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Interaction of *Neisseria meningitidis* with a Polarized Monolayer of Epithelial Cells

CÉLINE PUJOL,[1] EMMANUEL EUGÈNE,[1] LUC DE SAINT MARTIN,[2] AND XAVIER NASSIF[1]*

Laboratoire de Microbiologie, INSERM U411, Faculté de Médecine Necker-Enfants Malades, 75730 Paris cedex 15,[1] *and CHU de la Cavale Blanche, 29609 Brest cedex,*[2] *France*

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An important step in the pathogenesis of *Neisseria meningitidis* is the crossing of two cellular barriers, one in the nasopharynx and one in the brain. To approach the mechanisms by which this bacterium can achieve these goals, we studied the interactions between *N. meningitidis* and a monolayer of polarized tight junction-forming T84 cells grown on filter units. A capsulated, piliated, Opa$^-$, and Opc$^-$ *N. meningitidis* strain is shown to be capable of adhering to and crossing this monolayer several orders of magnitude more efficiently than an isogenic nonpiliated derivative. This bacterial interaction does not affect the barrier function of tight junctions, as assessed by (i) the absence of modification of the transepithelial resistance, (ii) the lack of increase of [^{3}H]inulin penetration across the monolayer, and (iii) the absence of delocalization of ZO-1, a tight junction protein. Electron microscopy studies and confocal examinations demonstrated that *N. meningitidis* (i) induces cytoskeletal rearrangements with actin polymerization beneath adherent bacteria, (ii) is intimately attached to the apical membrane of the cells, and (iii) can be internalized inside cells. Immunofluorescent staining with antipilus antibodies showed evidence that meningococcal piliation was dramatically reduced at later time points of bacterial cell interaction compared to the early phase of this interaction. In addition, adhesive bacteria recovered from an infected monolayer are piliated, capsulated, Opa$^-$, and Opc$^-$, a phenotype similar to that of the parental strain. Taken together, these data demonstrate that following pilus-mediated adhesion, *N. meningitidis* is involved in an intimate attachment which requires a bacterial component different from Opa and Opc and that meningococci cross a monolayer of tight-junction-forming epithelial cells by using a transcellular pathway rather than a paracellular route.

Neisseria meningitidis (also referred to as meningococcus [MC]) is an extracellular pathogen responsible for meningitis and septicemia. MC colonizes the nasopharynx and spreads from person to person. Factors responsible for MC carriage are poorly understood. In only a small percentage of colonized people, MC gains entry into the bloodstream, where it causes meningococcemia and/or progresses to the cerebrospinal fluid (CSF) to cause meningitis after crossing the blood-brain barrier (BBB). Therefore, to reach the meninges from the throat, MC must interact with two cellular barriers, one in the nasopharynx and one in the brain (the BBB). The BBB is composed of two different structures. The first corresponds to the endothelium of the capillaries. At this level, the endothelial cells differ from the cells present in peripheral capillaries by the presence of tight junctions with extremely high electrical resistance (18). The second structure responsible for the BBB is the choroidal plexus, which is the major site of CSF synthesis and is located in the ventricles (12). The endothelial cells in the choroidal plexus are fenestrated and have a peripheral structure. The BBB in this region is formed by tight junctions at the ventricular surface of the epithelial cells. Evidences indicate that, functionally and chemically, BBB endothelial and epithelial cells share many characteristics with polarized epithelia (6). The components at the BBB tight junctions are a select subset of standard components of peripheral polarized epithelia (2, 9, 10, 24). This suggests that mechanisms whereby bacteria interact with polarized epithelial cells may have relevance to the invasion of the CSF (20).

Most of the studies performed to elucidate the mechanisms of MC interactions with human cells have used subconfluent monolayers of nonpolarized epithelial and endothelial cells which lack tight junctions. By using such models, it has been shown that pilus-mediated adhesion is the only means by which capsulated strains are capable of interacting with endothelial and epithelial cells (16). Beside piliation, other bacterial attributes, such as Opc and the class 5 (Opa) proteins, have been identified as playing a role in the ability of the bacterium to interact with cells (22, 23). These bacterial attributes have been shown to mediate MC-cell interactions only in noncapsulated isolates. The role of these attributes in capsulated isolates remains to be determined.

Recently, a tissue culture model using epithelial and endothelial cell layers separated by a microporous membrane has been developed (3). However, the epithelial cells used in this model do not become polarized. To address the mechanisms by which a pathogen like MC can cross a cellular monolayer, it is necessary to study bacterium-cell interactions in assays using polarized cells forming tight junctions. Recently, using T84 cells, which are human polarized epithelial colonic cells, Merz et al. showed that *N. meningitidis* was capable of crossing a monolayer without destroying tight junctions (15). In this work, using a similar model, (i) we observed that following pilus-mediated adhesion, bacteria spread at the apical surface of the monolayer, reduce their piliation, and are involved in an intimate attachment with cytoskeletal modifications not related to Opa and Opc; (ii) we confirmed that MC cross the barrier of polarized cells without disrupting tight junctions; (iii) we demonstrated that during this process bacteria can be localized inside the cells and not between cells.

* Corresponding author. Mailing address: Laboratoire de Microbiologie, INSERM U411, Faculté de Médecine Necker-Enfants Malades, 156 Rue de Vaugirard, 75730 Paris cedex 15, France. Phone: 33 140615678. Fax: 33 140615592. E-mail: nassif@necker.fr.

MATERIALS AND METHODS

Strains and reagents. MC strains were grown on GCB medium containing the supplements described by Kellogg (11a), 3 μg of vancomycin per ml, 20 μg of colistin per ml, and, when necessary, 100 μg of kanamycin per ml. 8013 is a serogroup C class 1 strain. Clone 12 is a capsulated, Opa$^-$, and Opc$^-$ derivative of 8013 expressing a highly adhesive SB pilin variant (17). The *pilE*::Km mutation, which when introduced into MC strain 8013 abolishes pilin production, has been described previously (14). MC piliation was monitored by electron microscopy as previously described (14).

Monoclonal antibodies SM1, S3573, and 11B12 are specific for class I, Opc, and class 5 proteins, respectively (1, 21). The anti-PilC antiserum was polyclonal (16). Immunoblots were performed with total protein extracts as previously described (16). The sequencing of the *pilE* locus has been reported elsewhere (17).

Cell cultures, adhesion assay, and quantification of bacterial penetration across polarized T84 monolayer. T84 cells are human polarized intestinal epithelial cells (13). They were routinely grown at 37°C under 5% CO_2 in medium containing a 1:1 mixture of Dulbecco's minimal essential medium and Ham's F-12 supplemented with 10% fetal bovine serum, L-glutamine (200 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml).

For experimental assays, cells were grown on permeable supports with an area of 0.33 cm^2 (Costar, Cambridge, Mass.). The medium described above was used except that no antibiotic was added. Cells were seeded at a density of 5×10^5/well and after 4 to 5 days were assessed for the formation of tight junctions by measuring the transepithelial resistance (TER) with a Millicell-ERS (Millipore Corp., Bedford, Mass.) apparatus. Monolayers were used with a TER of between 500 and 2,000 ohms · cm^2; this resistance was usually achieved 5 days after seeding.

Assays were performed with overnight cultures of a single frozen stock of bacteria in order to avoid any secondary variations. Before the assay, the medium was removed from both compartments of the transwell unit and 5×10^4 MC were added at the top. The number of cell-associated CFU was determined after extensive washing and lifting off the monolayer by scraping and then vortexing the monolayer in order to dissociate the bacteria. This number represents both adhesive and invasive bacteria. To quantify bacterial invasion of cells, the monolayers were washed six times with phosphate-buffered saline (PBS) to remove nonadherent bacteria and incubated with fresh medium containing gentamicin (150 μg/ml) at 37°C. After 60 min of incubation, the medium was removed, the monolayer washed again twice with PBS, and cells were lysed at room temperature for 15 min with 1% saponin in PBS. Intracellular bacteria were enumerated by plating dilutions onto GCB agar.

To study the crossing of the monolayer, cells grown on transwells were infected as described above with 5×10^4 bacteria in the upper compartment. After 8 h of incubation, filter units were transferred into a well containing fresh uninfected medium. The lower surface between the two compartments was washed several times by removing the medium contained in the lower chamber, and bacteria were allowed to cross the monolayer for another hour. Appropriate dilutions of the basolateral medium were then plated onto selective GCB agar plates in order to determine the number of CFU which crossed the monolayer in 1 h. The same procedure was repeated at 23 h. The TER was determined 9 and 24 h after infection.

Measurement of [^{3}H]inulin penetration. [^{3}H]inulin measurements were performed as described by Canil et al. (4). After infection of a T84 monolayer grown on transwell filters units, 10^6 cpm of [^{3}H]inulin (molecular weight, 5,200; Amersham) in 200 μl of growth medium was added to the apical surface. The monolayer was further incubated at 37°C for 1 h, and 100 μl of the basolateral fluid was then removed and placed in 1 ml of scintillation fluid prior to counting. The permeability of the cell layer was defined as the percentage of counts per minute in the basolateral medium relative to counts per minute added. In some experiments, cytochalasin D (1 μg/ml; Sigma Chemical Co.) was added 30 min prior to infection and was present throughout the assay in order to alter the barrier permeability of the tight junctions.

Immunofluorescence microscopy. T84 cells were grown on transwell filter units until polarized and infected as described above. After measurement of the TER and assessment of the crossing of the monolayer by the bacteria, cells were washed six times with PBS and the cytoskeleton was stabilized with cold METM (50 mM morpholineethanesulfonic acid, 3 mM EGTA, 5 mM $MgCl_2$, 0.5% Triton X-100 [pH 7.4]) for 10 s. The samples were then fixed with cold 2.5% paraformaldehyde for 20 min, washed with PBS, and after neutralization with 0.1 M glycine for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 1 min. Before staining, samples were saturated twice in 0.2% gelatin in PBS. All immunoreagents were diluted in 0.2% gelatin–PBS.

Bacteria were labeled by indirect immunofluorescence. Cells infected with MC were usually incubated for 30 min in a 1/500 dilution of a rabbit antiserum directed against whole MC which has been previously described (17). Alternatively a polyclonal antibody raised in mice directed against the MC group C polysaccharide was used at a dilution of 1/500. This antiserum was a generous gift of M.-J. Quentin-Millet (Pasteur-Mérieux-Conaught, Marcy L'Etoile, France). After three washes in PBS, bacteria were revealed in a 1/100 dilution of goat anti-rabbit fluorescein-conjugated immunoglobulin G or in a 1/100 dilution of goat anti-mouse fluorescein-conjugated immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.). In some experiments, bacteria were stained with ethidium bromide at a final concentration of 3.3 mg/liter for 30 min.

For staining of F-actin, rhodamine-labeled phalloidin (Molecular Probes, Inc., Junction City, Oreg.) was diluted and used at a concentration of 8 U/ml. Tight junctions were stained with an anti ZO-1 antibody raised in rabbit diluted at 1/100 (Zymed Laboratories, Inc., San Francisco, Calif.) and were revealed with a 1/100 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G. Specific staining of MC pili was obtained by using monoclonal antibody 20D9 at a final concentration of 13.2 mg/liter. This monoclonal antibody was raised by injecting purified pili from clone 12 into mice. In an enzyme-linked immunosorbent assay, this antibody has been shown to recognize solely the SB pilin variant of clone 12 and not to react with another pilin variant expressed by another derivative of strain 8013. It is therefore likely that monoclonal antibody 20D9 reacts with an epitope located in the variable region of the SB pilin.

Immunostained filters were excised, placed in mounting medium (DABCO; 10 mg/ml; Sigma) on a glass slide, and sealed with nail polish under a coverslip. Labeled filters were observed in a confocal laser scanning microscope (TCS4D; Leica). Confocal sections were generally taken at intervals of 0.4 μm.

Electron microscopy. For scanning electron microscopy (SEM), cells were grown on coverslips seeded at a density of 5×10^5 cells/cm^2 and used 2 days later. Cultures were infected as described above. Monolayers were fixed with a 3% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.3). Preparations were then coated with gold palladium after critical point drying. Examination was performed with a JEOL 840A at the Centre Interuniversitaire de Microscopie Electronique (Paris, France).

For transmission electron microscopy (TEM), cells were grown in transwells as described above and were fixed in 1:1 mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate sucrose buffer (0.1 M cacodylate, 0.1 M sucrose, 5 mM $CaCl_2$, 5 mM $MgCl_2$, [pH 7.2]) at 4°C overnight. Monolayers were post-fixed in a mixture of 1% OsO_4 and 0.15% ruthenium red for 1 h and placed for 1 h in 1% uranyl acetate. After dehydration in graded series of alcohols, the cells were embedded with polyester filter in Epon. Thin sections were obtained by using an Ultracut ultramicrotome. For electron microscopy, sections were stained with uranyl acetate and lead citrate.

TABLE 1. Adhesion and invasion of clone 12 and its nonpiliated derivative onto a monolayer of T84 cells

Strain	Cell-associated CFU[a] at 9 h	Invasion[b]	
		9 h	24 h
Clone 12	7.0 ± 0.2 (30)	2.9 ± 0.7	3.8 ± 0.9
P$^-$[c]	4.8 ± 0.5 (0.024)	0.8 ± 1.2	2.1 ± 0.08

[a] Each value is the mean ± standard deviation of the log of at least three different experiments. In parentheses are the ratios of cell-associated CFU to total CFU.

[b] Reported as the log of cell-associated CFU after 60 min of incubation in a medium containing 150 μg of gentamicin per ml. Each value is the mean ± standard deviation of at least three different experiments.

[c] The *pilE*::Km derivative of clone 12.

RESULTS

Piliation is required for adhesion, penetration, and crossing of a T84 monolayer. It has previously been demonstrated that piliation is required for MC adhesion to nonpolarized epithelial and endothelial cells. To demonstrate that piliation was necessary for binding onto a polarized monolayer of epithelial cells, T84 were grown on transwells, and once they reached a TER of at least 500 ohms · cm^2, the upper compartment was infected with 5×10^4 CFU of MC. After 9 h of incubation, the number of cell-associated CFU was determined as described in Materials and Methods. Results of this experiment are reported in Table 1. As expected, the number of piliated clone 12 which are associated with the monolayer is several orders of magnitude higher than that of its nonpiliated derivative, thus confirming the role of pili in the interaction of MC with the apical surface of polarized epithelial cells.

We next determined whether MC was capable of penetrating a monolayer of polarized cells. Monolayers grown as above were apically infected with 5×10^4 CFU of either piliated clone 12 or its *pilE*::Km derivative. At 9 and 24 h, gentamicin

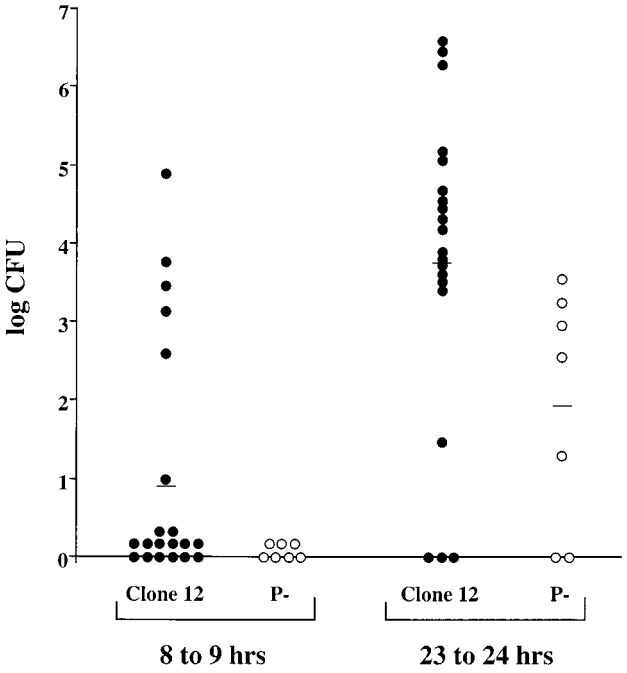

FIG. 1. Crossing of the T84 monolayer by clone 12 and its nonpiliated derivative. Each dot corresponds to the number of CFU which have crossed a monolayer during 1 h after 8 or 23 h of infection. P^- is a *pilE*::Km derivative of clone 12. Horizontal bars represent averages.

was added in both compartments for 60 min. The number of bacterial CFU present in the monolayer was then determined after lysis of cells. Results (Table 1) demonstrate that a small fraction of adhesive MC invade the monolayer. In addition, this number was higher at 24 h than at 9 h.

To assess whether MC was capable of crossing a monolayer of tight-junction-forming epithelial cells, T84 cells grown on transwells were infected as described above. During the period from 8 to 9 and from 23 to 24 h, the number of bacteria crossing the monolayer was assayed by placing the transwell in fresh medium and determining the number of CFU which had reached the lower chamber. Figure 1 shows that after 9 h, clone 12 crossed the monolayer in few infected wells, whereas at 24 h, bacteria crossed 17 of the 20 infected monolayers. In addition, at 30 h, bacteria which did not show any passage at 24 h crossed two of the three monolayers (data not shown). On the other hand, at 9 h, none of the monolayers infected with the nonpiliated strain were crossed by bacteria. At 24 h, the passage of nonpiliated bacteria was on average 2 orders of magnitude less efficient than that observed with the wild-type strain. The data obtained at 24 h were subject to statistical analysis using the Mann-Whitney test, and the difference observed between piliated and nonpiliated strains was found to be highly significant, with a *P* value of less than 0.005. These data demonstrate that MC crossed in a specific manner a monolayer of tight-junction-forming T84 cells and that this step requires piliation for maximum efficiency.

Infection of a T84 monolayer by *N. meningitidis* does not affect the barrier function of tight junctions. One explanation for the foregoing observation is that bacterial interactions with cells alter tight junctions, thus allowing the pathogen to cross the monolayer by using the paracellular pathway. To address this point, three kinds of experiments were performed.

(i) Resistance of the monolayer was determined at 9 and 24 h after bacterial infection (Table 2). The TER at these time points was not on average significantly different from that observed initially.

TABLE 2. TER of polarized T84 monolayers infected with *N. meningitidis*

Strain	TER (ohms · cm^2)[a]		
	0 h	9 h	24 h
Clone 12	1,300 ± 555	1,100 ± 372	1,025 ± 416
P^{-b}	1,012 ± 395	1,391 ± 361	1,089 ± 253

[a] Each value is the mean ± standard deviation of all monolayers for which data are reported in Fig. 1.
[b] The *pilE*::Km derivative of clone 12.

(ii) Flux of [^{3}H]inulin through the monolayer was measured after 24 h of infection (Table 3). As expected, [^{3}H]inulin did not penetrate uninfected monolayers which had a high electrical resistance. Monolayers in which tight junctions had been artificially destroyed by preincubation with cytochalasin D allowed a flux of inulin. Monolayers infected by either clone 12 or its nonpiliated derivative did not increase inulin penetration, thus arguing for the absence of alteration of the barrier function of an infected monolayer.

(iii) If tight junctions were destroyed by MC infection, one would expect a delocalization of proteins such as ZO-1 which are involved in the formation of these complexes. Figure 2A demonstrates that no such delocalization is visible on infected monolayers. Furthermore, no alteration in the underlying actin belts is visible.

Taken together, these findings demonstrate that MC crosses the monolayer of T84 cells without altering tight junctions.

***N. meningitidis* induces localized actin polymerization and can be internalized inside cells.** To determine the different events which follow bacterial attachment to T84 cells, three sets of experiments were performed.

(i) SEM experiments were done at different time points (Fig. 3). Four hours after infection, adhesion of MC is localized and bacteria adhered, forming clumps inside which pili can be observed (Fig. 3A). At later time points, bacteria express a diffuse pattern of adherence and the apical surface is covered by a single monolayer of diplococci instead of localized clumps (Fig. 3B).

(ii) Infected T84 cells grown on transwell units were stained with a rabbit polyclonal antibody directed against whole MC and rhodamine-labeled phalloidin, which labeled polymerized actin. Results are shown in Fig. 2C and D. In infected monolayers, spots of fluorescence corresponding to polymerized actin can be visualized. These spots colocalize with bacteria.

TABLE 3. [^{3}H]inulin penetration through an infected monolayer of T84 cells

Strain	TER (ohms · cm^2) at 24 h[a]	[^{3}H]inulin penetration (%)[a,b]
None	1,575 ± 195	0.25 ± 0.05
Clone 12	1,041 ± 44	0.26 ± 0.07
8013.*pilE*::Km	988 ± 81	0.24 ± 0.007
Clone 12 + cytochalasin D[c]	149 ± 94	1.5 ± 0.58

[a] Each value is the mean ± standard deviation of at least three experiments.
[b] Defined as the percentage of counts per minute in the basolateral medium relative to counts per minute added.
[c] Cytochalasin D was added 30 min prior to infection and was present throughout the assay in order to disrupt the tight junctions.

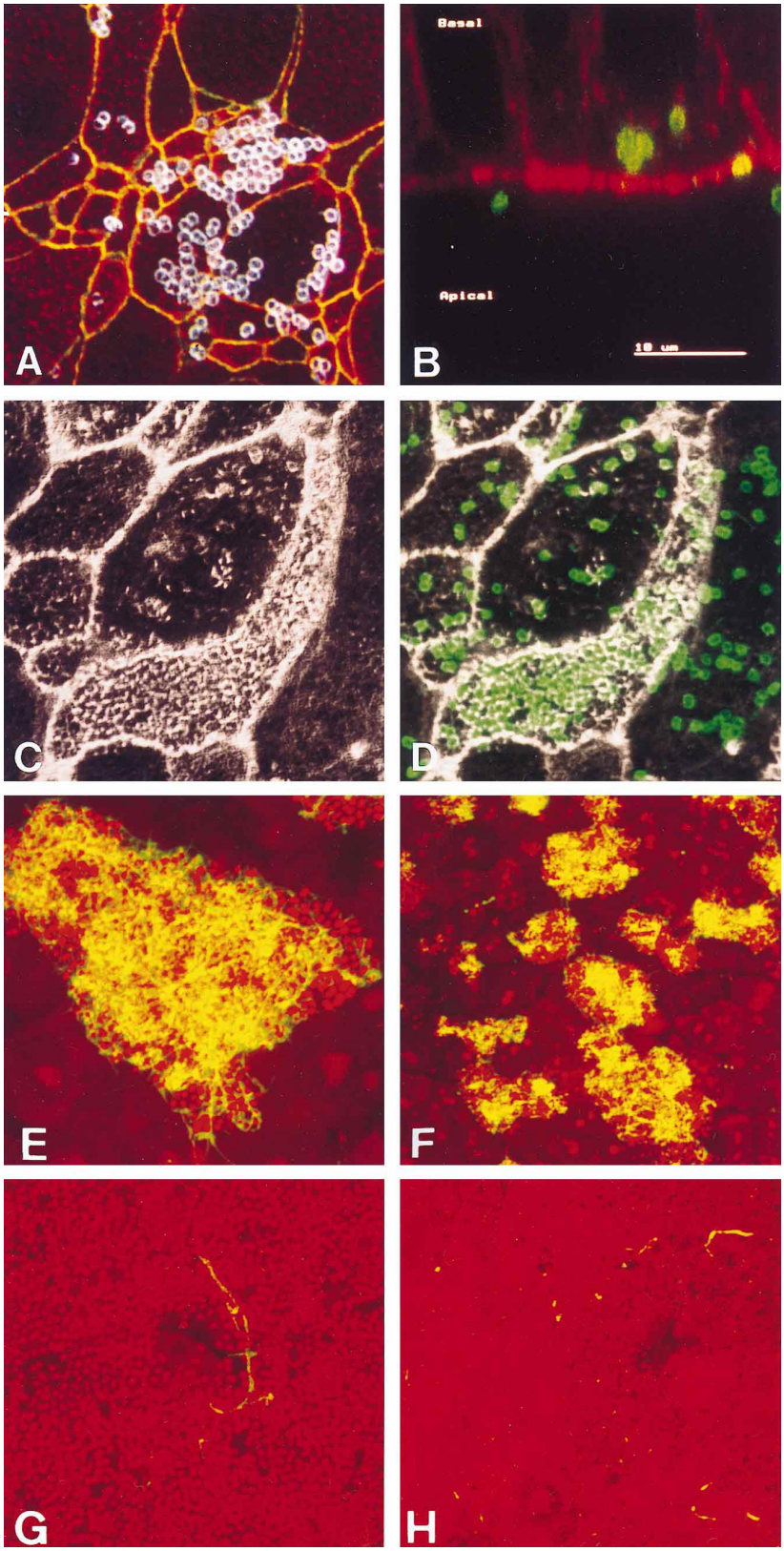

FIG. 2. Fluorescent confocal microscopy of permeabilized T84 monolayers grown on a transwell. (A) Images reconstructed from confocal sections of the apical region of a T84 cell monolayer infected for 9 h and stained with anti-ZO-1 (green), rhodamine-labeled phalloidin (red), and anticapsule antibody (purple) (superimposition of all the individual images). Yellow corresponds to colocalization of ZO-1 and actin staining. (B) Images reconstructed from confocal *xz* sections of an infected monolayer and stained with rhodamine-labeled phalloidin (red) and anti-MC antibodies (green). (C and D) Images reconstructed from confocal sections of the apical region of an infected monolayer stained with rhodamine-labeled phalloidin (grey) and anti-MC antibodies (green). (C) Actin staining; (D) the superimposition of both stainings. (E to H) Immunofluorescence labeling of pili. Images reconstructed from confocal sections of T84 monolayers infected for 4 h (E and F) or 9 h (G and H). Magnifications: ×170 (E and G) and ×54 (F and H). Cells and bacteria were stained with ethidium bromide (red). Monolayers were fluorescently labeled with monoclonal antibody 20D9, which specifically stains pili (green). The superimposition of both stainings is shown. Numerous pili are seen associated with bacteria at 4 h (E and F). At 9 h, no or very few pili are present (G and H) although cells are covered by a monolayer of bacteria.

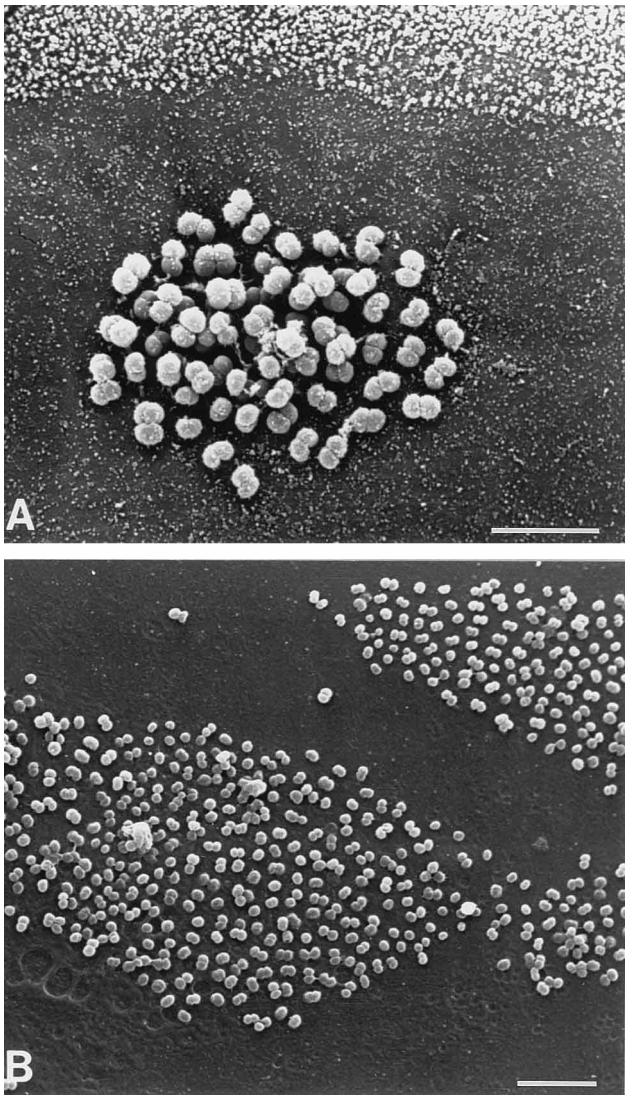

FIG. 3. SEM examination of a T84 monolayer grown on coverslip after 4 h (A) and 9 h (B) of infection by clone 12 (bar, 5 μm).

F-actin accumulation followed strictly the outline of adherent bacteria, giving a typical footprint-like appearance. These pictures suggest that cytoskeletal rearrangements occur at the apical surface of infected cells. In addition, optical sectioning of the infected cells was performed to determine the existence and the location of bacteria inside the monolayer. Figure 2B shows bacteria inside the cells which do not colocalize with the actin wall. These data strongly suggest that bacteria cross the monolayer by using the transcellular pathway. In addition, it should be pointed out that intracellular bacteria were not surrounded by polymerized actin.

(iii) TEM was performed on a T84 monolayer grown on a transwell and infected for 9 h with MC. As shown in Fig. 4B, bacteria became very intimately associated with cells. Cup-like invaginations and occasionally adherence pedestals were observed (Fig. 4A), thus confirming the cytoskeletal modifications at the apical surface of the cells. In occasional circumstances, MC were located inside cells in vacuoles (Fig. 4C). The membrane of the vacuole and the bacterium were not labeled by ruthenium red, unlike the apical membrane and the cell surface-located bacteria, thus arguing that these vacuoles correspond to true internalization.

MC reduces its piliation when involved in cellular interactions. As already mentioned, pilus-mediated adhesion plays a major role in MC interaction with polarized cells. However, at later time points, the very close interaction observed between MC and the apical membrane of the cells strongly suggests that other components are involved in this process (Fig. 4). Furthermore, the disappearance of clumping (Fig. 3B) suggests that bacteria are not as piliated at 9 h as they are at 4 h. This conclusion is reinforced by a close examination of bacteria interacting with cells, which do not show any bundle of pili at late stage of infection, whereas such structures are initially clearly visible (Fig. 3A). To confirm this loss of piliation, immunofluorescence staining of infected monolayers was performed with monoclonal antibody 20D9. This monoclonal antibody specifically stains SB pilins expressed by clone 12 (data not shown). Pilus staining was performed on monolayers infected for 4 and 9 h (Fig. 2E to H). After 4 h of infection, numerous pili are associated with bacterial clumps (Fig. 2E and F). On the other hand, very few pili are present at 9 h, although bacteria covered the cells (Fig. 2G and H). These data demonstrate that bacterial piliation is dramatically reduced at later time points.

To determine whether this reduction of MC piliation was related to a phase variation event, 10 cell-associated colonies obtained from an infected monolayer of T84 cells grown on transwell were taken for further study. These colonies were piliated, capsulated, Opa^-, Opc^-, and $PilC1^+$, like the parental strain (data not shown). Furthermore, the sequence of the *pilE* gene of two of these clones was determined and shown to correspond to that of SB pilin, which is the one expressed by the parental clone 12 (data not shown). These results suggest that a phase variation event is unlikely to be responsible for this decrease in piliation. In addition, these data indicate that pilus-mediated adhesion cannot be responsible for the close interaction between MC and cells. Since the cell-associated colonies of clone 12 do not express any class 5 or Opc protein, other bacterial attributes yet unidentified must be responsible for this very close bacterium-cell interaction.

DISCUSSION

In this study, we have analyzed the interaction of *N. meningitidis* with an epithelial monolayer of human polarized cells presenting organized tight junctions. As with other pathogens (7), MC interaction with T84 monolayers progresses in several steps. The first one is a localized adherence which is pilus mediated and leads to the formation of small colonies on the surface of the apical membrane. Following this step, bacteria spread onto the apical surface and form a monolayer covering the cells. At this stage, bacteria adhere intimately and firmly. Bacterial attributes involved in this intimate attachment have not yet been identified. Reduction of bacterial piliation suggests that pili are not responsible for this intimate attachment. Furthermore, cell-associated CFUs are Opa^- and Opc^-, thus suggesting that these outer membrane proteins are not involved in the intimate attachment. Some other, yet unidentified components, possibly upregulated by the bacterium-cell interactions, are likely to mediate this process. The mechanism by which these bacteria reduce their piliation when involved in a cellular process remains unknown. This event is not due to phase variation since bacteria recovered from an infected polarized monolayer are piliated. A cross talk between bacteria and cells could lead to the down-expression of genes involved

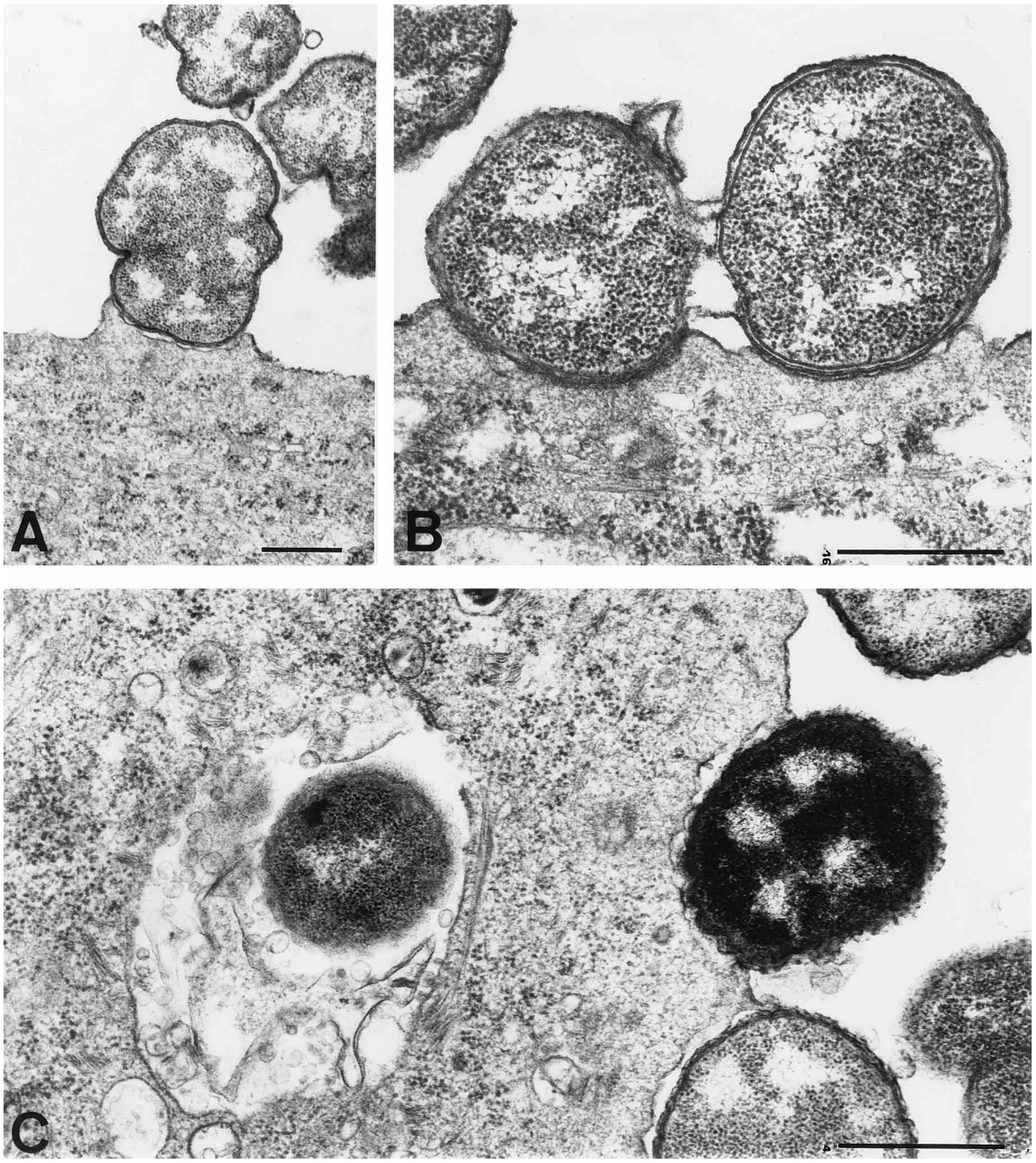

FIG. 4. TEM examination of T84 cells grown on a transwell infected for 9 h by clone 12. In panel C, note that ruthenium red stains the outer membrane of bacteria adhering to cells but not that of the bacterium contained in the vacuole (bar, 0.5 μm).

in piliation; this may correlate with the expression of bacterial attributes responsible for intimate attachment.

The intimate attachment generates cytoskeletal modification which occurs before the crossing of the monolayer by *N. meningitidis*, thus suggesting that these modifications are important to achieve this step. Considering that (i) the tight junctions are not altered and (ii) bacteria can be visualized inside cells by confocal examination and electron microscopy, we conclude that MC cross the T84 cellular monolayer by using the transcellular pathway. The crossing of a cellular barrier by *N. meningitidis* without destruction of tight junctions has recently been reported (15); however, the intracellular location of bacteria was not determined. These findings are consistent with earlier report by Stephens et al. (19). These authors, using a human nasopharyngeal organ culture system, have demonstrated that MC enter at the apical site of the epithelial cells, transcytose, and exocytose through the basolateral side of the cells.

The mechanism by which a nonpiliated derivative is capable of penetrating (Table 1) and crossing (Fig. 1) monolayers, even at a low frequency, remains unexplained. Our data clearly demonstrate that other bacterial components beside piliation are involved in MC-cell interaction. Without pili, these other mechanisms seem to be very inefficient but could still be re-

sponsible for the low level of bacterial crossing which is observed.

Different results for studies using similar models were reported for other pathogens. *Salmonella typhimurium* causes a progressive decrease in TER concomitant with increased paracellular inulin flux and with delocalization of ZO-1 (11). Enteropathogenic *Escherichia coli* is responsible for a decrease in TER by disrupting a transcellular pathway but does not alter the intercellular tight junctions (4). On the other hand, some other pathogens, like *Helicobacter pylori* (5), are unable to traverse such a monolayer and do not alter the barrier function, even though they are involved in an intimate attachment with actin reorganization.

The crossing of a cellular barrier is important in MC pathogenesis, considering that after colonization of the throat, MC will have to translocate first into the bloodstream and then from the bloodstream to the CSF. We believe that our data suggest that to achieve these goals, MC transcytose through cells and do not penetrate through opened tight junctions.

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