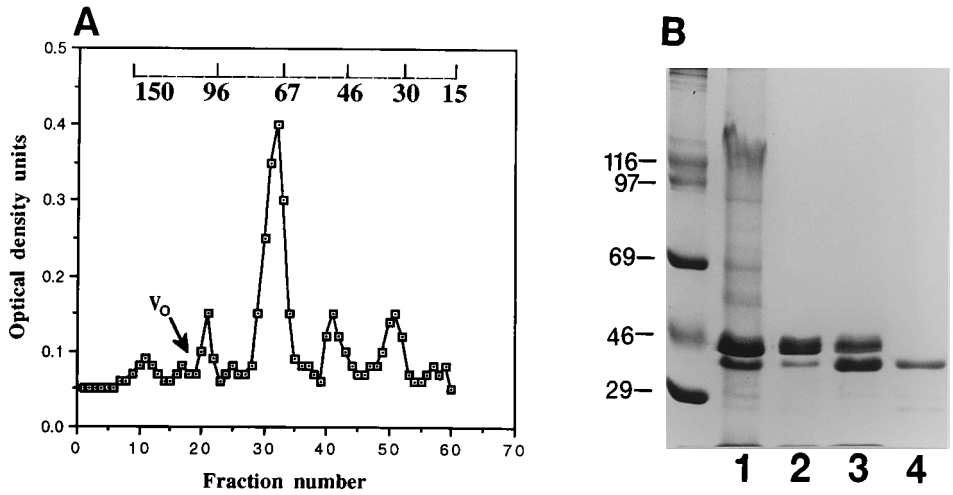

FIG. 3. Purification of *E. coli* OmpA. (A) The 3% LDS-soluble OmpA$^+$ strain E69 outer membrane proteins were loaded onto a preequilibrated Sephacryl S-300-HR column and eluted with 0.1% LDS–0.4 M LiCl–10 mM Tris-Cl (pH 7.5) buffer. The elution of proteins was monitored with a UV spectrophotometer at 280 nm. The elution of standard proteins was carried out with the same buffer without LDS, and the positions of the eluted proteins (in kilodaltons) are indicated at the top. (B) SDS-PAGE patterns of some fractions of OmpA. Lanes: 1, Sarcosyl-soluble membrane proteins; 2, 0.3% LDS-soluble membrane proteins; 3, 3% LDS-soluble proteins; 4, purified OmpA protein by Sephacryl S-300-HR. The molecular size markers (in kilodaltons) are indicated on the left.

To further demonstrate that E69 invasion of BMEC is specific to OmpA, we examined the effect of anti-OmpA antibodies on the invasion. These anti-OmpA antibodies reacted with intact OmpA$^+$ *E. coli* strains on colony blots but not with OmpA$^-$ strains (data not shown). Fab fragments of anti-OmpA antibodies were prepared to minimize nonspecific interaction of the Fc portion of antibodies with endothelial cells and examined for their ability to block the E69 invasion into BMEC. As shown in Fig. 4, the anti-OmpA antibodies inhibited the E69 invasion of BMEC by more than 80% whereas other unrelated antibodies (e.g., anti-S-fimbria antibody and anti-group B streptococcal antibody) did not show any inhibition. These results suggest that extracellular OmpA domains recognized by the anti-OmpA antibody may contribute to *E. coli* invasion of BMEC.

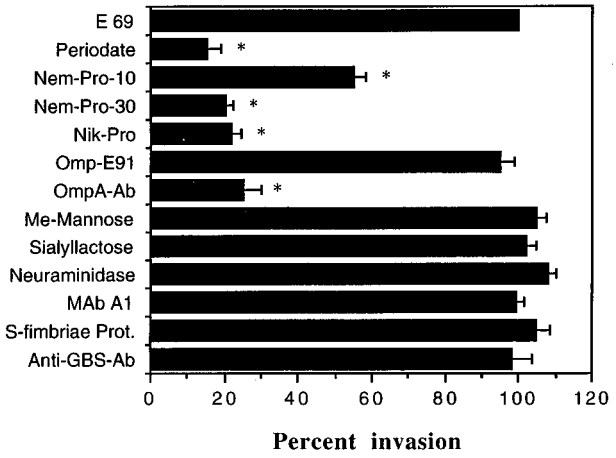

FIG. 4. Inhibition of OmpA$^+$ *E. coli* E69 invasion of BMEC by various reagents. For inhibition studies the BMEC monolayers were treated with 10 mM periodate for 10 min or incubated with either 10 μg (Nem-Pro-10) or 30 μg (Nem-Pro-30) of proteoliposomes containing OmpA isolated as described in Materials and Methods or proteoliposomes containing 30 μg of OmpA obtained from H. Nikaido (Nik-Pro) or outer membrane proteins of OmpA$^-$ strain E91 (Omp-E91) or purified S-fimbria proteins (20 μg) for 1 h before the bacteria were added. Similarly, Fab fragments of either anti-OmpA antibodies (Ab) or monoclonal antibodies (MAb) A1 or anti-GBS antibodies were incubated with BMEC, and invasion assays were carried out as described in the text. Simple sugars, methyl (Me) mannose and sialyl lactose (50 mM final concentration) were incubated with bacteria for 1 h before being added to the BMEC monolayers. The results are presented as the percentage of E69 invasion ($1.23 \times 10^4 \pm 0.2 \times 10^4$ CFU per well or approximately 0.12% of the inoculum) with medium alone and are the means for at least three different experiments; error bars indicate standard deviations. *, $P < 0.05$ by two-tailed, unpaired *t* test.

Characteristics of BMEC receptors involved in OmpA$^+$ *E. coli* invasion. Since all the *E. coli* strains listed in the Table 1 were found to possess both type 1 fimbriae (specific for α-mannose) and S-fimbriae (specific for α-2,3-sialyl lactose), we examined the effect of fimbriae on invasion. The invasion assays were carried out either with bacteria incubated with α-methyl-mannoside or sialyl lactose (50 mM, final concentration) or by treating the endothelial cell monolayer with neuraminidase from *Vibrio cholerae* preferentially specific for α-2,3-sialic acid or from *Arthrobacter ureafaciens*, specific for both α-2,3- and α-2,6-sialic acid. None of the treatments significantly affected the invasion of E69 (Fig. 4). Moreover, the monoclonal antibody to S-fimbrial adhesin, which completely blocks the binding of S-fimbriated *E. coli* to BMEC (33), did not show any inhibition of E69 invasion. These results suggest that neither type 1 fimbriae nor S-fimbriae play an important role in the invasion of OmpA$^+$ *E. coli* into BMEC and that sialic acid is not necessary for the interaction with BMEC surface molecules. To further determine whether any other carbohydrate moieties of BMEC are involved in the invasion, endothelial cells were treated with *meta*-periodate (5 mM sodium *meta*-periodate for 10 min in the dark), which cleaves free vicinal hydroxyl groups of sugar residues on oligosaccharides. The periodate treatment inhibited the E69 invasion of BMEC by >80% (Fig. 4), suggesting that the oligosaccharide portion of either glycoproteins or glycolipids could be involved in the OmpA-mediated interaction. A possible involvement of glycolipids was excluded since both E69 and E91 bound to the same glycolipids, i.e., galactosyl ceramide and sulfatide, a characteristic feature of the major S-fimbrillin protein (data not shown) (28). These results suggest that OmpA of E69 mediates the interaction with a carbohydrate epitope of glycoproteins of

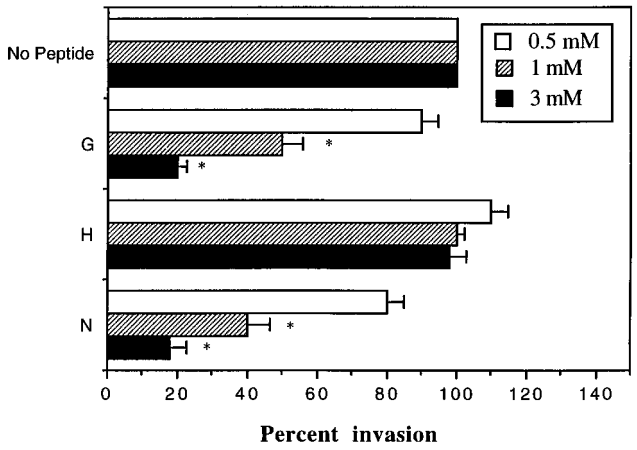

FIG. 5. Inhibition of E69 invasion of BMEC with synthetic peptides. Different concentrations of synthetic peptides were incubated with BMEC monolayers for 1 h before the bacteria were added, and the invasion assays were carried out as described in the text. The results are presented as the percentage of E69 invasion ($1.0 \times 10^4 \pm 0.35 \times 10^4$ CFU per well or approximately 0.1% of the inoculum) with medium alone and are the means for at least three different experiments; error bars indicate standard deviations. *, $P < 0.001$ by two-tailed, unpaired *t* test.

BMEC which is susceptible to periodate treatment and independent of the S-fimbria binding domain.

Inhibition of *E. coli* K1 invasion of BMEC by synthetic peptides. We showed that OmpA interacts with WGA-specific GlcNAc1-4GlcNAc epitopes of BMEC glycoproteins (29). Lucas et al. showed that the tumor necrosis factor (TNF) has a similar lectin-like activity for GlcNAc1-4GlcNAc moieties, which mediates trypanolytic activity and also identified the TNF domains involved in the recognition of carbohydrate epitope (21). On the basis of the amino acid compositions and sequences of the WGA and TNF domains involved in the recognition of GlcNAc1-4GlcNAc moieties, we identified the amino acid sequences present on extracellular loops of OmpA and synthesized two peptides: Asn-27–Gly–Pro–Thr–His–Glu-32 (N), and Gly-65–Ser–Val–Glu–Asn-69 (G). Another synthetic peptide, His-19–Asp–Thr–Gly-22 (H), was used as a control. These peptides were incubated with BMEC at different concentrations in experimental media for 1 h before the bacteria were added, and the invasion assays were carried out as described above. As shown in Fig. 5, none of the peptides was effective at a 0.5 mM concentration; however, at 1 and 3 mM concentrations, peptides G and N showed significant ($P < 0.001$) inhibition of E69 invasion into BMEC whereas peptide H did not show any inhibitory activity. These peptides showed no effect on the viability of the bacteria under the experimental conditions used.

DISCUSSION

Inadequate knowledge of pathogenesis and pathophysiology of neonatal *E. coli* meningitis has contributed to the significant mortality and morbidity associated with this disease. For example, it is unclear how circulating *E. coli* penetrates BMEC, which constitute the blood-brain barrier. Since *E. coli* OmpA has been shown to have sequence homology with Opa proteins involved in the invasion of *Neisseria gonorrhoeae* into cultured epithelial cells, we speculated that OmpA might play a similar role in *E. coli* K1 invasion of BMEC. In this study we demonstrated that OmpA promotes invasion of *E. coli* K1 into BMEC. This was shown by (i) an approximately 25- to 50-fold-higher frequency of invasion of BMEC by $OmpA^+$ strains than by $OmpA^-$ strains, (ii) loss of OmpA expression resulting in considerable attenuation (approximately 25-fold decrease) in the invasive ability into BMEC, (iii) restoration of the invasive capability of $OmpA^-$ strains to the level of the $OmpA^+$ strain by complementation with the *ompA* gene, and (iv) inhibition of $OmpA^+$ *E. coli* invasion into BMEC by purified OmpA proteins as well as by the anti-OmpA antibody.

Of interest, strain E58, in which the *ompA* gene was inactivated by Tn*phoA*, was found to exhibit approximately 50 to 70% invasive capability into BMEC. This strain was previously reported as an $OmpA^-$ strain which was unable to produce a 35-kDa OmpA protein as assessed by anti-C-terminal OmpA antibody immunoblotting (39). Since the Tn*phoA* insertion in strain E58 was identified 400 bp from the 3′ end of the *ompA* gene, we hypothesized that E58 might produce an approximately 20-kDa truncated OmpA protein. This hypothesis was based on the concept of Bremer et al., who have shown that OmpA fragments missing up to 122 carboxy-terminal amino acids can be efficiently incorporated into the outer membrane, suggesting that the entire *ompA* gene is not required for translocation (3). Our findings are in agreement with this prediction; i.e., E58 expressed a 20-kDa truncated OmpA protein, which reacted with the anti-N-terminal OmpA antibody as well as the anti-OmpA antibody used in our study and most likely contributed to the 50 to 70% invasive capability. Similarly, strain E109, which contained an approximately 27-kDa truncated OmpA protein lacking 53 C-terminal amino acids showed a 50 to 80% invasive capability compared with that of strain E105, which contained the entire *ompA* gene. These findings suggest that N-terminal portion of OmpA, not the C-terminal portion, may in part be involved in *E. coli* invasion of BMEC. An explanation for the reduced invasive capabilities of strains E58 and E109 compared with that of E69 may be that deletion of the C-terminal portion of OmpA could have altered the orientation of the N-terminal portion, resulting in inefficient interaction of OmpA with BMEC and, subsequently, a reduction in invasive abilities. A similar concept was proposed by Klose et al. (17, 18), who have shown that deletion of amino acid residues 154 to 180 of OmpA (which include the last β-strand and some periplasmic tail) or insertion of proline residues into the transmembrane region of OmpA by site-directed mutagenesis causes alteration in the assembly of OmpA. In addition, it is possible that the level of expression of OmpA may be quantitatively less in strains E58 and E109 than in $OmpA^+$ strains E69, E44, and E105, which is responsible for the reduced invasive capabilities of the E58 and E109 strains. Nevertheless, we cannot rule out the possibility that modifications of the outer membrane resulting from the absence of OmpA may contribute to reducing the ability of $OmpA^-$ strains to invade BMEC.

Our previous studies have shown that the degree of binding of S-fimbriated *E. coli* to BMEC is significantly higher than that of nonfimbriated bacteria and that S-fimbria-mediated binding is completely abolished by neuraminidase treatment of BMEC (33), suggesting the prerequisite for sialic acid in binding. The role of S-fimbria-mediated binding in the invasion of $OmpA^+$ strains into BMEC, however, was eliminated because (i) both $OmpA^+$ and $OmpA^-$ strains examined in this study were found to possess S-fimbriae and (ii) the monoclonal antibody to S-fimbriae, the sialyl lactose (S-fimbria receptor analog), the isolated S-fimbriae proteins, and the neuraminidase treatment of BMEC failed to affect the invasion frequency of $OmpA^+$ strains into BMEC. It is therefore likely that the role of S-fimbriae may be to provide a more intimate contact of circulating *E. coli* to BMEC in vivo to withstand blood flow,

which may be required for subsequent crossing of the blood-brain barrier. Studies are in progress to investigate the role of S-fimbriae in the pathogenesis of *E. coli* meningitis.

OmpA-enhanced invasion of *E. coli* into BMEC appeared to involve glycoproteins on BMEC as suggested by complete inhibition of E69 invasion with periodic acid. Sialic acid was not the determinant in this interaction as neuraminidase treatment of BMEC showed no effect on E69 invasion of BMEC. We recently showed that OmpA$^+$ *E. coli* invasion of BMEC was inhibited by incubating the endothelial cells with WGA, specific for the GlcNAc1-4GlcNAc epitope, and that the invasion was prevented by incubating the bacteria with GlcNAc1-4GlcNAc oligomers prior to the addition to BMEC, indicating that GlcNAc1-4GlcNAc moieties of BMEC glycoproteins are involved in the invasion of OmpA$^+$ *E. coli* (29). Of interest, TNF has also been shown to have a similar lectin-like affinity for *N*,*N*′-diacetylchitobiose, which mediates the trypanolytic activity (21). This activity is mediated by a region of TNF (Thr-105–Pro–Glu–Gly–Ala–Glu-110 in human TNF) which differs from the domain implicated in the tumoricidal effect of TNF. Thr and Glu residues are considered critically important in TNF binding to chitobiose. High-resolution electron density studies also revealed that amino acid residues Ser-62, Tyr-73, and Glu-115 are important in the WGA protein sequence for interaction with chitobiose (40, 41). On the basis of these observations, glutamic acid appeared to play an important role in both TNF and WGA protein sequences. We, therefore, synthesized the peptides representing extracellular loops of OmpA that contain glutamic acid. The synthetic peptide, N (Asn-27–Glu-32) represents a part of the first loop of OmpA, whereas G (Gly-65–Asn-69) represents the tip of second loop of OmpA (37). The synthetic peptide H, His-19–Gly-22, which represents a part of first, third, and fourth loops of OmpA, was used as a control. The inhibitory effect of synthetic peptides G and N present on two separate loops of OmpA might be due to the formation of a pocket-like structure that interacts with the carbohydrate epitope on BMEC. The extracellular fragment of OmpA consisting of amino acid residues 1 to 177 is folded across the membrane in eight antiparallel β-strands forming a β-barrel with four outside turns. These topographic structures of OmpA may fulfill the requirement of hydrogen bonding between two neighboring strands to form a receptor binding groove. A similar cooperative formation of a receptor binding pocket has been postulated for *Neisseria gonorrhoeae* outer membrane loops of Opa proteins (20).

It is important to note that the invasion frequency of BMEC by OmpA$^+$ *E. coli* (approximately 0.1%) was considerably less than the reported epithelial cell invasion frequencies by other gram-negative bacteria such *Shigella* and *Salmonella* species (usually 1 to 10% [9]). However, we have recently shown in the infant rat model of experimental hematogenous meningitis that *E. coli* with invasive capabilities (approximately 0.1% invasion frequency) was able to enter the central nervous system, while isogenic but noninvasive *E. coli* ($<$0.001% invasion frequency) failed to enter the central nervous system (12). In addition, we showed in the same animal model that GlcNAcβ1-4GlcNAc oligomers were able to prevent the entry of OmpA$^+$ *E. coli* into the central nervous system (29). These findings support our hypothesis that the 0.1% invasion frequency observed for OmpA$^+$ *E. coli* is related to enhanced invasiveness into the central nervous system in vivo and thus is biologically relevant.

Our findings have identified, for the first time, OmpA as an essential *E. coli* structure that enhances invasion of BMEC. Two short amino acid sequences, Asn-27–Gly–Pro–Thr–His–Glu-32 and Gly-65–Ser–Val–Glu–Asn-69, represent the essential domains of OmpA that were able to inhibit OmpA *E. coli* invasion of BMEC.

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