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A Tissue Culture Bilayer Model To Study the Passage of *Neisseria meningitidis*

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A tissue culture bilayer system has been developed as a model to study the mechanisms of attachment and invasion involved in the pathogenesis of *Neisseria meningitidis*. The model incorporates epithelial and endothelial cell layers separated by a microporous membrane and makes it possible to observe and quantify the passage of bacteria through the multiple layers and to study the mechanisms by which they make this passage. This model is adaptable to a wide variety of microbial pathogens and can be modified by substituting any physiologically relevant eucaryotic cells for the component layers. The system's makeup of cells of human origin and its reproducibility give it advantages over animal and primary organ culture models, while the added complexity of multiple layers allowing cell-to-cell communication makes it a more realistic human tissue model than standard cell monolayers.

Meningococcal meningitis continues to be a serious health problem worldwide. The etiologic agent, *Neisseria meningitidis*, has caused recent epidemics in South America, Africa, and the Middle East and remains a principal cause of morbidity and mortality in young children in developing countries where the disease is endemic (2). It is expected that global dissemination of outbreak-associated strains will become even more common as international travel increases (18). Pharyngeal carriage of *N. meningitidis* is common, but the mechanism by which the organism penetrates the mucosal surface and enters the bloodstream is still largely unknown. A number of animal models have been used to study the many facets of meningococcal pathogenesis; these models include monkeys (8), chicken embryos (5), mice (10, 12), neonatal mice (15), genetically variant mice deficient in lipopolysaccharide response (32), and guinea pigs with subcutaneously implanted chambers (3). While these models have been useful in answering many experimental questions, the fact that *N. meningitidis* is an exclusively human pathogen limits the relevance of animal models in the study of its pathogenesis.

The nasopharyngeal organ culture system developed by Stephens et al. (22) permits study of the interaction between bacteria and the intact mucosal surface as it would occur in a natural infection. However, these tissues are difficult to obtain, are variable from donor to donor, require the initial use of antibiotics, and have limited viability. Human buccal epithelial cells have been used by many researchers to study attachment of meningococci (16, 20); these cells are readily available but vary greatly in age, size, and viability. Mammalian tissue culture monolayers have also been used extensively in the study of neisserial pathogenesis. These studies have employed HeLa human cervical carcinoma cells (4, 14), HecIB human endometrial carcinoma cells (19), Chang conjunctival cells (27), HEp-2 larynx carcinoma cells (30), and human umbilical vein endothelial cells (28). Monolayer studies have added much to current knowledge about meningococcal attachment and invasion. However, when infecting the human host, the bacterium is required to react with more than a monolayer of cells. A more relevant model might well incorporate the added complexity of the cell-to-cell interaction associated with multiple layers.

In this study we have developed an artificial tissue system incorporating epithelial and endothelial monolayers on a microporous membrane as an approach to examine the process of attachment and passage that must occur as the bacterium makes its way from the mucosal surface through the epithelial cells and into the vascular system. The layering of one cell type over another allows the cells to communicate and interact as they might in vivo. We examined attachment and invasion by meningococci by using several epithelial cell monolayers, including HecIB, HEp-2, and HeLa cells. While the bacteria were found to invade all cell types, larger numbers of bacteria were found to attach to and invade the HecIB cells. On the basis of these preliminary data, we chose to use HecIB cells, an endometrial carcinoma cell line frequently used to study gonococcal attachment and invasion, as the epithelial layer. We chose HMEC-1 cells, a human microvascular endothelial cell line, as the second layer in our model. Using this system, we have examined a variety of meningococcal strains, including epidemic and sporadic case and carrier isolates, strains with and without pili or capsule, and other spontaneous and transposon-induced mutants. We have found marked differences among the strains, in terms of their abilities to pass through the bilayer, that may correlate with differences in virulence. We have also seen microscopic evidence that the bacteria are passing from the apical surface through the layer of epithelial cells and through the membrane to the endothelial layer below without causing damage to the epithelial cells. This resembles what one observes in the human host, where extensive tissue damage in the nasopharynx is only rarely reported. We have also examined several other genera of bacteria whose interaction with eucaryotic cells has been well characterized; bilayer assay results for strains of *Haemophilus influenzae* type b, *Salmonella typhimurium*, *Shigella flexneri*, and *Yersinia enterocolitica* showed differences but were consistent with previously published results on invasion and attachment. On the basis of these preliminary findings, the bilayer system appears to be a

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useful model for the study of the attachment and invasion factors contributing to meningococcal pathogenesis and will lend itself to similar studies with other microbial pathogens.

MATERIALS AND METHODS

Bacterial strains. Strains of *N. meningitidis* obtained from patients with clinical disease were designated case strains, and strains from asymptomatic carriers were designated carrier strains. Serogroup A strains were from an outbreak in Kenya; these were case strains F8187, F8188, and F8229 and carrier strains F8239, F8240, and F8243; and all were characterized as belonging to the III-1 clonal group of serogroup A by multilocus enzyme electrophoresis (12a). Serogroup B case strain NMB was from Pennsylvania (25), and M7 was a Tn*916*-induced mutant of NMB which produced no group B capsular polysaccharide (25). Code 2 was a serogroup B case strain from Georgia. FAM 18+ and FAM 18− were, respectively, piliated and nonpiliated serogroup C strains (originally obtained from Janne Cannon, University of North Carolina, Chapel Hill, and provided by David Stephens, Emory University, Atlanta, Ga.). Strains B92-2177, CI-416b, G1161, G7026, and G8052 were serogroup C case strains from the United States and Canada. G2881, G2938, R251, and B534 were nongroupable (with standard typing antisera) meningococcal carrier strains from the United States. *H. influenzae* type b (KC 1050), *S. typhimurium* (B2247), *S. flexneri* (3015-94), and *Y. enterocolitica* (3468-85) were patient isolates from the culture collections of the Division of Bacterial and Mycotic Diseases, Centers for Disease Control and Prevention. All strains were stored in Luria-Bertani broth with 20% glycerol at −70°C. Meningococcal strains were grown on chocolate agar plates (BBL, Cockeysville, Md.) at 37°C in 5% CO_2; other strains were grown on heart infusion agar with 5% rabbit blood (BBL) at 37°C.

Bilayer construction and infection. A Transwell-COL insert (Costar, Cambridge, Mass.) with 3.0-μm pores was placed in each well of a six-well tissue culture cluster plate. HMEC-1 endothelial cells (10^5 cells per ml) were suspended in endothelial basal medium (Gibco, Grand Island, N.Y.) with 7% fetal bovine serum, and 3-ml aliquots were added to each upper chamber (above the membrane). Endothelial basal medium plus 7% fetal bovine serum (without cells) was added to the lower chamber (beneath the Transwell insert) in a quantity sufficient to completely cover the membrane. The cells were incubated at 37°C in 5% CO_2 for 8 days, allowing formation of a continuous monolayer. The cell suspension and the medium in the lower chamber were removed, and a similar suspension of HecIB epithelial cells was added to the upper chamber. Fresh tissue culture medium was added to the lower chamber. At 7-day intervals all medium was removed from both chambers and replaced with fresh medium. As demonstrated by histological analysis, the bilayer was completely formed and ready for use after 15 to 20 days of incubation of the epithelial cell layer. Fluorescence microscopy was used to determine the orientations of the cell layers in relation to the membrane. All cells were stained with Bodipy 581/591 phalloidin (Molecular Probes, Inc., Eugene, Oreg.), which stained the F actin of both cell layers. The entire filter was then stained with epithelial membrane antigen (DAKO, Carpinteria, Calif.) labelled with fluorescein isothiocyanate. Actin in all cells fluoresced red; the outer membrane of the epithelial cells fluoresced green.

Before infection of the bilayer, all medium was removed, the bilayer was washed once with phosphate-buffered saline, and endothelial basal medium with 15% human serum was added (1 ml each in the upper and lower chambers). In some experiments cytochalasin D (5 μg/ml) was added to the medium in both chambers 30 min prior to infection and maintained throughout the experiment. Trypan blue assays of cell viability in HecIB and HMEC monolayers showed minimal cell death after 21 h in the presence of the same amounts of cytochalasin D.

Bacteria were grown overnight on chocolate agar and suspended in tissue culture medium to an optical density at 600 nm of 0.5, or 10^8 to 10^9 bacteria per ml. This suspension was diluted to add approximately 10^3 bacteria to each upper chamber, and the dish was incubated at 37°C in 5% CO_2. At 15, 18, and 21 h after infection, medium was removed from each lower chamber and dilutions were spread on agar plates to determine the number of viable bacteria passing through the bilayer. The first assay, performed 15 h following infection, determined the number of bacteria that had been able to travel through both cell layers and the porous membrane in this time period. Fresh medium was then added to the lower chamber. The second assay, at 18 h, measured the number of bacteria emerging from the cell layers in the preceding 3 h; these bacteria may have travelled the entire distance in this time or may have been making their way through the tissue during most of the 18 h. Likewise, the number of viable organisms found at the third time point, 21 h after infection, showed the number of organisms emerging from the tissue during the final 3 h of the assay. Because the numbers at 21 h were more variable from strain to strain, they were the numbers routinely used to compare strains. In some strains virtually no bacteria were able to make their way through the cell layers until the 21-h time point. Samples of the upper-chamber contents were plated at selected time points to determine bacterial growth over the course of the experiment. Following the last assay, all medium was removed and the insert was fixed for 4 to 12 h in 10% neutral buffered formalin. The bilayer was then sectioned and processed for microscopic observation.

Histological processing. Each chamber was removed with forceps from the formalin and placed on its own 47-mm-diameter, 0.2-μm-pore-size Vericel (Gelman Sciences, Ann Arbor, Mich.) membrane filter dampened with distilled water. A sharp-pointed scalpel blade was used to cut the cell growth and its supporting Costar membrane away from the inside of the chamber, at the same time cutting all the way through the underlying Vericel membrane. Supported by the larger Vericel membrane, the small stack of cell growth and artificial membranes was compactly rolled up with forceps. The original upper side of the Costar membrane thus reliably corresponded to its concave surfaces once it was rolled up. Two 2-mm cross sections were cut from the middle of the roll with a sharp blade and placed on edge on a square piece of lens paper. The lens paper was then folded over the two membrane ensembles, and the wrapped specimens were placed in a perforated plastic cassette containing the appropriate pathology accession number. Cassettes were immersed in 70% ethanol for transfer. Specimens were processed for 16 h in a Fisher Histomatic (Fisher Scientific, Pittsburgh, Pa.) tissue processor, embedded in Polyfin (Triangle Biomedical Sciences, Durham, N.C.) embedding medium, and sectioned at 4 μm on a Leitz 1512 microtome. Blocks were trimmed deeply enough to compensate for any retraction of the cell layer from the edges of the membranes. Sections were floated on a 44°C water bath, collected on aminosilane-coated glass microscope slides (3 by 1 in. [ca. 7.5 by 2.5 cm]), warmed in a 65°C paraffin oven for 20 min, and stained with Harris' hematoxylin and eosin. For species other than *N. meningitidis*, Steiner silver staining was also done.

Processing for transmission electron microscopy. Transwells were prepared for transmission electron microscopy by a modification of the procedure of the manufacturer (Costar). Growth medium was removed, and membranes were fixed with 2% glutaraldehyde for 1 h at room temperature. Fixative was removed and replaced with collidine buffer. The specimen was either stored at 4°C for later processing or immediately postfixed with 1% osmium tetroxide for 45 min at 4°C. Osmium was removed and replaced with uranyl acetate. The specimen was held at 4°C overnight and was then dehydrated through a graded series of ethanol (EtOH) concentrations, i.e., 35%, 50%, 70%, 95%, 100%, and 100% EtOH each for 10 min. Specimens were then infiltrated with complete embedding resin (LR White medium grade) as follows: 75% EtOH and 25% resin for 60 min, 50% EtOH and 50% resin for 60 min, and 25% EtOH and 75% resin for 60 min. The membrane was then cut from the Transwell, placed in a small glass jar, and put in complete resin overnight (16 h) at room temperature. At 2 days prior to fixation and infiltration of the membrane, complete resin was poured into an aluminum dish to a depth of 2 to 3 mm and allowed to polymerize in a 60°C oven. The membrane was placed on top of the hardened resin, carefully unrolled, and opened as flat as possible. Additional resin was poured over the membrane to cover it. The preparation was placed in a 60°C oven and allowed to polymerize for 72 h. The aluminum dish was removed, and the area of the resin containing the membrane was cut with a jeweler's saw. The piece was trimmed, and thick sections made and examined. Standard transmission electron microscopy protocols were followed for the remaining steps.

RESULTS

When epithelial cells were added to the chambers after the endothelial monolayer was established on the top side of the membrane, the endothelial cells migrated through the 3-μm pores to the basal side of the membrane, where they remained. The artificial membrane appeared to function as a basement membrane for both the epithelium and the endothelium. As the epithelial cells grew, this layer became several cells thick, and, in some cases, the cells differentiated into a columnar or cuboidal form. Determination of bilayer integrity was based on microscopic observation (Fig. 1) and on the minimal passage of inert uncharged beads through the bilayer system. Approximately 10^7 colored polystyrene beads, 0.833 μm in diameter (Seradyn, Indianapolis, Ind.), were added to 1 ml of tissue culture medium in the upper chamber. After 3 h, the medium in the lower chamber was removed and centrifuged. Fewer than 0.01% of the beads were observed to have passed into the lower chamber, suggesting that no large gaps existed in the cell layers.

Results of bacterial infections of the bilayer described below are summarized in Table 1. Replication in the upper chamber over 21 h resulted in numbers ranging from 1.1×10^7 to 2.5×10^9. To factor in this variation in growth rate, we have calculated ratios of the number of organisms passing into the lower chamber in the last 3 h to the final number in the upper chamber (LC/UC ratio), which reflects the percentage of organisms in the upper chamber able to pass into the lower

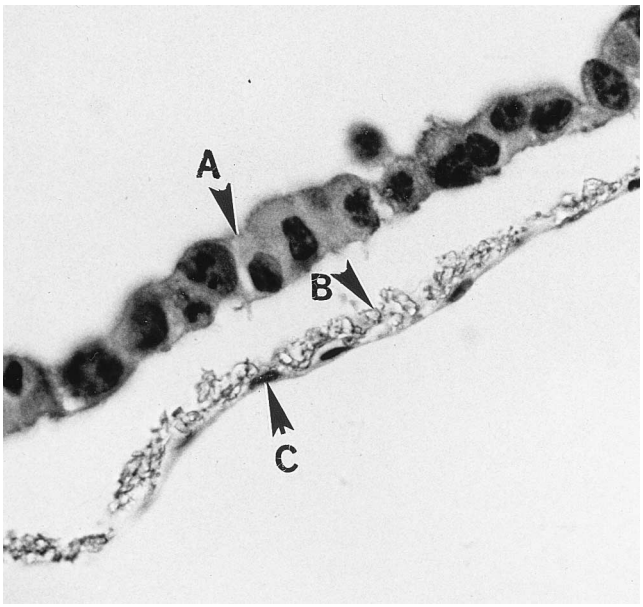

FIG. 1. Uninfected bilayer with columnar HecIB epithelial cells (A) above the microporous membrane (B) and thin, flat HMEC-1 cells (C) beneath the membrane. Magnification, ×400. HMEC cell suspensions were added to the Transwell chamber and allowed to grow for 8 days before being removed and replaced with HecIB cell suspensions; bilayers were used 17 to 20 days after HecIB addition. Bilayers were prepared for microscopic observation as described in Materials and Methods.

TABLE 1. Passage of *N. meningitidis* through tissue culture bilayer[a]

Strain	No. of bacteria[b] in:		LC/UC ratio (%)
	LC[c]	UC[d]	
Serogroup A			
F8187	$(2.5 \pm 0.2) \times 10^8$	$(1.5 \pm 0.4) \times 10^9$	16.7
F8188	$(9.6 \pm 0.5) \times 10^7$	$(2.0 \pm 0.3) \times 10^9$	4.8
F8229	$(1.0 \pm 0.4) \times 10^8$	$(1.1 \pm 0.5) \times 10^9$	9.1
F8239[e]	$(2.5 \pm 2.4) \times 10^4$	$(3.2 \pm 2.2) \times 10^7$	0.08
F8240[e]	$(2.0 \pm 1.1) \times 10^7$	$(8.8 \pm 0.3) \times 10^8$	2.3
F8243[e]	$(1.3 \pm 0.8) \times 10^8$	$(2.5 \pm 1.1) \times 10^9$	5.2
Serogroup B			
NMB	$(7.6 \pm 4.5) \times 10^6$	$(4.5 \pm 2.2) \times 10^8$	1.7
M7	$(2.6 \pm 2.5) \times 10^3$	$(1.1 \pm 1.2) \times 10^7$	0.02
Code 2	$(2.0 \pm 0.5) \times 10^8$	$(2.3 \pm 0.3) \times 10^8$	80.0
Serogroup C			
FAM 18+	$(2.3 \pm 1.3) \times 10^7$	$(4.0 \pm 1.1) \times 10^8$	5.7
FAM 18−	$(5.1 \pm 2.9) \times 10^7$	$(7.0 \pm 1.0) \times 10^8$	7.3
CI-416b	$(4.1 \pm 1.9) \times 10^5$	$(1.1 \pm 0.4) \times 10^9$	0.04
G7026	$(5.5 \pm 2.5) \times 10^7$	$(2.2 \pm 0.3) \times 10^9$	2.5
G8052	$(1.7 \pm 0.2) \times 10^5$	$(4.5 \pm 1.1) \times 10^8$	0.04
G1161	$(3.0 \pm 0.1) \times 10^5$	$(6.0 \pm 0.5) \times 10^8$	0.05
B92-2177	$(2.4 \pm 2.3) \times 10^6$	$(4.0 \pm 1.1) \times 10^8$	0.6
Nongroupable			
G2881[e]	$(6.9 \pm 3.1) \times 10^2$	$(4.6 \pm 2.7) \times 10^8$	<0.01
G2938[e]	$(1.7 \pm 1.6) \times 10^5$	$(2.0 \pm 2.0) \times 10^9$	0.01
B534[e]	$(6.8 \pm 6.0) \times 10^3$	$(1.0 \pm 1.2) \times 10^8$	<0.01
R251[e]	$(1.7 \pm 1.6) \times 10^5$	$(2.5 \pm 0.5) \times 10^9$	<0.01

[a] LC, lower chamber; UC, upper chamber.
[b] Numbers represent the means of at least three independent assays ± standard errors.
[c] Number of bacteria reaching LC between 18 and 21 h postinfection.
[d] Number of bacteria 21 h after infection with $\sim 10^3$ bacteria.
[e] Carriage strains; others (with the exception of mutant M7) are case strains.

chamber during this 3-h time period. This ratio is a measure of invasiveness in this model and allows comparison and grouping of strains based on these numbers. Among the serogroup A strains tested, numbers of viable organisms in the lower chamber at 21 h ranged from 2×10^7 to 2.5×10^8, with ratios of 2.3 to 16.7%. There appeared to be no significant difference between case and carrier strains in this group. The exception was a single carrier strain, F8239, with an average viable count of 2.5×10^4 and a ratio of 0.08%. Like the serogroup A strains, serogroup B case strain Code 2 showed viable counts of above 10^8 in the lower chamber at the 21-h time point, a ratio of 80%. The counts for NMB, a case isolate multiply passaged on artificial media, averaged 7.6×10^6 (ratio, 1.7%), while those for the transposon-induced mutant of strain NMB, M7, were considerably lower at 2.6×10^3 (0.02%). Within serogroup C, the piliated and nonpiliated variants of FAM 18 gave similar numbers, i.e., greater than 10^7 in the lower chamber with ratios of 5.7 and 7.3%. Numbers for other serogroup C case isolates ranged from 1.7×10^5 to 5.5×10^7, or ratios of 0.04 to 2.5%. Among the nongroupable strains tested numbers ranged from 6.9×10^2 to 1.7×10^5, giving ratios of 0.01% or less; thus, results for these strains were generally lower than those for any of the groupable strains but as a group were not statistically different.

A time course experiment in which bilayer chambers were removed for microscopic observation at 2, 12, 15, 18, and 22 h after infection showed that meningococci migrated from the apical surface down through and between the epithelial cells to the basal surface. At 12 h after infection, the bacteria were seen in contact with the upper surface of the epithelial cells (Fig. 2A). Electron microscopic observation suggests that this attachment is via actin pedestals (Fig. 3). At points along the epithelial surface, meningococci began to invade between adjacent cells, either moving in to fill an existing indentation or forcing a break in the cellular tight junction (Fig. 2). After 15 h, extracellular bacteria were often observed extending in vertical columns entirely through several layers of separated cells (Fig. 2B and 4). By electron microscopy bacteria were seen between cells, disrupting the integrity of the host cell tight junctions (Fig. 5). In some cases, where the epithelium had become stratified, the bacteria travelled in a lateral direction, spreading between the stacked layers of epithelial cells (Fig. 6). In addition, throughout the time course experiment meningococci were seen internalized by the host epithelial cells and appeared to be within vacuoles, perhaps moving through the cell towards the basal surface (Fig. 3 and 4). Meningococci emerged from the epithelial cell layer into the space below between 15 and 22 h following infection (Fig. 7A). Having passed through the epithelial layer by either the intracellular or intercellular route, the bacteria were then able to pass through the porous membrane and into and through the endothelial layer beneath, probably via the same mechanisms, finally emerging in large numbers on the basal surface (Fig. 7B). These figures illustrate typical results; the experiments were done with several strains, and there were no notable differences between strains. Viable count data suggest that the majority of bacteria travelled within the cells of the epithelial layer, since in the presence of cytochalasin D, an inhibitor of host cell phagocytosis, the numbers of bacteria passing through the bilayer were reduced by 95 to 99% (data not shown).

To compare the passage of meningococci through the bilayer system with those of other bacteria whose attachment and invasion mechanisms have been well characterized, we used the same protocol to examine passage of *H. influenzae* type b, *S. typhimurium*, *S. flexneri*, and *Y. enterocolitica*. The results are summarized in Table 2. The numbers of organisms reaching the lower chamber between 18 and 21 h following

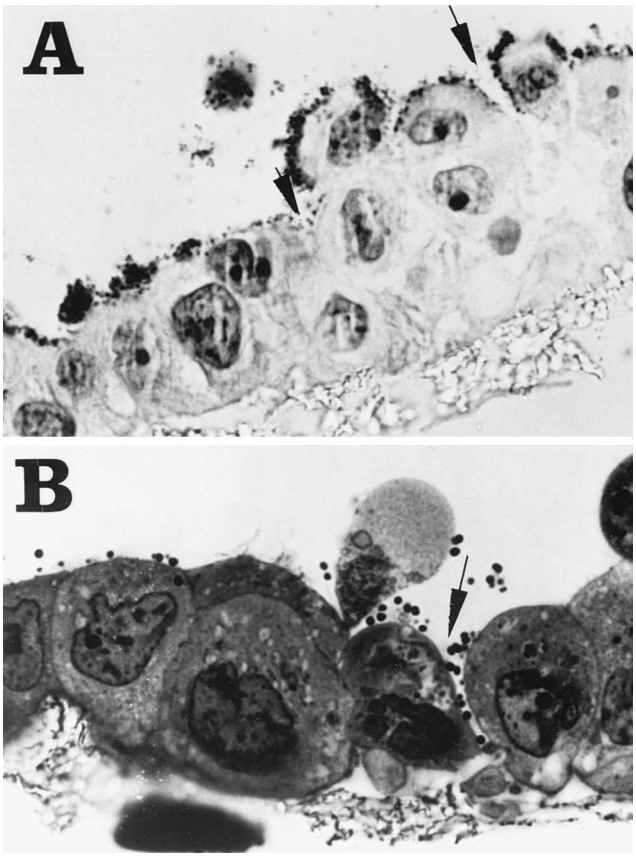

FIG. 2. (A) *N. meningitidis* NMB attached to the epithelial cell surface 12 h after infection with 10^3 bacteria; a few bacteria are seen beginning to make their way between adjacent cells (arrows). Magnification, ×1,000. (B) Meningococcal cells (strain F8229) moving through tight junction between adjacent HecIB epithelial cells (arrow) 21 h after infection. Magnification, ×3,000.

infection varied from 5×10^3 for *Y. enterocolitica* to 1.5×10^8 for *S. typhimurium*, with numbers of *H. influenzae* and *S. flexneri* falling between these two values.

DISCUSSION

The bilayer model system has several advantages over the models currently being used to study meningococcal pathogenesis. As opposed to the simple monolayer systems, the bilayer system offers the added complexity of multiple layers which more closely resemble the host tissues through which the invading meningococci must pass. The system is certainly easier to obtain and to use than any of the animal models and, by making use of human cells, is much more relevant to the pathogenesis of an exclusively human disease. The model is consistently reproducible without the patient-to-patient variability inherent in systems such as human buccal cells and nasopharyngeal organ cultures. It can also be maintained without the use of antibiotics and remains both viable and usable for a longer period of time. The infectious process can be studied with a much lower inoculum in this system than in the organ culture system (10^3 versus 10^6 diplococci).

Perhaps a more important point is the modeling of cell-to-cell communication between and among epithelial and endothelial cells that occurs in vivo. Some cellular communication occurs in the bilayer model as evidenced by the fact that when epithelial cells are added to the system after the endothelial monolayer is established on the apical side of the membrane, the endothelial cells migrate through the 3-μm pores to the basal side of the membrane, where they reestablish a monolayer. Once the epithelial cell layer is confluent, it continues to grow, eventually becoming several cells thick, and in some cases, the cells differentiate into a columnar form. The central membrane appears to function as a basement membrane for both the epithelium and the endothelium.

While monolayers are useful for studying attachment and invasion, the bilayer adds the opportunity to look at the passage from the apical surface through both cell layers and the final emergence into the subendothelial space. The only other model system with which one could potentially follow such passage is the human nasopharyngeal organ culture model. Stephens et al. have seen large numbers of meningococci in phagocytic vacuoles in the apical portion of these epithelial cells, but they did not observe the vacuoles to traverse these cells or pass through the basement membrane into the subepithelial tissues (22). They did, however, observe meningococci in the subepithelial space 18 to 24 h after infection, suggesting that the meningococci had crossed the mucosal surface. Microscopic observation of the bilayer model shows passage of meningococci not only through the epithelial cells and the basement membrane but also through the underlying endothelium. In addition, the number of meningococci passing through the cells to the subendothelial space can be quantified in the bilayer system.

The inoculation of a single bilayer chamber or of several identical chambers allows the study of a number of virulence characteristics, including attachment, invasion, transcytosis, and exit or extracellular passage through tight junctions. One can also examine the effect of various inhibitors of bacterial or host cell function or of antibodies on each of these aspects of the infectious process. Thus, it is possible to study the effect of inhibitors of host cell microfilament and microtubule function, pinocytosis, and protein synthesis and to study exactly how the inhibitors interfere with the infectious process. For example, the addition of cytochalasin D prior to infection significantly reduced the numbers of organisms passing through the bilayer, indicating that host cell function is important in meningococcal invasion and transcytosis.

An additional asset of this bilayer system is its potential adaptability for the study of a wide variety of organisms. We have examined several epithelial cell lines as the second layer in this system, including Chang conjunctival cells, which have been used to show differences between a virulent and an avirulent strain of *H. influenzae* biogroup aegyptius (13a). For any organism to be examined, it is possible to construct a bilayer by using physiologically relevant epithelial and endothelial cell lines. The endothelial layer might be replaced with another vascular line, such as human umbilical vein cells, perhaps more relevant to the pathogenesis of the organism. It may also be possible to introduce immunological factors (e.g., antibody or phagocytic cells) into the system to examine the infectious process in an environment even more closely resembling what the organism encounters in vivo.

A number of bacterial factors have been examined to determine their importance in meningococcal pathogenesis. One of the most studied has been the role of pili in the attachment of *N. meningitidis* to human epithelial and endothelial cells (16, 26). Many consider pili the most important mediator of the attachment which must occur before invasion can take place (21). However, as Stephens and McGee (23) pointed out in an earlier study, the extreme difference in pathogenicity between commensal neisseriae and meningococci suggests virulence mechanisms other than pili. In our model we have found that

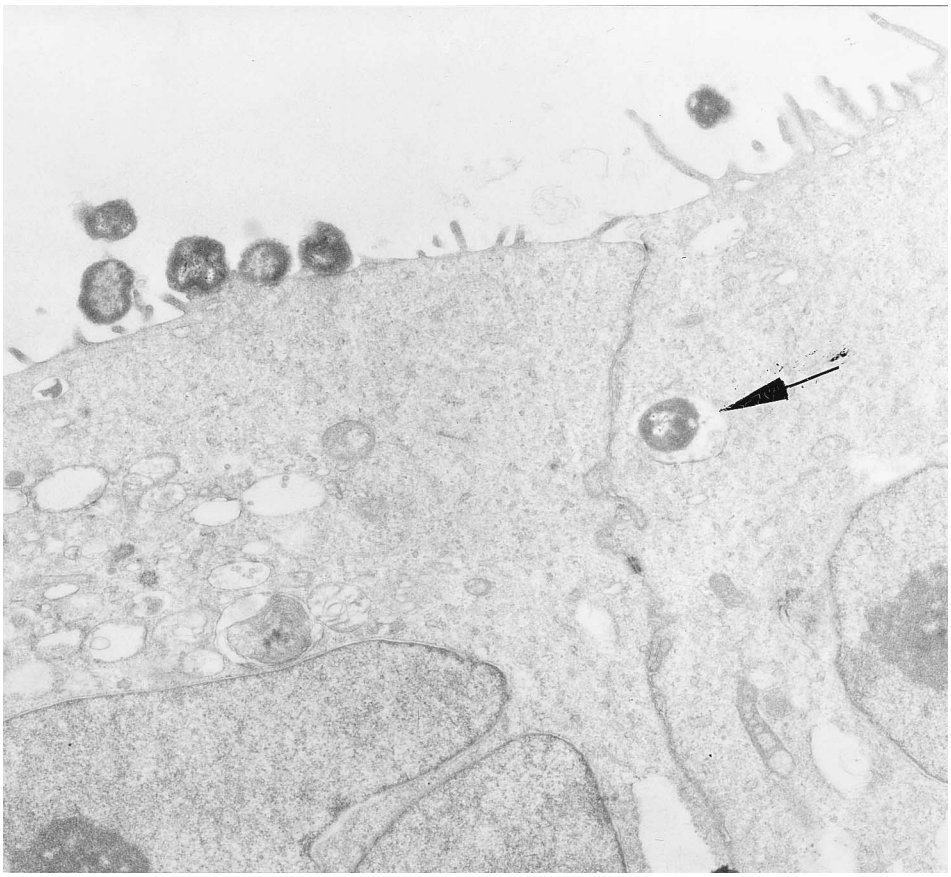

FIG. 3. Meningococci (strain F8229) attached to epithelial cell surface, possibly on actin pedestals; also visible is single meningococcal cell enclosed in vacuole (arrow). Magnification, ×10,500.

the serogroup C strain FAM 18+ and its nonpiliated variant pass through the bilayer system in almost equal numbers. We have also found that the serogroup A strain F8239 is the only strain other than piliated FAM 18+ that is consistently positive in a hemadsorption assay, a characteristic that correlates with the expression of pili on the meningococcal surface (13). F8239, however, passes through the bilayer in much lower numbers than any of the other serogroup A strains. In addition to pili, data suggest that the class 5 outer membrane proteins are also involved in attachment (1, 31). The strains studied here have not yet been examined for the presence of class 5 proteins. We are in the process of obtaining the necessary monoclonal antibodies to make these determinations.

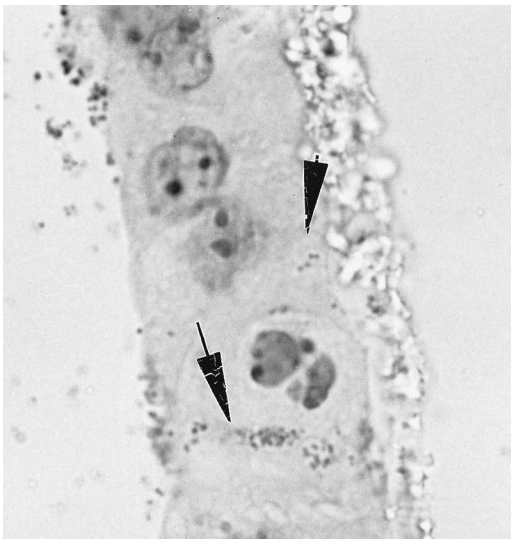

FIG. 4. Column of meningococci (strain NMB) travelling from apical to basal surface of epithelial cells (lower arrow); several meningococci are enclosed in a vacuole near the basal surface (upper arrow). Magnification, ×1,000.

While the initial cell attachment is necessary, it is not sufficient to cause disease (16); other factors must play the major role in invasion and transcytosis. One of the factors which plays a significant role in meningococcal virulence is the polysaccharide capsule. While encapsulation appears to be necessary for survival in the bloodstream, more-efficient attachment to epithelial and endothelial cells may occur with strains deficient in capsule; the capsule may mask pilus-binding sites and interfere with attachment (11, 23, 24, 28, 29). Our unpublished monolayer studies using human microvascular endothelial cells (HMEC-1) likewise showed decreased invasion by the capsulate strains compared with those known to be capsule deficient.

However, in the bilayer system there was a marked decrease in the number of bacteria passing through and into the lower chamber when the bacteria were capsule deficient or altered in capsule production. The correlation between invasiveness, as measured by passage through the bilayer, and the presence of capsule can be seen in Table 1. Serogroup A strain F8239, which does not agglutinate in the presence of group A capsular antisera, and the transposon-induced mutant of NMB (M7),

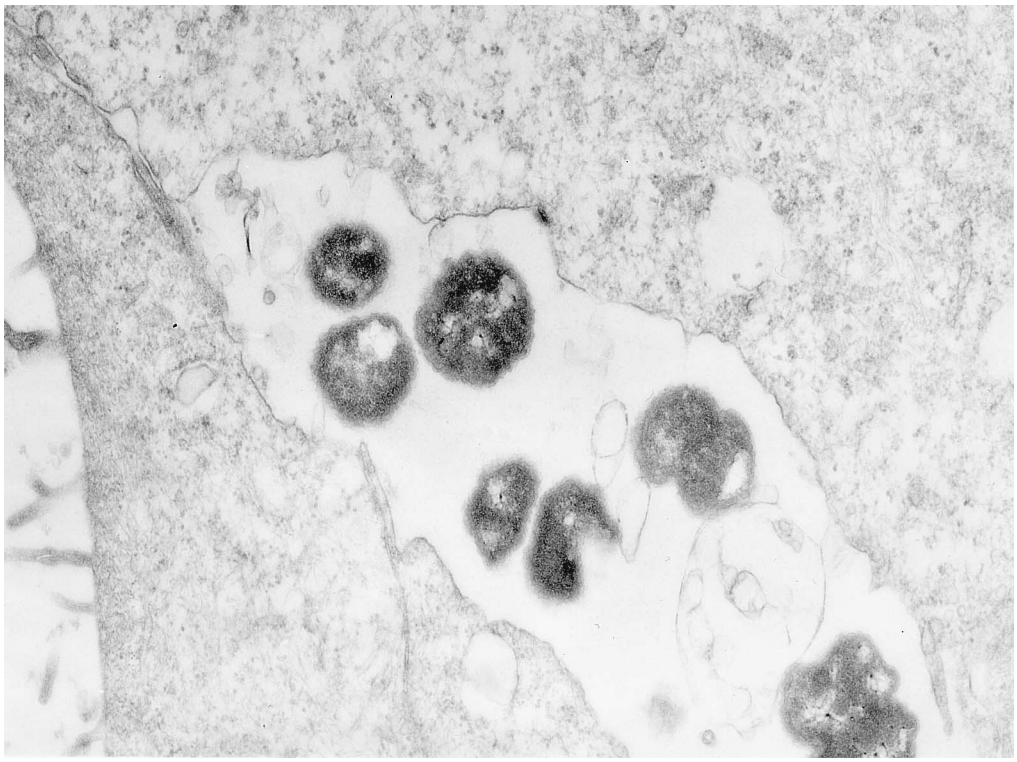

FIG. 5. Meningococci (strain F8229) moving between two epithelial cells disrupting a cellular tight junction. Magnification, ×19,000.

which does not produce group B capsular polysaccharide, have LC/UC ratios of less than 0.1, as do the four nongroupable isolates. In terms of their invasiveness, these six strains are either much less than or equal to the least invasive of the remainder of the strains, which are all groupable and therefore encapsulated.

As Falkow pointed out, many, if not all, invasive microbes encode several separate pathways for entry into cultured cells (6). When we added cytochalasin D, an inhibitor of host phagocytosis, to the system, we found that many fewer organisms were able to pass through the bilayer. This would support the previous epithelial and endothelial monolayer studies of Virji et al. (31) and could suggest that the intracellular route may be the primary route of passage. However, we also saw microscopic evidence of long columns of meningococci that appeared to be intercellular rather than within vacuoles. In electron micrographs we saw many more meningococci between cells than within them, and these intercellular organisms appeared to be, at least temporarily, disrupting the cellular tight junctions. Stephens and Farley (21) and Virji et al. (28) found that infection with meningococci produced cytotoxic effects after infection characterized by breakdown of epithelial cell tight junctions. Thus, this intercellular pathway, used primarily by the encapsulated bacteria, may be the alternate route taken by the meningococci. Although this route has been observed in *H. influenzae* type b passage through the nasopharyngeal organ culture (21), it has never been described in previous models for *N. meningitidis*. Cytochalasin D, which acts on host microfilaments, may interfere with contraction of the meshwork of microfilaments which regulate tension at the intercellular epithelial surface (5a), thus limiting bacterial passage. It is unclear at this time precisely what role the microfilaments are playing in passage of the meningococci through the bilayer. Experiments are currently under way to elucidate these mechanisms.

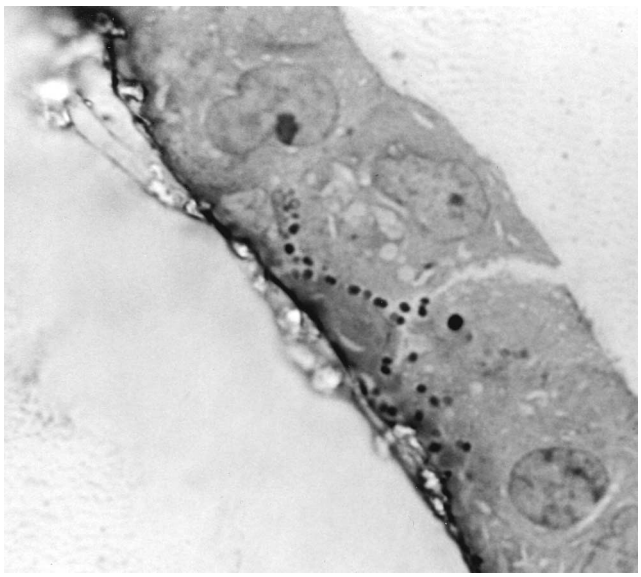

FIG. 6. Meningococcal cells (strain F8229) spreading laterally between cells of epithelial layer. Magnification, ×3,000.

An additional cytotoxic effect of the bacterial infection may be the deterioration of the endothelial layer observed microscopically 21 h after addition of the meningococci. With Huvec monolayers, Virji et al. also saw disruption of intercellular junctions, changes in normal cell morphology, and some loss of cells from the monolayers (28). This damage is consistent with

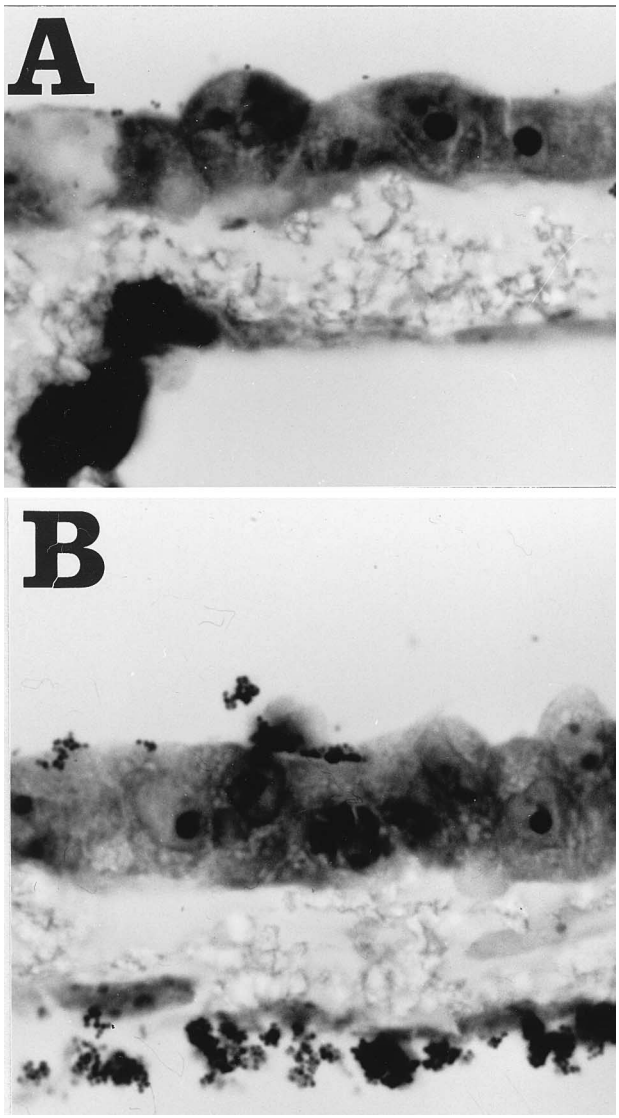

FIG. 7. (A) Meningococci (strain F8239) attached to basal surface of epithelial cells and in subepithelial space. (B) Large numbers of meningococci (F8239) attached to basal surface of endothelial cells. Magnification, ×1,000.

the extensive damage to the endothelial lining of the blood vessels seen in autopsy material from cases of human disease (9).

In a further effort to understand the mechanisms involved in meningococcal passage and, ultimately, those used by all invasive bacteria, we have looked at the passage through the bilayer model of several other organisms whose attachment and invasion mechanisms have been well characterized. As stated previously, *H. influenzae* type b is known to invade the epithelium by passing through the intercellular tight junctions. We saw rapid passage through the bilayer, with 3×10^7 organisms reaching the lower chamber in the first 15 h after *H. influenzae* type b infection. The invasive enteric pathogens *S. typhimurium* and *Y. enterocolitica* are phagocytized by eucaryotic cells and remain trapped in the phagocytic vacuole, while *S. flexneri* enters the cell and subsequently lyses the phagocytic vacuole to gain access to the host cell cytoplasm (17). Using polarized MDCK monolayers, Finlay et al. found that *Salmonella choleraesuis* cells transcytose the monolayer by 4 h after infection

TABLE 2. Passage of various bacteria through tissue culture bilayer[a]

Species	No. of bacteria in:		LC/UC ratio (%)
	LC[b]	UC[c]	
H. influenzae type b	9.4×10^7	2.5×10^9	3.80
S. typhimurium	1.5×10^8	9.5×10^8	15.80
S. flexneri	8.0×10^5	3.5×10^8	0.23
Y. enterocolitica	5.0×10^3	9.0×10^7	<0.01

[a] LC, lower chamber; UC, upper chamber.
[b] Number of bacteria reaching LC between 18 and 21 h postinfection.
[c] Each chamber was infected with $\sim 10^3$ bacteria.

and reach a maximal rate of 14 bacteria per MDCK cell per h after 9 h; *S. typhimurium* cells behave nearly identically (7). Although these studies examined only monolayers, we saw similar numbers emerging from the bilayer after infection with *S. typhimurium*, with an average of 2.5×10^6 at the 15-h time point. Intracellular trafficking of *Yersinia* and *Shigella* cells may be different from that of *Salmonella* cells, since far fewer organisms make their way through the bilayer in the first 15 h.

Using the bilayer system, one can see quantitative differences in passage which may be indicative of different mechanisms of transport across cell layers. Microscopic observation of the infected bilayers offers yet another perspective for more-detailed study and comparison of these mechanisms. Similarities in bilayer assay results between well-characterized bacterial species and meningococcal strains allow one to speculate on possible mechanisms of meningococcal passage and to design further studies to determine precise trafficking routes and eventually the responsible bacterial proteins and the genes which encode them. Also, by examining mutants and epidemic and sporadic case and carrier strains as they pass through the bilayer, it may be possible to determine which genes are turned on or off in response to changes in the environment and changes in the requirements for bacterial survival.

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