Interaction of *Neisseria meningitidis* with the Components of the Blood-Brain Barrier Correlates with an Increased Expression of PilC

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A fatal untreated case of fulminant meningococcemia was examined to investigate the crossing of the blood-brain barrier (BBB) by *Neisseria meningitidis*. Microscopic examination showed bacteria in vivo adhering to the endothelium of both the choroid plexus and the meninges. Comparison of the isolates cultivated from the blood and the cerebrospinal fluid (CSF) revealed no antigenic variation of the pilin or the class 5 protein, whereas the expression of the PilC protein was greater in the CSF and the choroid plexus than in the blood. This was due to an increased activity of one of the *pilC* promotors. This higher expression of PilC correlated in vitro with greater adhesiveness to endothelial cells. A mutation in the single *pilC* locus of this strain abolished in vitro pilus-mediated adhesion to endothelial cells. These data suggest that PilC plays an important role in the crossing of the BBB, likely through pilus-mediated adhesion.

Neisseria meningitidis is an extracellular pathogen responsible for meningitis and septicemia. The first step in the pathogenesis is the asymptomatic colonization of the nasopharynx. Factors responsible for this carriage are poorly understood. However, in a small percentage of colonized people, *N. meningitidis* gains entry into the bloodstream, where it can cause septicemia and invade the meninges to cause meningitis. Two lines of evidence suggest that *N. meningitidis* reaches the cerebrospinal fluid (CSF) via the bloodstream and is therefore capable of crossing the blood-brain barrier (BBB). First, *N. meningitidis* can be recovered from the CSF of rhesus monkeys after intravenous injection [1]. Second, *N. meningitidis* has been cultivated from the blood of patients before being recovered from the CSF [2]. However, in vivo interaction between *N. meningitidis* and the components of the BBB has never been demonstrated.

Two different structures are responsible for the formation of the BBB: the capillaries of the meninges and those of the choroid plexus. In the brain, the endothelial cells (EC) differ from those present in peripheral capillaries in two important respects: tight junctions with extremely high electrical resistance between cells [3] and a very slow rate of fluid-phase endocytosis. In essence, the combination of the limited paracellular and transcellular movement accounts for the existence of the BBB [4]. The second structure responsible for the BBB is the choroid plexus, which is the major site of CSF synthesis and is located in the cerebral ventricles. These are structures with a central core of blood vessels covered by epithelium. The EC in the choroid plexus are fenestrated and have structure like those found in peripheral capillaries. The BBB in this region is formed by tight junctions at the ventricular surface of the ependymal cells. A bacterial pathogen such as *N. meningitidis* can therefore cross the BBB either through the capillaries of the meninges or through the choroid plexus.

Type IV pili are the major ligand mediating adhesion of capsulated meningococci to EC (reviewed in [5]). *N. meningitidis* pili fall into two classes. Class I pili are very similar to gonococcal pili and react with the SM1 monoclonal antibody [6]. In class I *N. meningitidis*, as in gonococci, pilin is subject to antigenic variation because of changes in the nucleotide sequence of the *pilE* locus. Class II pili do not bind SM1. The molecular biology of class II pilin is unknown, including the gene encoding this pilin subunit. Pilin antigenic variation modulates adhesiveness to human cells [7–9], and the mechanism of this hyperadhesiveness is the ability of some pili to form bundles [10]. PilC proteins (PilC1 and PilC2) are two related proteins first described in gonococci, in which they have been shown to be tip-located adhesins [11]. The PilC proteins are subject to phase variation. This results from changes in the number of G residues within a tract of polyG located in the signal peptide–coding sequence [12]. We have identified two *pilC* loci, *pilC1* and *pilC2.* PilC1 and PilC2 have a similar if not identical function in *N. meningitidis* pilus biogenesis; that is, a double $PilC1^-/PilC2^-$ strain is nonpiliated, whereas a $PilC1^+$ or $PilC2^+$ strain is piliated. PilC1 appears to be a bifunctional protein, as it also affects adhesion through a mechanism unrelated to pilus biogenesis: Only $PilC1^+$ strains are adhesive, whereas $PilC1^-/PilC2^+$ strains are piliated but nonadhesive [13]. By analogy with data obtained with gonococci, the current model considers PilC1 as a tip-located adhesin. Besides pili,

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other outer membrane components such as class 5 proteins and Opc have been implicated in bacterial–host cell interaction [14]. Their contribution to the adherence of capsulated strains remains unclear.

To assess the importance of pilus-mediated adhesion in the crossing of the BBB, the virulence of appropriate mutants should be tested in animal models that faithfully reproduce the human disease. Because these bacteria interact only with cells of human origin, it is not possible to use animal models to test the role of *N. meningitidis* adhesion in CSF invasion. In this work, we used material obtained from a fatal case of meningococcemia to investigate the role of the PilC protein in the crossing of the BBB.

Materials and Methods

Strains and patient. *N. meningitidis* strains were grown on gonococcal base (GCB) medium containing the supplements described by Kellogg. Strain 8013 is a serogroup C class 1 strain; clone 12 is a derivative of this strain expressing a highly adhesive pilin variant [13]. Strain Z3685 is a serogroup A strain expressing large amount of Opc [15]. *Escherichia coli* strain DH5α was used for plasmid preparation. pMGC10, carrying the *ermAM* gene, has been described before [16]. When necessary, the GCB medium was made selective by adding 3 μg/mL vancomycin and 20 μg/mL colistin. Erythromycin was used at a concentration of 2 μg/mL for *N. meningitidis* selection and at a concentration of 200 μg/mL for *E. coli.* Meningococcal piliation was monitored by electron microscopy as described [7].

A meningococcus strain, designated ROU, was cultivated from the blood, the choroid plexus, and the CSF of a 2-month-old infant who was found dead unexpectedly one morning. The night before, he was not considered ill by his mother, as he did not have any fever or symptoms. This strain was serogrouped at the Unité des Neisseria (Institut Pasteur, Paris) and found to belong to the W135 capsular serogroup. Subsequent agglutination reactions to check the capsulated phenotype of the different isolates were done by use of an agglutinating serum obtained from Diagnostics Pasteur (Marnes-la-Coquette, France). To avoid secondary variations, bacteria isolated from the blood, the choroid plexus, and the CSF were initially frozen as pools without further subculturing. Each pool contained several hundred colonies from the primary cultures. Bacterial pools obtained from the blood, the choroid plexus, and the CSF were designated ROU_{bl}, ROU_{cp}, and ROU_{csf}, respectively. Biologic assays were performed with subcultures of these frozen stocks.

During the autopsy, the brain was removed. Samples of meninges and choroid plexus were placed in 0.2 *M* cacodylate, pH 7.4, with 2% glutaraldehyde for 2 h, postfixed in 2% aqueous osmium tetroxide for 1 h, dehydrated in graded ethanol solution, and embedded in Epon 812. Semithin sections were cut from the Epon-embedded material and stained with 1% toluidine blue. After sampling, the brain was fixed as well in 10% formalin solution; specimens from coronal sections were taken for standard histology. All of this material was paraffin-embedded and stained by a standard hematoxylin-eosin staining.

Bacterial adhesion to the vessels of the brain was estimated as the percentage of capillaries having at least 1 diplococcus clearly interacting with the EC. The mean number was obtained after observing all of the capillaries in 50 fields. The data reported were obtained by 2 independent investigators.

DNA and RNA techniques, protein preparation, and immunoblotting. All recombinant DNA protocols were from Sambrook et al. [17]. Immunoblots were done with total protein extracts as described [13].

Oligonucleotides used in this study are ERMAM1 (5′-GCAAACTTAAGAGTGTGTTGATAG-3′), ERMAM3 (5′-AAGCTTGCCGTCTGAATGGGACCTCTTTAGCTTCTTGG-3′), OPC1 (5′-CCGCTAATGAGTTTACCG-3′), OPC2 (5′-CGTATTCACGAATACTGG-3′), C1-8 (5′-CTGCCTTTTTAAAGTTTTATTCATCGT-3′), C1-354 (5′-GGATAACAGTAATATTCAAAGATTAT-3′), and CEXT3 (5′-TCTAGACCCGCCCCTCCCGAAAAACGCAAA-3′).

RNA extraction and primer extension were done as described elsewhere [18]. Labeled oligonucleotide C1-8 was used in primer extension experiments; the products were analyzed by electrophoresis with a dideoxy sequencing ladder.

The intragenic *pilC* probe was derived from the pJL1 plasmid described elsewhere [13]. This probe recognizes both *pilC* loci of strain 8013. Monoclonal antibodies SM1, S3734, S3409, S3573, and 4B12/C11 are specific for class I, class IIa, and class IIb pilins, Opc, and class 5 proteins, respectively [19–21]. The anti-PilC serum was polyclonal [13].

Cell cultures and adherence assays. Human brain EC (HBEC) were obtained from J. Nelson (Oregon Health Sciences University, Portland) [22]. These cells were isolated from temporal lobe tissue obtained from lobectomy. They were obtained in passage 3 and used between passages 5 and 8. HBEC were cultivated in endothelial SFM medium (Life Technologies GIBCO BRL, Gaithersburg, MD) containing 10% heat-inactivated normal human serum and supplemented with endothelial cell growth factors (50 μg/mL) and heparin (50 U/mL). Cultivation of human umbilical vein EC (HUVEC) has been reported elsewhere [13].

The day before inoculation, confluent monolayers were trypsinized and seeded into 24-well trays at a density of 2×10^5 cells/well. For adhesion assays, bacteria were resuspended in RPMI containing 10% heat-inactivated fetal calf serum at a density of 10^6 cfu/mL. One milliliter of this suspension was added to each well. The plates were incubated for 4 h at 37°C in 5% CO_2. The medium was then removed, and the number of colony-forming units (cfu) in the supernatant was calculated by plating dilutions onto GCB agar. Each well was then washed five times to remove nonadherent bacteria. The EC were harvested by scraping the cells into 1 mL of media. The number of cell-associated cfu was then determined by plating. The degree of adhesion was calculated as the ratio of cell-associated cfu/cfu present in the supernatant.

Construction of a pilC$^-$ derivative of the ROU strain. pNM2 is a *pilC1*-containing cosmid from a previously described library of *N. meningitidis* strain 8013 [17]. This latter strain has two *pilC* loci, designated *pilC1* and *pilC2,* whereas in the ROU strain the use of a *pilC*-specific probe identified only one locus. The *pilC1* allele was localized in pNM2 on a 3-kb *Hin*dIII-*Hin*dIII fragment. This fragment was subcloned into the *Hin*dIII site of pT7-7 to generate pTC1-17. The 3-kb *Hin*dIII-*Hin*dIII fragment cloned in pTC1-17 carries the entire PilC1 open-reading frame and contains two *Pvu*II sites located at both ends of the open-reading frame. The *Pvu*II fragment of pTC1-17 was deleted and replaced by the *ermAM* gene, which was amplified using pMG10 as target and

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ERMAM1 and ERMAM3 as primers; the resultant plasmid was designated pTC1-17Δ::*ery*. This deletion removed most of the PilC1-coding sequence. This plasmid was then linearized and transformed into the ROU strain. Transformants were selected on the basis of erythromycin resistance. It should be pointed out that the ERMAM3 primer contained an uptake sequence that is required for meningococcal transformation. Several erythromycin-resistant transformants were selected, purified, and examined for the correct insertion of the mutagenized allele by Southern blot by use of an erythromycin and a *pilC* probe. One transformant was selected for further study.

Results

N. meningitidis adhere to choroidal EC and to meningeal EC. The diagnosis of fulminant meningococcemia in the case described above was based on the suddenness of the death, the isolation of meningococci from the blood and the CSF, adrenal hemorrhage (at autopsy), and the histologic examination, which did not show acute inflammation in the meninges nor in any other part of the body. The infant did not have complement nor properdin deficiency. Considering that meningococci were recovered from the CSF and that death occurred very early, bacteria should be found in the brain of this patient associated with the component through which it crosses the BBB. Numerous meningococci were seen adhering to the EC of the capillaries of the meninges and the choroid plexus (figure 1). The number of infected capillaries was higher in the choroid plexus than in the meninges: 68% of the capillaries observed in the choroid plexus had adherent bacteria compared with only 7% of those in the meninges. The greater number of bacteria found attached to the vessels of the choroid plexus could argue for a preferential crossing of the BBB through this structure. However, since a careful examination of the semithin sections of the choroid plexus did not reveal adherence of bacteria to choroidal epithelial cells, it cannot be ruled out that meningococcus may pass directly through the meningeal capillaries to the CSF. Indeed, both routes may be used by this pathogen to reach the CSF.

The crossing of the blood-brain barrier is not associated with pilin antigenic variation or class 5 protein variation. Several outer membrane proteins are subject to genic variation events. Among these are pilin, PilC, which regulates pilus-mediated bacterial adhesion, and other components, such as class 5 proteins and Opc. These components have been implicated as being able to modulate bacterial adherence. In vitro, different variants express different functions, and it has been suggested that one of the roles of genic variation is to modulate the expression of some virulence factors. To assess whether the crossing of the BBB could be associated with antigenic variation of these proteins, we compared the phenotypes expressed by ROU_{bl} and ROU_{csf}. Using SM1, S3734, and S3409 monoclonal antibodies, which are specific for class I, IIa, and IIb pilin, respectively, we demonstrated that ROU is a class IIb pilin strain. The migration of pilin in SDS-PAGE expressed by isolates obtained from blood and CSF is identical (figure 2A, B), suggesting that ROU_{bl} and ROU_{csf} expressed similar variants. Comparison of class 5 proteins did not display any difference. As figure 2 shows, the blood and CSF isolates expressed one class 5 protein that had a similar SDS-PAGE migration pattern after solubilization at both 42°C (figure 2D) and 100°C (figure 2C). Opc protein was not detectable in a total cell extract as tested with the S3573 monoclonal antibody (data not shown). To determine whether this was due to phase variation or due to a lack of *opc* gene, a Southern blot of *Cla*I-digested chromosomal DNA was done with an *opc* probe. This probe was obtained by amplification of the entire open-reading frame of the *opc* gene by use of OPC1 and OPC2 as primers and total DNA of strain Z3685 as target. Using these primers, we could not detect *opc* in the ROU strain (data not shown). Taken together, these data do not support the hypothesis that the crossing of the BBB is related to an antigenic variation event involving pilin, Opc, or a class 5 protein.

The expression of PilC is higher in isolates obtained from CSF and the choroid plexus than in the blood isolate. Another bacterial surface structure subject to phase variation is the PilC protein. To define the *pilC* genotype of the ROU strain, a Southern blot of *Cla*I-digested DNA was done with a probe recognizing both *pilC* loci of strain 8013. In the ROU strain, only one *Cla*I fragment was identified by this probe, suggesting that this strain carries only one *pilC* allele, unlike what we previously reported with another strain. The introduction of the mutagenized *pilC* allele, constructed as described above, showed recombination events all occurring at the same chromosomal location, confirming that the ROU strain has only one *pilC* locus. A Western blot using an anti-PilC antiserum is shown in figure 2E. A 110-kDa protein reacting with the antiserum is present in all isolates (lanes 1–3) and absent from the $pilC^-$ mutant (lane 4), thus confirming that this protein is PilC. In addition, even though each lane in figure 2E was loaded with the same amount of protein, the level of expression of PilC in lane 1, which corresponds to protein extracts obtained from ROU_{bl}, is lower than that observed in the CSF and choroid plexus isolates. Since all of these isolates were frozen as pool from the primary culture, one explanation for this discrepancy is the presence of both $PilC^+$ and $PilC^-$ derivatives in the blood isolate. However, 10 isolated colonies of ROU_{bl} expressed similar low amounts of PilC (data not shown), and 10 isolated colonies of ROU_{csf} had the same high level of expression of PilC (data not shown). Furthermore, considering that PilC proteins are subject to phase variation and that this event is thought to be under the control of a frame-shift mutation within a polyG tract in the signal peptide–encoding region, we amplified the sequence corresponding to this region from total DNA prepared from both ROU_{bl} and ROU_{csf} using primers C1-354 and CEXT3 and determined the nucleotidic sequence of the promotor regions. With both strains, the sequence was identical and the number of G residues in the polyG stretch corresponded to an ''on'' phase (figure 3). These data rule out the possibility that this lower expression of the PilC protein was related to the

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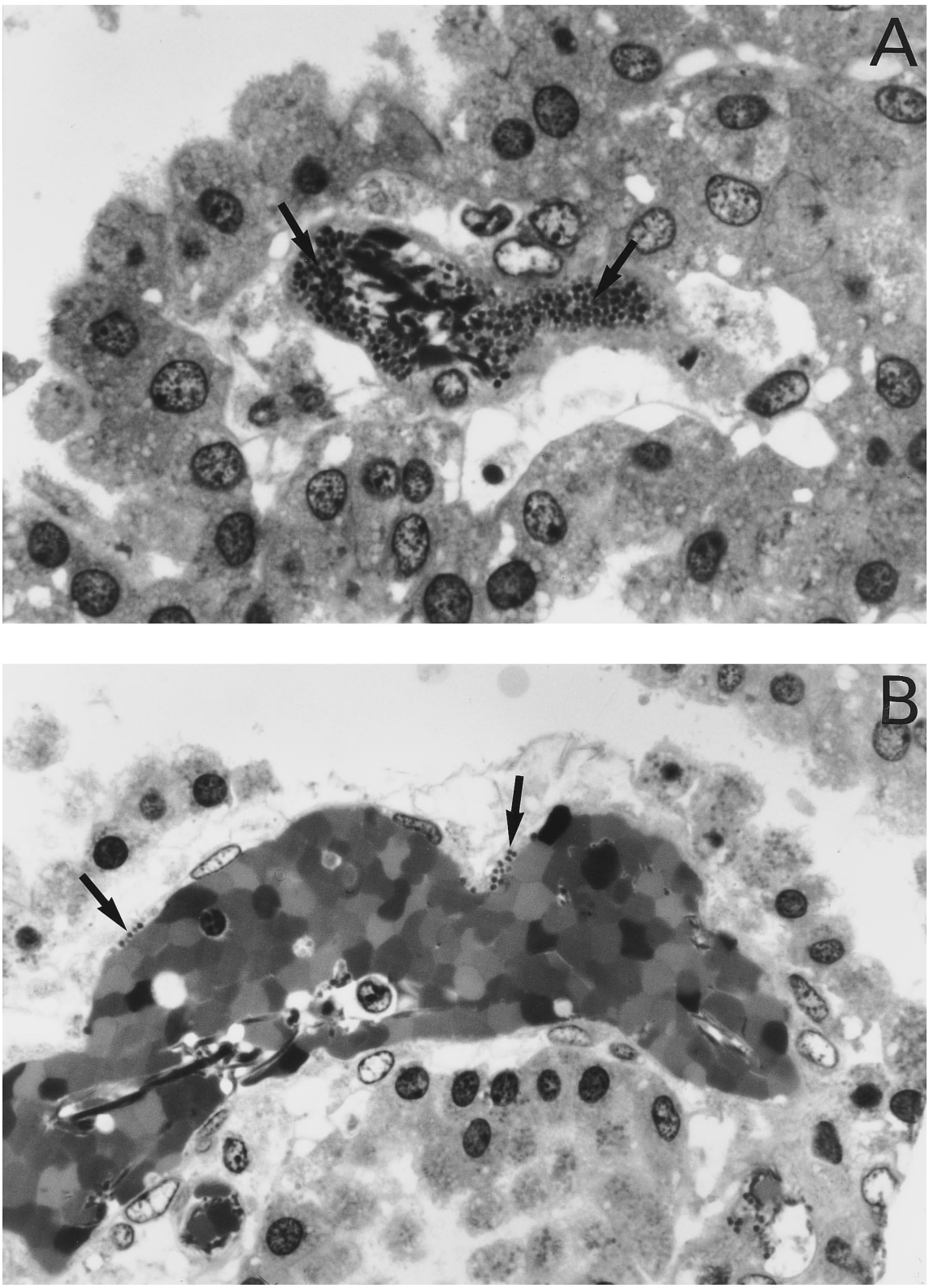

Figure 1. Sections of capillaries of choroid plexus stained with toluidine blue. Arrows identify clumps of bacteria. Field shown in **B** demonstrates that bacteria interact with endothelial cells.

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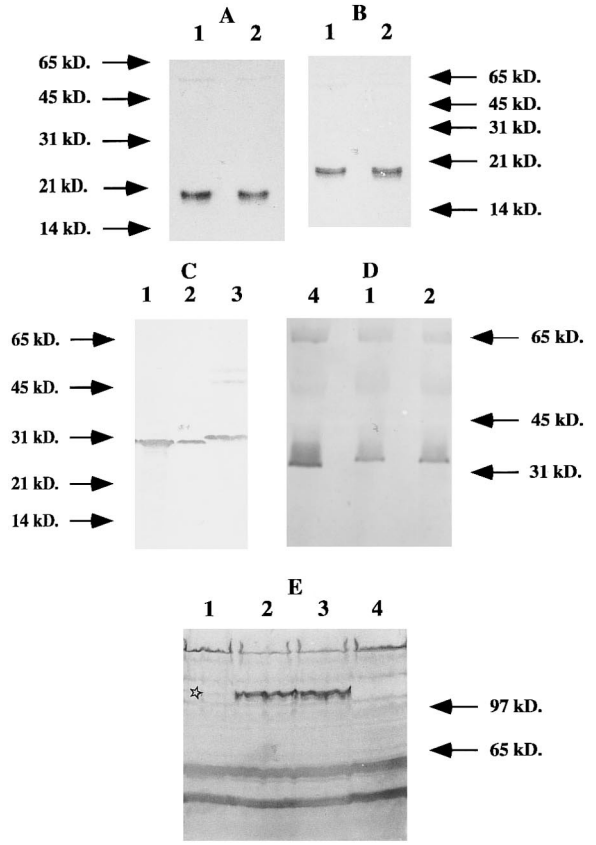

Figure 2. **A**, Immunoblot of total protein extracts done using S3409 monoclonal antibody. Proteins were separated by 15% SDS-PAGE. Lane 1: ROU_{bl}, lane 2: ROU_{csf}. **B**, Same as **A**, except SDS-PAGE in which protein extracts were separated contained 3 *M* urea. Urea totally denatures proteins and shows that both pilins have same migration pattern. **C**, Immunoblot of total protein extracts done using 4B12 monoclonal antibody. Samples were boiled at 100°C before loading. Lane 1: ROU_{bl}, lane 2: ROU_{csf}, lane 3: 7972, serogroup A strain expressing different class 5 protein. **D**, Same as C, except samples were heated at 42°C instead of 100°C. Lane 1: ROU_{bl}, lane 2: ROU_{csf}, lane 4: 7964, serogroup C strain expressing different class 5 protein. **E**, Immunoblot of total protein extracts done using anti-PilC polyclonal antiserum. Proteins were separated by 10% SDS-PAGE. Lane 1: ROU_{bl}, lane 2: ROU_{cp}, lane 3: ROU_{csf}, lane 4: *pilC*$^-$ derivative of ROU strain. Star in lane 1 points out location of PilC protein.

presence of a mixed PilC$^-$ and PilC$^+$ population in the bloodstream.

Considering that most of the choroidal vessels had meningococci adhering to the EC (figure 1), the high level of PilC expression in ROU_{cp} suggests that PilC may be required for the bacteria to interact with the EC.

The high level of expression of PilC is responsible for enhanced adhesiveness to human EC. As mentioned above, the expression of PilC is necessary for piliation and pilus-mediated adhesion. To address the relevance of the increased expression of PilC of ROU_{csf} and ROU_{cp} compared with ROU_{bl}, piliation and adhesiveness of these isolates were determined and compared with those of PilC$^-$ derivatives. These latter were obtained by retransforming total DNA of the Δ*pilC::ery* derivative into the ROU_{csf} and ROU_{bl} isolates. From each experiment, 100 erythromycin-resistant colonies were pooled. All biologic assays were done with subcultures obtained from these pools.

Negative staining and electron microscopic examination confirmed that the ROU_{csf}, ROU_{bl}, and ROU_{cp} isolates were piliated, whereas the PilC$^-$ derivatives did not express any pili (data not shown). All of the clinical isolates had well-individualized pili surrounding the bacteria, and bacteria obtained from CSF and the choroid plexus were surrounded by slightly more pili than was the isolate obtained from blood (data not shown). The pilus morphology was identical in all cases; the fibers were long and thin and not forming large bundles. This observation is consistent with the fact that none of these isolates formed large clumps when grown in liquid media.

Adhesion assays were done with two endothelial cell types, HBEC and HUVEC. This latter cell type mimics binding to choroidal EC, since capillaries at this level have a structure similar to that found in peripheral vessels. As shown in table 1, ROU_{bl}, ROU_{cp}, and ROU_{csf} isolates adhered to the surface of these cells. On the other hand, the nonpiliated PilC$^-$ derivatives were nonadhesive. Furthermore, isolates obtained from the CSF and the choroid plexus have a similar level of adhesiveness, which is several times higher, on both cell types, than the adhesiveness of the blood isolate. In addition, with the same inoculum and the same number of EC, adherence of all of these isolates was 10-fold less to HBEC than to HUVEC. This was also the case for the clone 12, a serogroup C isolate used as positive control in this experiment.

Taken together, these data demonstrate that pilus-mediated adhesion is the means by which the ROU isolates interact with EC in vitro and that the increased expression of PilC in the isolates obtained from the CSF and the choroid plexus correlates with a more abundant piliation and a higher adhesiveness to both endothelial cell types than that of ROU_{bl}. The fact that this increased production of PilC is already present in bacteria isolated from the choroid plexus, where most of the vessels had bacteria interacting with EC, is highly suggestive that this phenotypic change is selected for its ability to increase bacterial adhesiveness.

The activity of one of the pilC promotors is selectively increased in the isolate obtained from the CSF. To gain insights into the mechanism responsible for the high expression of PilC in the CSF and in bacteria isolated from the CSF and the choroid plexus, transcriptional analysis was done. In previous work using strain 8013, we showed that the two *pilC* alleles of this strain were transcribed differently [18]. *pilC2* was transcribed from only one promotor, whereas *pilC1* has three transcriptional start sites, designated PC1.1, PC1.2, and PC1.3. PC1.1 corresponds to the unique *pilC2* promotor. PC1.2 and PC1.3 are located in a region downstream of PC1.1. This region does not have a counterpart in *pilC2* and has been shown to contain a PilA-binding domain. As shown in figure 3, a similar region is found upstream of the unique *pilC* in strain ROU. Primer extension analysis using oligonucleotide C1-8 and mRNA prepared from ROU_{csf} and ROU_{bl} demonstrated that this locus is transcribed from only two

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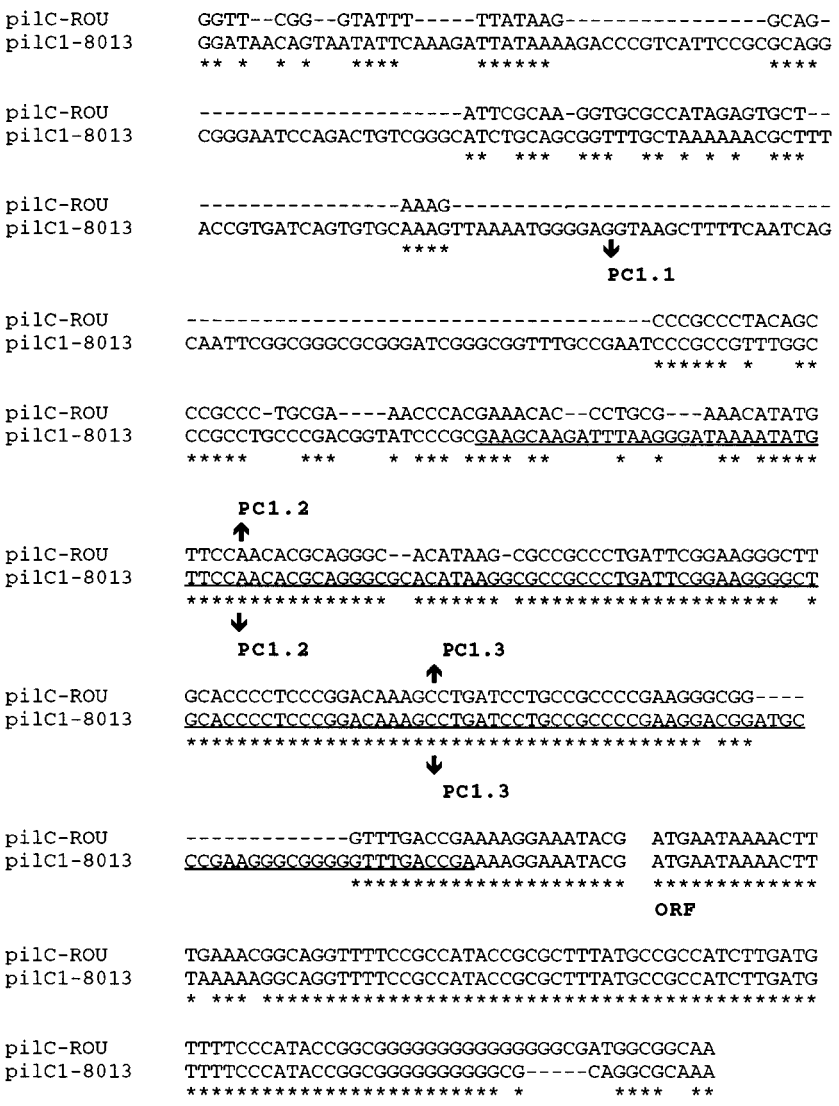

Figure 3. Sequence comparison of 5′ region of *pilC* allele of ROU strain with previously reported *pilC1* allele of strain 8013. Underlined region corresponds to *pilC1*-specific region containing PilA-binding domain. *pilC1* locus of strain 8013 is transcribed from 3 promotors, PC1.1, PC1.2, and PC1.3. Only PC1.2 and PC1.3 are present in ROU strain.

promotors localized in the region containing the PilA-binding domain. These promotors correspond to the previously designated PC1.2 and PC1.3 loci (figure 3). No transcriptional start site corresponding to PC1.1 was detected in the ROU strain. In addition, after the same amount of RNA was loaded, the *pilC* mRNAs corresponding to PC1.2 and PC1.3 were quantified by excising the bands corresponding to each start site and measuring their radioactivity. The ratios of ROU_{csf} to ROU_{bl} were 1.3 and 4, respectively, for PC1.2 and PC1.3. In addition, the ratios of PC1.3 to PC1.2 were 5.1 and 1.7 for ROU_{csf} and ROU_{bl}, respectively. These data show that PC1.3 is the stronger promotor in both isolates, and the higher expression of *pilC* in the CSF isolate is due to a higher transcription of the gene from the PC1.3 promotor.

Discussion

We investigated some aspects of invasion across the BBB by *N. meningitidis* in a case of fulminant meningococcemia in which death occurred before antibiotic treatment. Considering that the patient died after the onset of disease, before an acute inflammatory response, it is likely that it happened at a time when bacteria were crossing the BBB. At this stage, bacteria were found adhering to the EC of both the choroid plexus and the meninges, thus demonstrating that *N. meningitidis* is capable of interacting with the components of the BBB and that this interaction is likely to be required for CSF invasion. Regarding the route used by meningococci to invade the CSF, the fact that adhesiveness occurred preferentially inside the choroid plexus is in favor of a crossing of the BBB through this route. However, the lack of bacteria between the choroidal epithelial cells argues for a crossing directly through the capillaries of the meninges. However, a careful examination of the semithin sections of the choroid plexus did not reveal bacteria inside or between choroidal epithelial cells; therefore, it cannot be ruled out that meningococci may pass directly through the meningeal capillaries to the CSF. Indeed, both routes may be used by this pathogen to reach the CSF.

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Table 1. Adherence of meningococcal strains on endothelial cells.

	HUVEC		HBEC	
	Average	SD	Average	SD
Clone 12	0.415	0.035	0.039	0.018
ROU_{bl}	0.022	0.006	0.001	0.000
ROU_{csf}	0.073	0.020	0.007	0.005
ROU_{cp}	0.065	0.030	0.007	0.006
ROU_{bl} $PilC^-$	0.000	0.000	0.000	0.000
ROU_{csf} $PilC^-$	0.000	0.000	0.000	0.000

NOTE. HUVEC = human umbilical vein endothelial cells; HBEC = human brain endothelial cells. Averages and SDs were obtained from at least 3 individual experiments.

The fact that more vessels had adherent bacteria in the choroid plexus than in the meninges is striking. One explanation would be that the higher blood flow in the choroid plexus is responsible for this preferential binding. Alternatively, more receptors for meningococci could be present on the surface of the EC of the choroid plexus than on the meninges capillaries. In vitro adhesiveness demonstrated that *N. meningitidis* adhered better onto HUVEC (which are peripheral like EC) than onto HBEC, suggesting that the preferential binding to the EC of the choroid plexus could be related to the expression of a greater number of meningococcal receptors by these cells.

The bacterial attributes required for meningococci to invade the meninges remain unknown. Since different structures of the same protein may express different functions, antigenic variation has been implicated as a possible modulator of neisserial virulence [23]. In this work, the meningococci did not show any differences in pilin variants and class 5 protein variants expressed by isolates obtained from the blood and the CSF. On the other hand, we found that the isolates obtained from the CSF were expressing significantly more PilC protein than was the blood isolate. The fact that this phenotypic change is present on bacteria obtained from the choroid plexus, where they are interacting with a component of the BBB (see figure 1), rules out the possibility that the high expression of PilC is selected for once the bacteria have reached the CSF and suggests that this phenotypic change is required for the crossing of the BBB.

The mechanism for this high expression of PilC is an increase in activity of one of the *pilC* promotors. In addition, considering that this phenotype is stable, since isolated colonies of the ROU_{csf} isolate expressed a similar high level of PilC, this increase in transcriptional activity is likely to be due to a phase variation in a gene encoding an activator or repressor of this promotor. The ROU strain has only one *pilC* locus; this is distinct from what has been previously described with *N. gonorrhoeae* [12] and another *N. meningitidis* strain in which 2 loci, designated *pilC1* and *pilC2,* have been identified [13]. Recent data obtained with gonococci suggest that the PilC proteins are in fact the adhesins of type IV pili and are located at the tip of the pilus. The PilC proteins are also important for piliation and competence, since $PilC1^-/PilC2^-$ strains are nonpiliated and noncompetent. In the case of meningococci, a PilC1 allele is required for adhesion, since $PilC1^-$ derivatives are nonadherent. Transcriptional regulation is another difference between the two *pilC* alleles in *N. meningitidis:* The expression of PilC1 is under the control of PilA and a PilA-binding domain (a 150-bp sequence upstream of the open-reading frame) located in the promotor region. The presence of a similar sequence in the promotor region of the unique *pilC* allele of the ROU strain suggests that this allele is of the adhesive type.

It is generally assumed that pili and subsequently pilus-mediated adhesion play a major role in the crossing of the BBB [2]. The rationale for this comes from the fact that fresh clinical isolates are piliated. In this work we show that *N. meningitidis* adhere to the EC in the brain and that invasion of the CSF correlates with a higher expression of the PilC molecule, which is required for pilus-mediated adhesion. Taken together, these data strongly argue in favor of a role for pili in the crossing of the BBB. The mechanism by which the increased expression of PilC enhances bacterial adhesion is not clear. A likely explanation would be that the increase in piliation is responsible for this phenotypic change. However one cannot rule out the possibility that this increase in expression is responsible for more molecules of adhesins located at the tip of the pilus. Recently, Virji et al. [24] isolated a hyperadherent variant of a class 1 *N. meningitidis* strain with a high level of expression of PilC. This variant was able to withstand considerable shearing force and remained firmly attached to HUVEC. These authors suggested that a better anchorage of pili to bacterial surface could be responsible for this phenotype.

Two types of adhesion are mediated by pili, low and high adhesiveness. In a previous work, we showed that this difference in adhesiveness is influenced by pilin antigenic variation, some variants being responsible for high adhesiveness and others for low adhesiveness. We demonstrated that bundling of pili is the mechanism involved in high adhesiveness, by increasing bacteria-bacteria interactions and therefore allowing bacteria to grow as colonies on the surface of the cells [10]. Neither of the isolates of the ROU strains had pili assembled into bundles. This suggests that a colonial mode of growth is not necessary for meningococci to interact with the BBB.

A better understanding of the mechanisms responsible for bacterial pathogens' ability to cross the BBB will require the study of interactions with tight junction–forming cells, ideally using brain EC. Recently, a system using epithelial cells has been described [25], which could allow the more precise identification of the bacterial attributes beside pili that are involved in this step.

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