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Endothelial Cell GlcNAcβ1-4GlcNAc Epitopes for Outer Membrane Protein A Enhance Traversal of *Escherichia coli* across the Blood-Brain Barrier

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Inadequate knowledge of pathogenesis and pathophysiology has contributed to the high mortality and morbidity associated with neonatal *Escherichia coli* meningitis. We have shown previously that outer membrane protein A (OmpA) contributes to *E. coli* K1 invasion of brain microvascular endothelial cells. In this study we report that this OmpA$^+$ K1 *E. coli* invasion of brain microvascular endothelial cells was inhibited by wheat germ agglutinin and chitooligomers prepared from the polymer of 1,4-linked GlcNAc, chitin. The specificity of the interaction between OmpA and GlcNAcβ1-4GlcNAc epitopes was verified by the demonstration that chitotriose-bound OmpA and wheat germ agglutinin-bound brain microvascular endothelial cell membrane proteins inhibit *E. coli* K1 invasion. Of interest, OmpA$^+$ *E. coli* invasion into systemic endothelial cells did not occur, but invasion similar to that of brain microvascular endothelial cells was observed when systemic cells were treated with α-fucosidase, suggesting that the GlcNAcβ1-4GlcNAc moieties might be substituted with L-fucose on these cells. More importantly, the chitooligomers prevented entry of *E. coli* K1 into the cerebrospinal fluid of newborn rats with experimental hematogenous *E. coli* meningitis, suggesting that the GlcNAcβ1-4GlcNAc epitope of brain microvascular endothelial cells indeed mediates the traversal of *E. coli* K1 across the blood-brain barrier. A novel strategy with the use of soluble receptor analog(s) may be feasible in the prevention of devastating neonatal *E. coli* meningitis.

Escherichia coli is the most common gram-negative bacterium that causes meningitis during the neonatal period. The mortality and morbidity associated with this disease have remained significant despite advances in antimicrobial chemotherapy and supportive care (23). Case fatality rates range from 15 to 40%, and approximately 50% of the survivors sustain neurologic sequelae (6, 23). Inadequate knowledge of the pathogenesis and pathophysiology of the infection has contributed to this high mortality and morbidity. For example, most cases of neonatal *E. coli* K1 meningitis develop as a result of hematogenous spread, but the factors responsible for the neurotropism adapted by circulating *E. coli* to cross brain microvascular endothelial cells (BMEC), which constitute the blood-brain barrier, have not been determined.

Many surface structures expressed by *E. coli* in the interaction with carbohydrate epitopes, including mannose-specific type 1 fimbriae (10) and sialic acid-specific S-fimbriae (11), have been identified. We and others have previously shown that S-fimbriae mediate *E. coli* binding to BMEC, via SfaS adhesin specific to the NeuAcα2,3-galactose epitope of endothelial cell glycoproteins and SfaA specific to sulfated glycolipids (13, 15). However, binding via S-fimbriae was not accompanied by invasion (14), suggesting that other structures of *E. coli* are required for invasion of the BMEC. There is substantial evidence that the outer membrane proteins of gram-negative organisms contribute to their ability to invade cultured mammalian cells. For example, Kupsch et al. have demonstrated that a prominent outer membrane protein of neisseriae, Opa, is responsible for entry into epithelial cells (9). The structural similarities of *E. coli* outer membrane protein A (OmpA), a 35-kDa heat-modifiable protein, and the *Neisseria* Opa protein (8) suggest that OmpA may have a function in the pathogenesis of *E. coli* meningitis similar to that of Opa. We have recently shown that OmpA contributes to the invasion of *E. coli* K1 into BMEC (16) as demonstrated by (i) an approximately 50- to 100-fold increase in invasion of BMEC by OmpA$^+$ strains compared with that by OmpA$^-$ strains, (ii) restoration of the invasive capabilities of the OmpA$^-$ strain to the level of the OmpA$^+$ parent strain by replacement of the *ompA* gene, and (iii) inhibition of OmpA$^+$ *E. coli* invasion into BMEC by purified OmpA proteins as well as by anti-OmpA antibodies.

In the present study, we showed that the degree of OmpA$^+$ *E. coli* invasion of endothelial cells of brain origin was significantly higher than that of systemic endothelial cells. We also determined that *E. coli* OmpA interacts with the GlcNAcβ1-4GlcNAc epitopes of BMEC glycoproteins. These GlcNAcβ1-4GlcNAc epitopes on systemic endothelial cells appeared to be substituted with L-fucose, as α-fucosidase treatment of human umbilical vein endothelial cells showed OmpA$^+$ *E. coli* invasion to the level of BMEC. The biological significance of this interaction was demonstrated in a newborn rat meningitis model by the inhibition of the entry of OmpA$^+$ *E. coli* into the central nervous system by soluble GlcNAcβ1-4GlcNAc oligomers.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and chemicals. The strains used in this study (E69 and E91) were derived from a cerebrospinal fluid (CSF) isolate of *E. coli* K1 strain RS218 (serotype O18:K1:H7) as described previously (26) and were obtained from J. N. Weiser. Briefly, strain E69 (OmpA$^+$) was generated by P1 transduction of the *E. coli* K-12 *ompA* gene to an OmpA$^-$ mutant of RS218, and E91 (OmpA$^-$) is a mutant lacking the entire *ompA* gene (3). Other phenotypic characteristics of E69 and E91 were not affected, including expression of hemolysin and aerobactin as well as lipopolysaccharide, S-fimbriae, type 1 fim-

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briae, and the K1 capsule, which was tested by colony blotting with antibodies to lipopolysaccharide and S-fimbriae, by mannose-sensitive hemagglutination, and by the antiserum agar technique for the K1 capsule, respectively (7). 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. Lectins and chitin hydrolysate were obtained from Vector Laboratories (Burlingame, Calif.). All other reagents were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified.

Isolation of BMEC. Bovine BMEC were isolated as described previously (19). Briefly, the homogenates of bovine brain cortices were centrifuged in 25% bovine serum albumin (BSA), and the pellet containing crude microvessels was digested with collagenase-dispase. Microvascular capillaries were isolated by adsorption to a glass bead column, plated on collagen-fibronectin-coated dishes, and cultured in growth medium containing D-valine (to inhibit the growth of nonendothelial cells). Human cerebral cortex fragments were obtained by surgical resection of the cortices of children (less than 7 years old) with seizure disorders at Childrens Hospital Los Angeles. Human and rat BMEC were isolated as described above, except that 15% dextran was used instead of BSA for initial centrifugation. Human BMEC were further purified by fluorescence-activated cell sorting and were >99% pure (20). Bovine, human, and rat 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. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, Calif.), and human aortic arterial endothelial cells (HAAEC) were obtained from Coriell Cell Repositories (Camden, N.J.).

Fucosidase and PNGase F treatments of endothelial cells. Both BMEC and HUVEC monolayers were washed once with 50 mM phosphate buffer (pH 6.5) and incubated with 0.1 U of neuraminidase from *Vibrio cholerae* per ml for 1 h at 37°C in the same buffer (24). The monolayers were then washed four times with phosphate-buffered saline (PBS) and further incubated with 0.1 U of α-L-fucosidase from bovine epididymides per ml in 50 mM sodium acetate buffer (pH 5.5) for 1 h at room temperature. Similarly, another set of monolayers without neuraminidase treatment were also incubated with fucosidase enzyme. Control monolayers were treated with corresponding buffer solutions. The efficiency of digestion by these enzymes was tested by lectin Western blotting (immunoblotting) of the BMEC membranes with *Maackia amurensis* lectin (specific for NeuAcα2,3Gal) and *Ulex europaeus* agglutinin I (UEA-I, specific for L-fucose). Cleavage of asparagine-linked oligosaccharides from BMEC surface glycoproteins was carried out by using 5 U of peptide-*N*-glycosidase F (PNGase F) in 50 mM sodium phosphate buffer (pH 7.8) at 37°C overnight (18). The monolayers were then washed with PBS. Control monolayers were incubated with phosphate buffer without PNGase F. The cleavage of oligosaccharides on BMEC by PNGase F was evaluated by wheat germ agglutinin (WGA) lectin blotting of membrane proteins. The viability of the cells after these treatments was verified by trypan blue exclusion assay (4).

Invasion assays. The invasion assays were performed by a modification of the method of Tang et al. (21). Approximately 10^7 bacteria were added to confluent monolayers of BMEC in a 24-well plate coated with collagen (BMEC and bacterium ratio of approximately 1:100) in experimental medium (500 μl) containing M199-Ham F-12 (1:1) 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 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 in the same medium containing gentamicin (100 μg/ml) for 1 h at 37°C. The condition of the BMEC monolayers was assessed after each experiment under phase-contrast microscopy, and no detachment of cells was observed. Both strains of *E. coli* were susceptible to gentamicin (MIC of 8 μg/ml), and no viable bacteria were recovered after 1 h of incubation. 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. Each assay was run at least three times in triplicate. For inhibition experiments, various lectins (50 μg per well) were incubated with BMEC for 1 h at 37°C prior to the addition of the bacteria. Different concentrations of chitin hydrolysate and chitotriose; the glycoproteins fetuin (20 μg), thyroglobulin (200 μg) and oromucoid (30 μg); and simple sugars (50 mM, final concentration) were incubated with 10^7 bacteria for 1 h on ice before being added to the BMEC.

Preparation of endothelial cell membranes and WGA-affinity chromatography. BMEC and HUVEC were grown to confluence in T165 flasks (Costar, Cambridge, Mass.) and scraped from the flasks into medium containing protease inhibitors, aprotinin, and phenylmethylsulfonyl fluoride (PMSF). The cells were pelleted by centrifugation (10,000 × *g*) at 4°C and resuspended in 50 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100, 1 mM dithiothreitol, aprotinin, and PMSF. The cells were subjected to brief sonication (three 10-s pulses) and centrifuged at 4,000 × *g* to remove cellular debris. The supernatant was centrifuged at 105,000 × *g* at 4°C for 1 h. The membrane pellet was resuspended in Tris buffer containing 0.03% Triton X-100 and protease inhibitors. The membrane proteins (10 mg) were incubated separately in preequilibrated WGA-Sepharose (2-ml bed volume) for 1 h at 4°C and then washed with 10 bed volumes of Tris buffer containing 0.01% Triton X-100 and protease inhibitors. The bound proteins were eluted with 250 mM GlcNAc in Tris-HCl, (pH 7.4). The eluted proteins were dialyzed against Tris buffer extensively and concentrated in Centricon tubes. The WGA-bound endothelial cell membrane proteins (10 μg) were incubated with 10^7 $OmpA^+$ *E. coli* cells for 1 h on ice before being added to the BMEC monolayers, and the invasion assay was carried out as described above.

Isolation of *E. coli* membrane proteins and chitotriose-affinity chromatography. *E. coli* E69 and E91 were grown overnight in brain heart infusion broth containing the appropriate antibiotics. The bacteria were collected by centrifugation, washed three times with 50 mM PBS, and resuspended in PBS containing 0.5% Triton X-100 and the protease inhibitors PMSF and aprotinin. The bacterial suspension was briefly sonicated, frozen at −70°C, thawed again, and sonicated for 30 s. Freezing and thawing was repeated three times and followed by centrifugation at 8,000 × *g* to remove debris, and the supernatant was ultracentrifuged at 120,000 × *g* for 1 h at 4°C. The pellet was solubilized in PBS containing 0.1% Triton X-100 and protease inhibitors to a final concentration of 2 mg/ml. The membrane proteins (0.5 mg) from the $OmpA^+$ *E. coli* strain were loaded onto a preequilibrated chitotriose-Sepharose 4B (1-ml bed volume) column in PBS and incubated for 0.5 h at 4°C. The column was washed with 5 bed volumes of PBS containing 0.1% Triton X-100. Bound proteins were eluted with 0.5 M GlcNAc in PBS–0.1% Triton X-100, dialyzed for 24 h against PBS, and concentrated. The proteins (10 μg per well) were incubated with BMEC monolayers for 1 h before the bacteria were added, and the invasion assays were carried out as described above. Chitotriose-Sepharose-bound E91 membrane proteins were also isolated in a similar manner.

Western blotting. The proteins were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred onto Immobilon P nitrocellulose by using a Millipore electroblot. The blots were incubated with either 5% (for anti-OmpA antibodies) or 0.5% (for lectins) nonfat milk in PBS for 1 h. The blots were incubated with anti-OmpA antibodies (1:3,000 dilution in blocking buffer) for 2 h and then with goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase and washed five times with PBS–0.05% Tween. For lectins, the blots were incubated with 10 mg of biotinylated WGA or UAE-I per ml for 2 h, washed five times with PBS, and then incubated with streptavidin peroxidase (1:3,000 in blocking buffer) for 30 min. After the blots were washed, the color was developed with diaminobenzidine and hydrogen peroxide as the substrates.

Newborn rat model of hematogenous *E. coli* meningitis. *E. coli* bacteremia and meningitis were induced in 5-day-old rats by a previously described method (7). Briefly, outbred, pathogen-free, pregnant Sprague-Dawley rats with timed conception were purchased from Charles River Breeding Laboratories (Wilmington, Mass.); the rats delivered in our vivarium 5 to 7 days after arrival. At 5 days of age, all members of each litter were randomly divided into two groups to receive intracardiac injection of $OmpA^+$ strain E69 (10^6 CFU) in 50 μl of saline or saline containing 2 mg of chitin hydrolysate. One to two hours later, blood and CSF specimens were obtained as described previously (7) for quantitative cultures on agar containing kanamycin (40 μg/ml).

RESULTS

Invasion of endothelial cells derived from brain and systemic vessels by *E. coli* K1. We have previously shown that $OmpA^+$ *E. coli* invades BMEC at significantly higher rates than $OmpA^-$ strains (16). To define whether the interaction of $OmpA^+$ *E. coli* is specific to endothelial cells of brain origin, invasion assays were carried out with BMEC from cows, rats, and humans, as well as systemic endothelial cells, e.g., HUVEC and HAAEC. The invasion frequencies of $OmpA^+$ and $OmpA^-$ *E. coli* strains are summarized in Table 1. The percentage of $OmpA^+$ *E. coli* cells into bovine BMEC (0.11%), rat BMEC (0.04%), and human BMEC (0.08%) was 50- to 100-fold greater than that of the $OmpA^-$ mutant (≤0.001%). In contrast, invasion into non-brain endothelial cells (HUVEC and HAAEC) was negligible (<0.001%), regardless of the expression status of OmpA. This difference in the invasive strength of $OmpA^+$ and $OmpA^-$ strains did not arise from the differences in the binding of these bacteria to BMEC (16), which was consistent with our demonstration that both $OmpA^+$ and $OmpA^-$ *E. coli* strains possess S-fimbriae. Of interest, the degree of bacterial binding to systemic endothelial cells was significantly less than that to BMEC ($2.0 \times 10^4 \pm 1.0 \times 10^4$ versus $5.4 \times 10^5 \pm 1.2 \times 10^5$ CFU per well, respectively; $P < 0.001$). In addition, the number of cell-associated bacteria was approximately 10 times higher with $OmpA^+$ *E. coli* than

TABLE 1. *E. coli* invasion of endothelial cells derived from brain and systemic vessels

E. coli strain	Phenotypic characteristic	% Invasion (mean ± SD)				
		BMEC			HUVEC	HAAEC
		Bovine	Rat	Human		
E69	O18:K1:OmpA$^+$	0.11 ± 0.02[a]	0.04 ± 0.02[a]	0.08 ± 0.02[a]	0.001 ± 0.001	<0.001
E91	O18:K1:OmpA$^-$	0.001 ± 0.001	<0.001	<0.001	<0.001	<0.001

[a] $P < 0.001$ by two-tailed, unpaired t test compared with E91 results.

with OmpA$^-$ *E. coli*. Thus, the negligible invasion observed with systemic endothelial cells may, in part, be due to less binding. Taken together, these findings suggest that the invasion of BMEC by OmpA$^+$ *E. coli* may stem from a specific interaction of OmpA with structures present on BMEC but not on non-brain endothelial cells.

Identification of BMEC carbohydrate epitope involved in *E. coli* invasion by lectin inhibition studies. We also previously showed that the invasion of OmpA$^+$ *E. coli* into BMEC was completely abolished by treating the BMEC with periodate, suggesting the involvement of a carbohydrate epitope on BMEC (16). Various lectins (Table 2) were used to identify the BMEC carbohydrate structures involved in invasion. Lectins were incubated with BMEC monolayers before the bacteria were added. Only WGA, specific for GlcNAcβ1-4GlcNAc, blocked the invasion by >95% ($1.2 \times 10^4 \pm 0.2 \times 10^4$ for BMEC versus $1.3 \times 10^2 \pm 0.3 \times 10^2$ with WGA), whereas other lectins showed no significant effect (Fig. 1). Several investigators have shown that the binding of WGA to cells or glycopeptides is decreased after treatment with neuraminidase, implicating the role of sialic acid residues in WGA interactions (2). However, neither neuraminidase treatment of BMEC (Table 3) nor *M. amurensis* lectin (Fig. 1), specific for NeuAcα2,3-Gal (25), showed any significant effect on OmpA$^+$ *E. coli* invasion of BMEC. In contrast, pretreatment of BMEC monolayers with PNGase F, an enzyme that cleaves asparagine-linked (*N*-linked) high-mannose, hybrid, and complex oligosaccharides from glycoproteins (5), resulted in a marked decrease in invasion of OmpA$^+$ *E. coli* (Fig. 1). There was no apparent change in the protein or glycolipid pattern by the treatment of BMEC with PNGase F (data not shown). These results suggest that OmpA$^+$ *E. coli* recognizes the GlcNAcβ1-4GlcNAc epitopes linked to asparagine on the glycoproteins. GlcNAcβ1-4GlcNAc epitopes often contain α-fucose linked to asparagine-bound GlcNAc via an α-1,6 linkage, but neither *Pisum sativum* agglutinin or UAE lectin, which interacts with L-fucose, nor free fucose was able to block the invasion (Fig. 1). In fact, the OmpA$^+$ *E. coli* invasion of BMEC was not affected by treating the endothelial cells with α-fucosidase with or without neuraminidase (Table 3). These findings indicate that the GlcNAcβ1-4GlcNAc epitopes of complex oligosaccharides on BMEC glycoproteins interact with OmpA$^+$ *E. coli* for invasion of BMEC. The possibility of glycolipid involvement in this interaction was ruled out, as high-performance thin-layer chromatography immunoblotting showed that both OmpA$^+$ and OmpA$^-$ *E. coli* strains bound to the same glycolipids, galactosylceramide, and sulfatide (15) (data not shown).

Inhibition of OmpA$^+$ *E. coli* invasion of BMEC with sugars and glycoproteins. Since WGA, specific to the GlcNAcβ1-4GlcNAc epitope, blocked the *E. coli* invasion, we examined the effect of chitin hydrolysate containing GlcNAc polymers of various lengths in the invasion assays (Fig. 2). The results showed that chitin hydrolysate completely blocked *E. coli* K1 invasion of BMEC in a dose-dependent manner, whereas GlcNAc and mannose monosaccharides were ineffective. In addition, neither lactose nor cellobiose, a disaccharide of glucose linked via β1,4, showed any inhibitory activity. Since chitin hydrolysate contains a heterogeneous population of GlcNAc polymers, chitotriose was examined for its effect on *E. coli* K1 invasion. The trisaccharide reduced the bacterial invasion by approximately 50% at a concentration of 2 mg and almost completely (>95%) blocked the invasion at a concentration of 10 mg per well ($1.2 \times 10^4 \pm 0.2 \times 10^4$ for E69 versus $1.0 \times 10^2 \pm 0.2 \times 10^2$ with chitotriose; $P < 0.001$). Neither chitin hy-

TABLE 2. Lectins and glycoproteins used in this study

Source of lectin (lectin) or glycoprotein	Carbohydrate specificity or oligosaccharide structure(s)
Arachis hypogea (peanut agglutinin)	Galβ-1
Concanavalin enisiformis (concanavalin A)	α-methyl-mannose
Maackia amurensis (MAL)	NeuAcα2,3-galactose
Phaseolus vulgaris (phytohemagglutinin E)	Galβ1,4-GlcNAcα1,2-Man
Pisum sativum (*P. sativum* agglutinin)	Terminal α1,6-linked fucose
Triticum vulgare (WGA)	GlcNAcβ1,4-GlcNAc
Ulex europaeus (UEA-I)	L-fucose
Fetuin	NeuAc α2,3Galβ1,4-GlcNAcβ1,2Manα1,6 branch; NeuAcα2,6-Galβ1,4GlcNAcβ1,2-Manα1,3-Manβ1,4-GlcNAcβ1,4-GlcNAcβ1-Asn; NeuAcα2,3-Galβ1,4-GlcNAcβ1,4
Thyroglobulin-B and -P[a]	±NeuAcα2,6 Galβ1,4-GlcNAcβ1,2Manα1,6 branch; ±Galα1,3 Galβ1,4GlcNAcβ1,2-Manα1,3-Manβ1,4-GlcNAcβ1,4-GlcNAcβ1-Asn; Fucα1,6

[a] Thyroglobulin-B also contains unfucosylated structures of oligosaccharides.

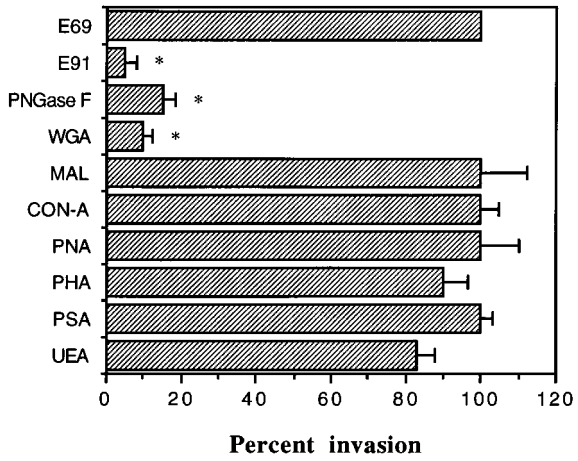

FIG. 1. Inhibition of OmpA$^+$ *E. coli* invasion of BMEC with different lectins. Bovine BMEC monolayers were either treated with PNGase F or incubated with 50 μg of each lectin (columns 4 to 10) for 1 h at 37°C, followed by the addition of OmpA$^+$ *E. coli*, E69. Invasion assays were done as described in Materials and Methods. The specificities of the lectins are given in Table 2. The columns for E69 and E91 represent controls with buffer alone and the value for 100% invasion of E69 into BMEC was 11,230 ± 531 CFU per well. The data represent the means of triplicate experiments. Error bars indicate standard deviations. *, $P < 0.001$ by two-tailed, unpaired *t* test.

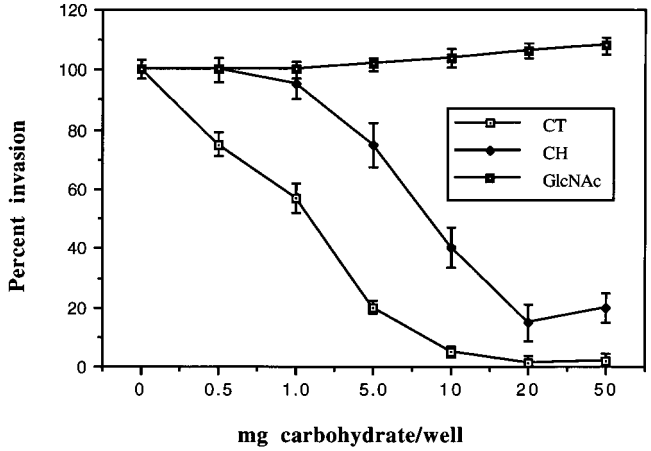

FIG. 2. Dose-dependent inhibition of OmpA$^+$ *E. coli* invasion of BMEC with chitotriose (CT), chitooligomers (CH), and GlcNAc. The OmpA$^+$ strain, E69, (10^7 CFU/ml) was incubated with different concentrations of carbohydrates for 1 h on ice before being added to the BMEC monolayers. All values represent the means of triplicate determinations; error bars indicate standard deviations.

drolysate nor chitotriose had any effect on the viability of either bacteria or BMEC under the experimental conditions employed.

To further assess the inhibitory properties of the GlcNAcβ1-4GlcNAc epitope, we analyzed the effect of glycoproteins containing different carbohydrate chains and simple mono- and disaccharides on *E. coli* K1 invasion of BMEC (Fig. 3). The concentrations of glycoproteins were chosen to give similar quantities of carbohydrate. Fetuin, a serum glycoprotein containing approximately 10% of triantennary complex-type oligosaccharides as a major species (Table 2), and asialofetuin were the only glycoconjugates that could significantly block the *E. coli* K1 invasion (70% ± 5.5% and 65% ± 3.9% reductions, respectively). Bovine thyroglobulin containing a mixture of α1,6-substituted and unsubstituted GlcNAcβ1-4GlcNAc in its oligosaccharide portion (25) showed only 25% ± 4.7% inhibition, whereas porcine thyroglobulin whose GlcNAc1-4GlcNAc epitopes are almost all replaced by L-fucose (27) did not exhibit any inhibition of invasion. The exclusive inhibitory activity of fetuin along with the chitooligomer data suggests that the receptor epitope for OmpA$^+$ *E. coli* K1 may be confined to the GlcNAcβ1-4GlcNAc moiety and that fucosyl substitutions at position 6 prevent the interaction with OmpA. These data corroborate with the lectin results.

Inhibition of *E. coli* invasion of BMEC with chitotriose-bound OmpA proteins and WGA-bound BMEC proteins. To examine whether the invasion of OmpA$^+$ *E. coli* is due to the interaction of OmpA with the GlcNAcβ1-4GlcNAc epitope of BMEC glycoproteins, the chitotriose-bound membrane proteins from OmpA$^+$ and OmpA$^-$ *E. coli* strains were purified and used in invasion assays. The Coomassie staining showed a 35-kDa protein reactive with the anti-OmpA antibody (blot not shown) which was found in chitotriose-bound E69 membrane proteins, while some nonspecific proteins were retained from the OmpA$^-$ E91 fraction (Fig. 4A). As shown in Fig. 4B, invasion of E69 was inhibited only when the endothelial cells were incubated with the chitotriose-bound 35-kDa protein obtained from E69 membrane proteins and not by proteins from E91 (10 μg of protein per ml per well). Similarly, the WGA-agarose-bound BMEC membrane protein fraction, which showed several proteins reactive to WGA on Western blots (data not shown), exerted significant inhibition of E69 invasion of endothelial cells at a concentration of 10 μg per well (Fig. 4B). Because of the small amount of HUVEC glycoproteins obtained from WGA-Sepharose column (<0.1% of total loaded proteins), inhibition experiments could not be carried out. The requirement of high concentrations of GlcNAcβ1-4GlcNAc (25 mM), compared with the inhibitory concentra-

TABLE 3. Effect of α-fucosidase treatment on cell-associated and intracellular OmpA$^+$ *E. coli* (strain E69) in BMEC and HUVEC

Treatment or strain	Bovine BMEC			HUVEC		
	Mean CFU ± SD/well		% Invasion[a]	Mean CFU ± SD/well		% Invasion[a]
	Cell-associated	Intracellular		Cell-associated	Intracellular	
Control with buffer	$(5.4 \pm 1.2) \times 10^5$	$(8.4 \pm 1.2) \times 10^3$	0.1*	$(2.0 \pm 1.0) \times 10^4$	55 ± 12	<0.001
α-Fucosidase	$(7.2 \pm 0.5) \times 10^5$	$(1.9 \pm 0.7) \times 10^4$	0.12*	$(7.2 \pm 0.2) \times 10^4$	$(6.3 \pm 0.7) \times 10^3$	0.07*
α-Fucosidase + WGA	$(5.2 \pm 0.4) \times 10^5$	$(1.0 \pm 0.4) \times 10^2$	<0.001	$(6.3 \pm 0.5) \times 10^4$	37 ± 12	<0.001
Neuraminidase	$(9.6 + 0.2) \times 10^5$	$(1.2 \pm 0.3) \times 10^4$	0.14*	$(2.5 \pm 0.3) \times 10^4$	$(5.8 \pm 0.3) \times 10^2$	<0.001
Neuraminidase + α-fucosidase	$(9.2 \pm 0.5) \times 10^5$	$(1.1 \pm 0.5) \times 10^4$	0.12*	$(6.8 \pm 0.6) \times 10^4$	$(6.9 \pm 0.2) \times 10^3$	0.08*
E91 (OmpA$^-$)	$(3.2 \pm 0.9) \times 10^5$	$(3.0 \pm 0.2) \times 10^2$	<0.001	$(2.0 \pm 0.7) \times 10^3$	10 ± 11	<0.001

[a] % invasion, (the number of bacteria internalized divided by the inoculum size) × 100. *, $P < 0.001$ by two-tailed, unpaired *t* test compared with either control or E91.

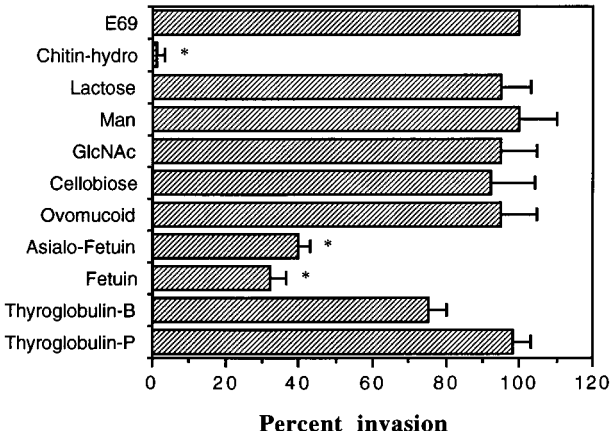

FIG. 3. Effect of sugars and different glycoproteins on OmpA$^+$ *E. coli* invasion of BMEC. The bacteria were incubated with chitin hydrolysate (2 mg/100 μl), mono- and disaccharides (50 mM), fetuin and asialofetuin (20 μg/ml), oromucoid, thyroglobulin-B, or thyroglobulin-P (200 μg/ml) for 1 h on ice before adding to the BMEC. The data represent the means of triplicate determinations, with error bars indicate standard deviations, and are expressed as the percent invasion of E69, which was 10,835 ± 675 CFU per well. *, $P < 0.01$ by two-tailed, unpaired *t* test.

tions of BMEC glycoproteins (10 μg), implies that conformation of the BMEC carbohydrate epitope may be important and/or additional structures such as the protein core may also be required for optimal interaction with OmpA.

Invasion of E69 into HUVEC after treatment with α-fucosidase. Since OmpA$^+$ *E. coli* showed higher levels of invasion into BMEC, but not into systemic endothelial cells, via interaction with GlcNAcβ1-4GlcNAc epitopes on BMEC surface glycoproteins, we initially examined whether these epitopes are available on non-brain endothelial cells. The GlcNAcβ1-4GlcNAc moieties are widely present on many eukaryotic cell surface glycoproteins, most often fucosylated via α1,6 linkage to Asn-linked GlcNAc. Thus, we speculated that the GlcNAcβ1-4GlcNAc epitopes on systemic endothelial cells might be substituted with L-fucose and thus might not be able to interact with OmpA. To verify this possibility, endothelial cell glycoproteins were analyzed by Western blotting with WGA and UAE-I lectins followed by antilectin antibodies. The bovine, human, and rat BMEC membrane proteins showed several strong bands with molecular masses ranging from 15 to 180 kDa that were reactive to WGA, whereas HUVEC and HAAEC showed a few very faint bands. In contrast, the UAE-I lectin showed little reactivity to BMEC glycoproteins; however, it reacted strongly with several proteins on systemic endothelial cells (blot not shown).

To further support the hypothesis that GlcNAcβ1,4-GlcNAc epitopes on HUVEC might be substituted with L-fucose and are not available for interaction with OmpA, HUVEC were treated with α-fucosidase to remove the fucosyl residues, and the invasion assays were carried out. BMEC were also treated with fucosidase as a comparison. To eliminate the possibility that S-fimbriae interaction with sialyl galactose epitopes on BMEC may contribute to *E. coli* invasion, we initially treated both BMEC and HUVEC with neuraminidase followed by fucosidase. As shown in Table 3, the invasion of E69 into HUVEC after fucosidase treatment, with or without prior digestion with neuraminidase, was significantly increased to the level of bovine BMEC invasion (0.07% for HUVEC versus 0.12% for bovine BMEC), whereas E91 showed no difference in invasion. In addition, the E69 invasion of fucosidase-treated HUVEC was blocked by incubation with WGA, suggesting that the GlcNAcβ1,4-GlcNAc epitopes on HUVEC glycoproteins might be available for WGA and OmpA interaction after the removal of L-fucose. Fucosidase treatment had no effect on E69 invasion of bovine BMEC. The enzyme treatments also had no effect on the viability of the endothelial cells.

Blocking of K1 *E. coli* entry into central nervous system with chitooligomers. We examined the biological relevance of the in vitro data in our well-established newborn rat model of experimental hematogenous meningitis. The development of techniques for atraumatic collection of blood and CSF specimens allows the use of this experimental model to examine the pathogenic mechanisms responsible for the development of hematogenous *E. coli* meningitis. Since 20 mg of chitin hydrolysate blocked the invasion of approximately 10^7 CFU of E69 into BMEC by >80%, we used 2 mg of carbohydrate to block the bacterial binding sites before injecting approximately 10^6

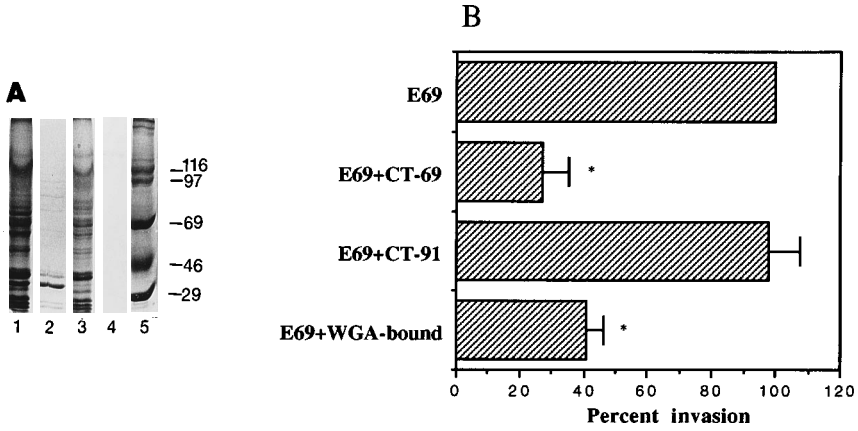

FIG. 4. (A) Coomassie-stained SDS-polyacrylamide gel electrophoresis of chitotriose-Sepharose-bound OmpA$^+$ and OmpA$^-$ *E. coli* membrane proteins. The membrane proteins from strains E69 (OmpA$^+$, lane 1) and E91 (OmpA$^-$, lane 3), chitotriose-Sepharose-bound membrane proteins from both strains (E69, lane 2; E91, lane 4), and standard molecular mass markers (lane 5) were separated on 10% gels. The molecular mass markers (in kilodaltons) are indicated on the right. (B) Inhibition of OmpA$^+$ *E. coli* invasion of BMEC by chitotriose-bound OmpA proteins and WGA-bound BMEC membrane proteins. The chitotriose-bound E69 (CT-69) and E91 (CT-91) membrane proteins were added to the BMEC monolayer in experimental media and incubated for 1 h at 37°C before the bacteria were added. Similarly, OmpA$^+$ *E. coli* E69 (10^7 CFU/ml) was incubated with WGA-bound BMEC membrane proteins (10 μg) for 1 h on ice before being added to BMEC monolayers. The data represent the means of triplicate determinations; error bars indicate standard deviations. *, $P < 0.03$ by two-tailed, unpaired *t* test.

TABLE 4. Comparison of bacterial counts in blood (mean ± standard deviation) and number of animals with positive CSF cultures between groups receiving strain E69 with normal saline or chitooligomers

Treatment group	*n*	Bacteremia (log CFU/ml of blood)	No. of animals with positive CSF culture (%)
Saline	22	6.08 ± 0.53	10 (44)
Chitooligomers	23	5.79 ± 0.48	3 (14)[a]

[a] $P = 0.023$ by Fisher's exact test.

CFU of E69 into the animals. As shown in Table 4, the magnitudes of bacteremia were similar between the two groups; however, the occurrence of meningitis shown by positive CSF cultures was significantly less in the animals receiving chitooligomers than in the control group. Thus, GlcNAcβ1-4GlcNAc epitopes of BMEC indeed appear to mediate the traversal of $OmpA^+$ *E. coli* across the blood-brain barrier both in vitro and in vivo.

DISCUSSION

E. coli meningitis usually develops as a result of hematogenous spread, but it is not clear how circulating *E. coli* cross BMEC, which constitute the blood-brain barrier. We have recently shown that OmpA of *E. coli* contributes to the invasion of BMEC, which is blocked by treating the BMEC with periodate, suggesting a role of the carbohydrate moieties of BMEC in invasion (16).

In the present study we showed that Asn-linked oligosaccharides played an important role in $OmpA^+$ *E. coli* invasion, as the treatment of BMEC monolayers with PNGase F resulted in significant inhibition of the invasion. Inhibition studies with several lectins indicated that GlcNAcβ1-4GlcNAc epitopes of BMEC glycoproteins were responsible for the interaction with OmpA in *E. coli* invasion. In contrast, treatment of BMEC with neuraminidase or bacteria with sialyl lactose did not affect the invasion (16), suggesting that NeuAcα2,3-galactose is not a critical component involved in $OmpA^+$ *E. coli* invasion of BMEC. Since neither the GlcNAc monosaccharide nor phytohemagglutinin E lectin, specific to Galβ1,4GlcNAcα1,2Man, showed any inhibitory activity, $OmpA^+$ *E. coli* might interact with the internal GlcNAcβ1-4GlcNAc epitopes linked to Asn and not with the GlcNAc moieties on two side chains of the bi- or triantennary oligosaccharides of the glycoproteins. This concept was supported by our demonstration that $OmpA^+$ *E. coli* invasion of BMEC was inhibited by fetuin but not by porcine thyroglobulin. Almost all GlcNAcβ1-4GlcNAc epitopes of porcine thyroglobulin are substituted with L-fucose, whereas GlcNAcβ1-4GlcNAc moieties of fetuin are unsubstituted (27). Thus, GlcNAcβ1-4GlcNAc moieties of fetuin might be readily accessible to OmpA and thereby able to inhibit the $OmpA^+$ *E. coli* invasion of BMEC. In contrast, porcine thyroglobulin might not be available to OmpA because of the presence of L-fucose in the GlcNAcβ1-4GlcNAc moiety. Although we used isogenic derivatives of $OmpA^+$ and $OmpA^-$ *E. coli* in this study to demonstrate that OmpA is responsible for invasion, it is possible that other surface molecules whose expression is closely dependent on OmpA may contribute to the interaction with GlcNAcβ1-4GlcNAc epitopes. However, the chitotriose affinity-purified proteins showed only a 35-kDa protein reactive to the anti-OmpA antibody, suggesting that OmpA is a very likely candidate for interaction with BMEC glycoproteins. Thus, OmpA interaction with GlcNAcβ1-4GlcNAc epitopes may be a key step for the invasion of *E. coli* into BMEC. Nevertheless, we cannot exclude the possibility that OmpA interaction with BMEC glycoproteins may induce the synthesis of putative adhesin molecules on the bacterial surface which subsequently contribute to the invasion by *E. coli.*

Despite the presence of GlcNAcβ1-4GlcNAc epitopes on many eukaryotic cell surface glycoproteins, we observed that $OmpA^+$ *E. coli* invasion occurred only in BMEC and not in the endothelial cells of systemic origin. This differential interaction of $OmpA^+$ *E. coli* with BMEC could be due to the presence of a higher number of GlcNAcβ1-4GlcNAc epitope-bearing receptors on BMEC than on non-brain endothelial cells. Another explanation, based on our observation that porcine thyroglobulin could not block the invasion of $OmpA^+$ *E. coli* into BMEC because of the presence of L-fucose on GlcNAcβ1-4GlcNAc attached to Asn, may be that GlcNAcβ1-4GlcNAc epitopes on non-brain endothelial cells might not be accessible for OmpA because of substitutions by L-fucose. In support of these two possibilities, we showed that there were large number of WGA-reactive proteins on BMEC and that they were unsubstituted, whereas systemic endothelial cells contained fewer WGA-reactive proteins, which appeared to be substituted with fucose. Immunocytochemical studies by other investigators (12, 17) have shown that WGA reactivity is far greater in the cerebral endothelium than in non-brain endothelia, as was the result in our study. Similarly, Belloni and Nicolson have shown by lectin Western blots that WGA-reactive glycoproteins were far more numerous in brain microvessels than in systemic vessels (1). Of interest, we also showed that $OmpA^+$ *E. coli* invaded HUVEC to the level of BMEC invasion after treatment of HUVEC with α-fucosidase and that WGA inhibited the $OmpA^+$ *E. coli* invasion of fucosidase-treated HUVEC, suggesting that OmpA-reactive epitopes were available only after the removal of fucose residues on HUVEC glycoproteins. Taken together, these findings suggest that $OmpA^+$ *E. coli* invasion of BMEC, not systemic endothelial cells, may be due to greater availability of GlcNAcβ1-4GlcNAc epitopes of glycoproteins on BMEC than on systemic endothelial cells. A similar concept of specific interaction with BMEC compared with that of non-brain endothelial cells has been reported for *Bordetella pertussis* filamentous hemagglutinin (22). *B. pertussis* filamentous hemagglutinin was shown to interact with polypeptides present only on cerebral microvessel extracts and not on aortic endothelial cells.

The importance of the GlcNAcβ1-4GlcNAc epitope was also demonstrated by our finding that chitooligomers and chitotriose-bound OmpA proteins inhibited the invasion of *E. coli* into BMEC. A biological relevance of the OmpA and Glc NAcβ1-4GlcNAc interaction was further strengthened by the demonstration that chitooligomers blocked the entry of $OmpA^+$ *E. coli* into CSF in the newborn rat model of experimental hematogenous meningitis. As shown previously (7), this animal model has several important similarities to the pathogenesis of human *E. coli* meningitis, such as the hematogenous infection of the meninges without the need for direct inoculation of bacteria into the CSF. We have previously shown that the degree of bacteremia is a primary determinant for meningeal invasion by *E. coli* K1 (7). The magnitude of bacteremia between the two groups of animals receiving $OmpA^+$ *E. coli* with or without chitooligomers was similar, indicating that the inability of *E. coli* to enter the CSF in the animals receiving chitooligomers did not reflect decreased bacterial viability in the bloodstream and that chitooligomers indeed interfered with bacterial entry into the central nervous system.

In summary, we have demonstrated that *E. coli* OmpA interacts with GlcNAcβ1-4GlcNAc epitopes of BMEC glycopro-

teins and that bacterial entry into the central nervous system is blocked by chitooligomers in the newborn rat model of hematogenous meningitis. Further investigations of BMEC receptors for OmpA and other critical microbial determinants involved in invasion may provide important insights into the pathogenesis of *E. coli* meningitis and may prove useful for developing novel strategies for prevention of this devastating disease.

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REFERENCES

1. **Belloni, P. N., and G. L. Nicolson.** 1988. Differential expression of cell surface glycoproteins on various organ-derived microvascular endothelia and endothelial cell cultures. J. Cell. Physiol. **136:**398–410.
2. **Bhavanandan, V. P., and A. Katlic.** 1979. The interaction of wheat germ agglutinin with sialoglycoproteins: the role of sialic acid. J. Biol. Chem. **254:**4000–4008.
3. **Bremer, E., T. J. Silhavy, M. Maldener, and S. T. Cole.** 1986. Isolation and characterization of mutants deleted for the *sulA-ompA* region of the *E. coli* K12 chromosome. FEMS Microbiol. Lett. **33:**173–178.
4. **Cook, J. A., and J. B. Mitchell.** 1989. Viability measurements in mammalian cell systems. Anal. Biochem. **179:**1–7.
5. **Cummings, R. D., and S. Kornfeld.** 1982. Fractionation of Asn-linked oligosaccharides by serial lectin-agarose affinity chromatography. J. Biol. Chem. **257:**11235–11240.
6. **Gladstone, I. M., R. A. Ehrenkranz, S. C. Edberg, and R. S. Baltimore.** 1990. A ten-year review of neonatal sepsis and comparison with previous fifty-year experience. Pediatr. Infect. Dis. J. **9:**819–825.
7. **Kim, K. S., H. Itabashi, P. Gemski, J. Sadoff, R. L. Warren, and A. S. Cross.** 1992. The K1 capsule is the critical determinant in the development of *E. coli* meningitis in the rat. J. Clin. Invest. **90:**897–905.
8. **Klugman, K. P., E. C. Gotschlich, and M. S. Blake.** 1989. Sequence of structural gene (rmpM) for the class 4 outer membrane protein of *Neisseria meningitidis*. Infect. Immun. **57:**2066–2071.
9. **Kupsch, E. M., B. Knepper, T. Kuroli, I. Hener, and T. F. Meyer.** 1993. Variable opacity (Opa) outer membrane proteins account for the cell tropism displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. EMBO J. **12:**641–650.
10. **Mirelman, D., and J. Ofek.** 1986. Introduction to microbial lectins and agglutinins, p. 1–19. *In* D. Mirelman (ed.), Microbial lectins and agglutinins, properties and biological activity. J. Wiley & Sons, New York.
11. **Moch, T., H. Hoschutzky, J. Hacker, K. D. Krocke, and K. Jann.** 1987. Isolation and characterization of α-sialyl-β-galactosyl-specific adhesin from fimbriated *E. coli*. Proc. Natl. Acad. Sci. USA **84:**3462–3466.
12. **Nag, S.** 1985. Ultrastructural localization of lectin receptors on cerebral endothelium. Acta Neuropathol. **66:**105–110.
13. **Parkkinen, J., T. K. Korhonen, A. Pere, J. Hacker, and S. Soinila.** 1988. Binding sites of the rat brain for *E. coli* S-fimbriae associated with neonatal meningitis. J. Clin. Invest. **81:**860–865.
14. **Prasadarao, N. V., M. F. Stins, C. A. Wass, and K. S. Kim.** 1994. Role of S-fimbriae and outer membrane protein in *E. coli* binding to and invasion of endothelial cells, abstr. 406. FASEB J. **8:**A70.
15. **Prasadarao, N. V., C. A. Wass, J. Hacker, K. Jann, and K. S. Kim.** 1993. Adhesion of S-fimbriated *E. coli* to brain glycolipids mediated by sfaA gene-encoded protein of S-fimbriae. J. Biol. Chem. **268:**10356–10363.
16. **Prasadarao, N. V., C. A. Wass, J. N. Weiser, M. F. Stins, S.-H. Huang, and K. S. Kim.** 1996. Outer membrane protein A of *Escherichia coli* contributes to invasion of brain microvascular endothelial cells. Infect. Immun. **64:**146–153.
17. **Simionescu, M., N. Simiocescu, and G. E. Palade.** 1982. Differential microdomains on the luminal surfaces of capillary endothelium: distribution of lectin receptors. J. Cell Biol. **94:**406–413.
18. **St. Geme, J. W., III.** 1994. The HMW 1 adhesin of nontypeable *Haemophilus influenzae* recognizes sialylated glycoprotein receptors on cultured human epithelial cells. Infect. Immun. **62:**3881–3889.
19. **Stins, M. F., N. V. Prasadarao, L. V. Ibric, C. A. Wass, P. M. Luckett and K. S. Kim.** 1994. Binding of S-fimbriated *E. coli* to brain microvascular endothelial cells. Am. J. Pathol. **145:**1228–1236.
20. **Stins, M. F., N. V. Prasadarao, and K. S. Kim.** 1994. Development of a human brain microvascular endothelial cell line with blood-brain barrier characteristics: transfection with SV-40 large T. Mol. Biol. Cell (Suppl.) **5:**245a. (Abstract.)
21. **Tang, P., V. Foubister, M. G. Pucciarelli, and B. B. Finlay.** 1993. Methods to study bacterial invasion. J. Microbiol. Methods **18:**227–240.
22. **Tuomanen, E. L., S. M. Prasad, J. S. George, A. M. Hoepelman, P. Isben, I. Heron, and R. M. Starzyk.** 1993. Reversible opening of the blood-brain barrier by anti-bacterial antibodies. Proc. Natl. Acad. Sci. USA **90:**7824–7828.
23. **Unhanand, M., M. M. Mustafa, G. H. McCracken, and J. D. Nelson.** 1993. Gram-negative enteric bacillary meningitis: a twenty-year experience. J. Pediatr. **122:**15–21.
24. **Wang, J. R., and M. W. Stinson.** 1994. Streptococcal M6 proteins binds to fucose containing glycoproteins on cultured human epithelial cells. Infect. Immun. **62:**1268–1274.
25. **Wang, W. C., and R. D. Cummings.** 1988. The immobilized leukoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex-type Asn linked oligosaccharides containing terminal sialic acid-linked α-2,3 to penultimate galactose residues. J. Biol. Chem. **263:**4576–4585.
26. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:**2252–2258.
27. **Yamamoto, K., T. Tsuji, T. Irimura, and T. Osawa.** 1981. The structure of carbohydrate unit B of porcine thyroglobulin. Biochem. J. **195:**265–275.

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