Roles of PilC and PilE Proteins in Pilus-Mediated Adherence of Neisseria gonorrhoeae and Neisseria meningitidis to Human Erythrocytes and Endothelial and Epithelial Cells

INA SCHEUERPFLUG,1,2† THOMAS RUDEL,2 ROLAND RYLL,1 JASMINE PANDIT,1 and THOMAS F. MEYER1,2,*

Abteilung Infektionsbiologie, Max-Planck-Institut für Biologie, 72076 Tübingen,1 and Max-Planck-Institut für Infektionsbiologie, 10117 Berlin,2 Germany

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Unlike other type 4 pili, the neisserial pilus consists of at least two distinct proteins, the highly variable major subunit PilE forming the pilus fiber and the tip-associated adhesin PilC. PilC protein purified either from gonococci or from Escherichia coli interacted with different human epithelial cell lines, primary epithelial and endothelial cells. The binding of PilC protein efficiently prevented the attachment of piliated Neisseria gonorrhoeae and Neisseria meningitidis to these cell types. Fluorescent beads coated with pilus prepared from pilus-deficient wild-type N. gonorrhoeae also adhered to these cells, in contrast to beads coated with pilus prepared from a piliated PilC-deficient mutant. In the latter case, the binding of fluorescent beads was restored after pretreatment of the pilus-loaded beads with purified PilC. Piliated wild-type N. gonorrhoeae, the piliated PilC-deficient mutant, and N. gonorrhoeae pilus assembled in Pseudomonas aeruginosa agglutinated human erythrocytes, while nonpiliated gonococci did not. Consistently, purified PilC did not agglutinate or bind to human erythrocytes, suggesting that N. gonorrhoeae PilE is responsible for pilus-mediated hemagglutination.

Both Neisseria gonorrhoeae and Neisseria meningitidis primarily infect mucosal cell surfaces of humans, their exclusive host. N. meningitidis colonizes the nasopharyngeal epithelium and rarely disseminates via the vascular system to penetrate the blood-brain barrier. N. gonorrhoeae causes the sexually transmitted disease gonorrhoea, which has a low incidence of dissemination. Although the primary infection routes for N. gonorrhoeae and N. meningitidis are different, the binding specificities for human tissues are similar. For both pathogens, the initial colonization step is mediated by type 4 pili, and pilus-mediated binding also appears to be critical in defining host specificity. The type 4 pilus function as adhesins for epithelial and endothelial cells.

Only recently has the nature of the pilus adhesive been elucidated. The adhesin critical for the binding of N. gonorrhoeae to human epithelial cells has been identified as PilC (37). The PilC protein is produced in small quantities and is encoded by two variant genes in N. gonorrhoeae MS11 (12). The expression of PilC is controlled by short variable G stretches affecting the translational reading frame and the expression of each pilC gene (12) and thus pilus-mediated adherence to epithelial cells (26, 28, 38). The PilC protein has been located at the tip of type 4 pili as well as on the surface of N. gonorrhoeae (33, 36). Surface-bound PilC is involved in DNA uptake (36) and probably also in pilus transport (4). Purified PilC binds to human epithelial cells in vitro (37). Interestingly, the binding of PilC to epithelial cells prevents the binding of both N. gonorrhoeae and N. meningitidis, irrespective of the variant of PilC produced, indicating that both pathogens recognize related or identical receptors on human epithelial cells. Similar studies have been performed with an N. meningitidis strain which produces so-called class II type 4 pili, thus generalizing the function of PilC as a neisserial pilus adhesin (39).

Several previous researchers suggested a role for the major pilus subunit PilE in receptor recognition (28, 34, 38, 41, 46, 49). We recently proposed that N. gonorrhoeae pilus may comprise at least two distinct binding specificities, one for epithelial cells which is dependent on PilC and another for human erythrocytes (38). The ability of a piliated PilC-deficient mutant to agglutinate human erythrocytes and its lack of binding to epithelial cells already have suggested the possibility that PilE is a hemagglutinin (35); however, the question remains as to whether other factors contribute to hemagglutination. Only the use of purified components would identify the agglutinin for human erythrocytes. The concept of two different binding specificities located in two different components of the pilus is complicated by the fact that PilE undergoes antigenic variation (24), which influences epithelial cell-specific adherence (18, 38, 50) rather than erythrocyte binding (38).

The molecular basis of pilus-mediated binding of pathogenic Neisseria to human endothelial cells has not been elucidated so far. Virji et al. (48) demonstrated an efficient interaction of piliated variants of N. meningitidis and N. gonorrhoeae with human umbilical vein endothelial cells (HUVEC). In contrast to adherence to epithelial cells, the pilus-mediated binding of N. gonorrhoeae to HUVEC was not substantially influenced by antigenic variant PilE proteins (48). Also, N. meningitidis derivatives which expressed either class I or class II pilin adhered similarly to HUVEC, suggesting that a common epitope of Neisseria pilus was involved in the pilus-endothelial cell interaction.

In this study, we investigated the role of PilC purified either from N. gonorrhoeae or from Escherichia coli in the pilus-mediated binding of N. gonorrhoeae and N. meningitidis to epithelial and endothelial cells. We demonstrated the binding of purified PilC proteins to different cell types and performed adherence competition experiments by using purified PilC with
N. gonorrhoeae and N. meningitidis strains. Recombinant Pseudomonas aeruginosa derivatives forming gonococcal pili were examined, demonstrating that PilE was the erythrocyte-specific adhesin of N. gonorrhoeae. Further evidence for the distinct adherence functions of PilE and PilC was provided by a novel in vitro binding assay with purified components.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** All pathogenic bacterial strains used in this work are depicted in Table 1. E. coli strains were grown in Luria broth (LB) medium supplemented with ampicillin (100 μg ml⁻¹) or erythromycin (250 μg ml⁻¹) as needed. Gonococcal and meningococcal strains were grown on GC agar base with vitamin supplements (Becton Dickinson) at 37°C in 5% CO₂. Recombinant gonococci were grown on GC agar base supplemented with tetacycline (10 μg ml⁻¹), erythromycin (7 μg ml⁻¹), chloramphenicol (12 μg ml⁻¹), or isopropyl-β-D-thiogalactopyranoside (IPTG) (100 μg ml⁻¹) as needed. P. aeruginosum K2P8 X90 was grown at 37°C on LB plates and on GA agar base containing carbenicillin at 500 μg ml⁻¹. Plasmids pTR81, pTR102, pIS25, and pHIS26 (Table 2) were maintained in E. coli K-12 strain DH5α grown at 37°C.

**Construction of strains and plasmids.** All plasmids used in this work are described in Table 2. All enzymes were used in accordance with manufacturer instructions, and the remaining cloning procedures were carried out by standard methods (40). The basis for the construction of the pilC2His6 (see below) gene was pilC2His6 and was inserted in Hermes-6a, which contains the invariant pilC2 gene in a Hermes vector. This plasmid was used for PCR, which resulted in two fragments. A second PCR with those two fragments resulted in a fragment encoding the N-terminal domain of PilC with six histidines (His6) inserted (pC2His6) (37). This N-terminal fragment with the histidine insertion was exchanged for the N-terminal portion of PilC encoded by pTR31, generating pHIS26.

**Transformation and conjugation of N. gonorrhoeae and E. coli.** Transformation and conjugation of gonococci were carried out as described by Rudek et al. (38). E. coli K-12 was transformed by the method of Messing and Vieira (21).

**Purification of PilC2His6 protein.** Gonococcal strain N505 was induced overnight on GC agar plates containing tetracycline at 10 μg ml⁻¹ and IPTG at 100 μg ml⁻¹ at 37°C in 5% CO₂. E. coli H2627 was grown overnight on LB plates with ampicillin (100 μg ml⁻¹). PilC protein expression was induced in liquid cultures by adding IPTG at 100 μg ml⁻¹ for 2 h at 37°C. The bacteria were pelleted, suspended in 50 mM Tris-Cl (pH 8.0)–150 mM NaCl, lysed by sonication. The suspension was centrifuged at 4,000 rpm in a Sorvall centrifuge for 20 min at 4°C to separate the bacterial membranes. The membranes were harvested by centrifugation at 35,000 × g for 1 h at 4°C. The PilC2His6 (PilC2 with His6 attached) protein was dissolved by incubating the membranes in 2% N,N-dimethyl/dodecylamine-N-oxide (LDAO) in 50 mM Tris-Cl (pH 8.0)–150 mM NaCl and lysed by sonication. The suspension was centrifuged at 4,000 rpm in a Sorvall centrifuge for 20 min at 4°C to separate the bacterial membranes. The membranes were harvested by centrifugation at 35,000 × g for 1 h at 4°C. The PilC2His6 protein was eluted by a shift of the pH from 8.0 to 4.0 with 10 mM sodium citrate buffer containing 150 mM NaCl.

**Transformation of pili.** The gonococcal and pseudomonal pili were isolated by the method of Brinton et al. (2). The pilC2His6 mutant was grown for 18 h on GC agar plates. After harvesting the bacteria in 50 mM Tris-Cl (pH 8.0)–150 mM NaCl, the bacteria were washed twice and resuspended in 0.15 M carbonate buffer (pH 10.5). The pili were sheared off in a Sorvall Omnimixer at 5,000 rpm for 60 s on ice. The cell debris was removed by centrifugation at 13,000 × g for 30 min at 4°C. The supernatant was dialyzed against PBS (pH 7.4) overnight at 4°C. The pili crystalized and could be collected by centrifugation at 15,000 × g for 60 min at 4°C in a Sorvall centrifuge. After resuspension of the pellet in carbonate buffer, the suspension was centrifuged at 20,000 × g for 30 min at 4°C to remove insoluble outer membrane proteins. Afterward, the supernatant was dialyzed against PBS (pH 7.4) overnight at 4°C. The crystallization and solubilization steps were repeated three times to obtain pili of a high purity.

**Covaspheres.** The purified PilC2His6 protein and the purified pili were valued by Covaspheres MX fluorescent particles (0.5 μm) (Duke Scientific Corporation) by the method described by the manufacturer. Fluorescent beads (100 μl) were mixed with 20 μg of purified PilC2His6 protein, the same amount of purified pili, or fetuin at a ratio of 1:2. For coupling, the probes were

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**TABLE 1. Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Recipient strain, relevant genotype, and autonomous plasmid</th>
<th>Plasmid(s) used for gene replacement</th>
<th>Relevant phenotype*</th>
<th>Reference or source</th>
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<td></td>
<td>P' PilC* PilE1 Opa-*</td>
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<tr>
<td>N138 N. gonorrhoeae MS11 variant F3; ΔpilE2</td>
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<td>P' PilC* PilE3 Opa-*</td>
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<td>N300 N219; opa-50; pTH7</td>
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<td>pC20, Hermes-7</td>
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<td>pC20, pEMK55</td>
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* P' PilC* PilE1 Opa-* with six histidines (His6) inserted (pC2His6). This work

**TABLE 2. E. coli plasmids used in this study**

<table>
<thead>
<tr>
<th>Plasmid</th>
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<th>Source or reference</th>
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<td>Bluescript KS Hermes-8</td>
<td>Cloning vector</td>
<td>Stratagene 16</td>
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<td>Promoterless cat gene in SfiI site of opa-50 locus</td>
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<tr>
<td>pEMK55</td>
<td>pVHIS26 pilC1; library clone</td>
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<tr>
<td>pTR25</td>
<td>pTR25; ermC in SmaI site</td>
<td>35</td>
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<tr>
<td>pTR32</td>
<td>pTR27; cat-1 between SmaI and SmaI sites</td>
<td>35</td>
</tr>
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<td>pTR36</td>
<td>pTR25; cat-1 in SmaI site</td>
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<td>pTR45</td>
<td>pTR27; cat-1 between XhoI and XhoI sites</td>
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<td>pTR81</td>
<td>pilC2ΔermC in Bluescript KS</td>
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<tr>
<td>pIS25</td>
<td>pilC2ΔermC in Bluescript KS</td>
<td>This work</td>
</tr>
<tr>
<td>pPHIS26</td>
<td>pilC2ΔermC in Hermes-8</td>
<td>This work</td>
</tr>
<tr>
<td>pRH117</td>
<td>pPAHI121; pilE1</td>
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* pilC2ΔermC, pilC2ΔermC with His6 coding sequence.
rotated for 1 h at room temperature and pelleted by centrifugation. The supernatant was collected, and the protein concentrations before and after coupling were calculated on a sodium dodecyl sulfate (SDS) gel after silver staining. The unoccupied sites on the Covaspheres were saturated by incubation in 20 mM Tris-Cl (pH 7.5)–1% formalin for 10 min.

Cell cultures. Tissue culture reagents were obtained from Gibco-BRL. The epithelial cell lines used in the adherence experiments were ME-180 human cervix carcinoma (ATCC HTB13), Hec-1B human endometrium carcinoma (ATCC HTB133), RT112 human urinary bladder carcinoma (kindly provided by W. W. Franke, German Cancer Research Center, Heidelberg, Federal Republic of Germany [FRG]), Chang human conjunctiva (ATCC CCL20.2), MDCK (ATCC CCL34), MDBK, and PSEK (kindly provided by H.-J. Rziha), and NIH 3T3 mouse fibroblast (ATCC CRL1658). The RT112 cells were routinely maintained in Waymouth’s MB752/1 medium supplemented with 10% fetal calf serum (FCS), the MDBK and NIH 3T3 cells in were grown in Dulbecco minimal essential medium with 10% FCS, and the PSEK cells were grown in minimal essential medium with nonessential amino acids and 5% FCS at 37°C in 10% CO₂. All the other epithelial cell lines were cultured in RPMI medium supplemented with 5% FCS at 37°C in 5% CO₂. The endothelial cells used were HUVEC (Promocell, Heidelberg, FRG), which were cultured in endothelial cell growth medium (Promocell) at 37°C in 5% CO₂. Cultures of primary cornea epithelium were maintained as previously described (44).

Adherence and adherence competition experiments. For the adherence and adherence competition experiments, epithelial cells were cultivated on glass coverslips in 24-well plates until preconfluent. The HUVEC were placed on glass coverslips coated with 0.2% gelatin to mediate adherence of the endothelial cells to the glass slides. All cells were preincubated for competition experiments by adding purified PilC2His₆ protein at 600 ng ml⁻¹ to epithelial and endothelial cells, incubating the mixture for 20 min at 37°C, and washing the mixture twice with PBS-Ca²⁺–Mg²⁺. The cells were infected with 5 × 10⁵ bacteria in 0.5 ml of medium without FCS per well for 1 h at 37°C in 5% CO₂. To stop the infection and to remove nonbond bacteria, the cells were washed five times with PBS-Ca²⁺–Mg²⁺. Then, the cells were fixed with 2% paraformaldehyde in PBS for 30 min and stained with crystal violet. The number of adherent bacteria was established and compared with the adherence of wild-type strain N138, which is set at 100% adherence. Representative photographs were taken to show the adherence patterns of the bacteria. All gonococci used in this assay lacked the expression of pilC₂ (38) (Fig. 2b, d, f, and h). Furthermore, strong adherence and adherence competition experiments, epithelial cells irrespective of whether it was derived from recombinant E. coli or from recombinant N. gonorrhoeae. Indeed, PilC₂His₆ from both preparations bound to ME-180, Hec-1B, and RT112 human epithelial cells, which were shown before to be good substrates for the adherence of pilated gonococci (38) (Fig. 2b, d, f, and h). Furthermore, strong binding was detected with human primary cornea epithelial cells (Fig. 2k), in contrast to the same cell type from sheep, bovine, or porcine origin (data not shown). Also, no binding of the protein to nonhuman cell lines which did not interact with (PilC₂His₆) by affinity chromatography through a Ni²⁺-nitrilotriacetic acid column (10). The latter modification, which generated pilS25, was achieved by substitution of the 5’ region of pilC₂ in pTR81. To produce PilC₂His₆ in a PilE-free environment, the BamHI-HindIII fragment of pilS25 was subcloned into the Hermes-8 shuttle vector (16), generating pilS26, which was used to transform N219 to generate N879 containing pHS126, the recombinant pmetM₂5.2 plasmid encoding PilC₂His₆. N879 was used as a donor for the conjugal transfer of pHS126 into N655, a mutant of strain MS11 carrying deletions in pilE and both pilC genes (35, 38), yielding the final PilC-overproducing strain, N656.

The recombinant PilC₂His₆ protein was purified from IPTG-induced cultures as described in Materials and Methods. To exclude any contaminating gonococcal proteins or LPS, both individual and pooled fractions were tested on silver-stained gels (Fig. 1). Furthermore, the PilC₂His₆ protein could also be purified from recombinant E. coli H2627 carrying pilS25. PilC₂His₆ protein produced in E. coli was purified by essentially the same procedure; however, this preparation was slightly contaminated by some unidentified E. coli proteins (Fig. 1). The functional integrity of PilC₂His₆ was tested by genetic complementation of the pilC double mutant N621 with plasmid pH126, yielding strain N881.

Binding of purified PilC₂His₆ to human epithelial cells and competitive inhibition of pilus-mediated adherence of N. gonorrhoeae and N. meningitidis. We demonstrated previously the binding of purified PilC₂His₆ protein to ME-180 human cervix carcinoma epithelial cells but the lack of binding to MDCK cells (37). Here we show that the binding of the recombinant PilC₂His₆ protein exhibited the same pronounced specificity for epithelial cells irrespective of whether it was derived from recombinant E. coli or from recombinant N. gonorrhoeae. Indeed, PilC₂His₆ from both preparations bound to ME-180, Hec-1B, and RT112 human epithelial cells, which were shown before to be good substrates for the adherence of pilated gonococci (38) (Fig. 2b, d, f, and h). Furthermore, strong binding was detected with human primary cornea epithelial cells (Fig. 2k), in contrast to the same cell type from sheep, bovine, or porcine origin (data not shown). Also, no binding of the protein to nonhuman cell lines which did not interact with

FIG. 1. Demonstration of PilC₂His₆ proteins purified from N. gonorrhoeae and E. coli. The protein gel shows pure silver-stained PilC₂His₆ proteins from recombinant E. coli H2627 (lane 1) and from N. gonorrhoeae (lane 2). Lane M, molecular weight markers (in thousands).
FIG. 2. Binding of purified PilC2His6 proteins to different human epithelial cell types. (Left panels) Untreated epithelial cells. (Right panels) Epithelial cells after treatment with 600 ng of pure PilC2His6 protein from N. gonorrhoeae ml⁻¹, except that panels c and d show cells not treated (c) and treated (d) with PilC2His6 purified from E. coli. The bound proteins were stained with rabbit anti-PilC serum (AK217) and fluorescein isothiocyanate-labelled secondary antibody. Epithelial cells were ME-180 (a to d), Hec-1B (e and f), RT112 (g and h), and human primary cornea epithelial cells (i and k). Bars, 10 μm.
piliated gonococci, such as MDCK, MDBK, PSEK, and NIH 3T3, was observed (data not shown). Thus the PilC2His6 protein purified from \textit{E. coli} showed the same binding specificity for epithelial cells as the protein prepared from gonococci (Fig. 2d).

To test whether \textit{E. coli} PilC2His6 was capable of blocking the binding of piliated \textit{N. gonorrhoeae} to the epithelial cell receptor(s), we performed adherence competition experiments. PilC protein indeed blocked the binding of piliated \textit{N. gonorrhoeae} N137 (PilE\textsubscript{F3}) (Fig. 3A, panel b), N137 (PilE\textsubscript{E1}), and N200 (PilE\textsubscript{A}) (data not shown) and of piliated \textit{N. meningitidis} N862 to several human epithelial cell lines (Fig. 3B, panels b, d, and f). The most efficient binding of piliated wild-type gonococci was observed with human primary cornea epithelial cells (Fig. 3C, panel a). The individual primary cornea epithelial cells differed in their capacities to bind gonococci, probably due to their differentiation status. While some of the cells were completely covert, others bound less of the piliated gonococci. To inhibit the attachment of the wild-type strain N138 (PilEF\textsubscript{3}) to primary cornea epithelial cells, which have a high affinity for piliated gonococci, an approximately 20-fold-larger amount of purified PilC2\textsubscript{His6} protein was required, compared to the amount required to inhibit the binding of \textit{N. gonorrhoeae} to permanent epithelial cell lines (Fig. 3C, panel b). This finding might indicate a higher density of accessible PilC receptors on the primary cells.

Treatment of the target cells with an unrelated His\textsubscript{6}-tagged antibody fragment, the elution buffer without PilC protein, or the heat-treated PilC2\textsubscript{His6} protein did not affect the attachment of either \textit{N. gonorrhoeae} or \textit{N. meningitidis} to any of the epithelial cells, consistent with previous results (37). Treatment of Chang conjunctiva epithelial cells with PilC2\textsubscript{His6} had no influence on the non-pilus-mediated adherence conferred by the phase-variable Opa adhesin; for example, the ability of the Opa50-expressing, nonpiliated strain N303 (19) to adhere was not affected (data not shown). Thus, the inhibition of gonococcal adherence by PilC2\textsubscript{His6} protein is pilus specific.

Preincubation of the epithelial cells with pili purified from wild-type strains or piliated pilC double mutant N556 (35) at concentrations of about 5 \(\mu\)g/ml or more could not prevent the adherence of wild-type \textit{N. gonorrhoeae} to epithelial cells (data not shown). Since the amount of PilC in pilus preparations from wild-type strains is below 1% compared with the amount of PilE, the PilC concentration in these experiments was at least 50-fold lower than that required for competitive inhibition of adherence by PilC. This experiment thus provides evidence that PilE is not an efficient competitor for the binding of pilated \textit{Neisseria} strains.

Purified PilC protein binds to HUVEC and inhibits the pilus-mediated adherence of gonococci and meningococci. Pili constitute important determinants for the association of \textit{N. meningitidis} and \textit{N. gonorrhoeae} with human endothelial cells (26, 28, 48). In order to define the role of PilC in the interaction of \textit{Neisseria} with endothelial tissue, PilC2\textsubscript{His6} binding and adherence inhibition assays similar to those described for ep-
ithelial cells were performed. First, several pathogenic Neisse-
ria strains were tested for adherence to HUVEC. Piliated 
gonococcal strains N137 (PilC1 PilEE1) and N138 (PilC1 
PilEF3) and meningococcal strain N862 strongly bound to 
HUVEC (Fig. 4). In contrast, the piliated pilC double mutant 
N556 did not interact with HUVEC (Fig. 4e). This finding 
suggests that PilC is involved in the pilus-dependent adherence 
of N. gonorrhoeae to HUVEC. Consistently, after treatment of 
HUVEC with purified PilC2 His6 protein at a concentration of 
600 ng/ml, the binding of gonococcal as well as meningococcal 
strains was prevented (Fig. 4b, d, and h). Furthermore, efficient 
binding of PilC2His6 to HUVEC was demonstrated by immu-
nostaining of bound protein on the cells with the specific PilC 
antiserum (Fig. 4m). Hence, PilC proteins from different 
N. gonorrhoeae and N. meningitidis strains constitute endothelial 
cell-specific adhesins.

PilC is able to mediate the binding of purified pili to human 
epithelial cells. To address the question of whether PilE is able 
to bind to epithelial cells, we set up an in vitro binding assay 
with purified components. Pili isolated from wild-type strain 
N137 and from the piliated pilC double mutant N556 and 
purified PilC2His6 protein alone were coated on Covasphere 
MX fluorescent particles and analyzed for binding to ME-180 
epithelial cells and MDCK epithelial cells. Covaspheres coated 
with pili from strain N137 or with isolated PilC2His6 protein 
bound well to ME-180 cells (Table 3 and Fig. 5), but essentially 
no binding was observed for MDCK cells, with the exception of 
apparently dead cells (data not shown). Interestingly, the pat-
terns of binding of the two samples to ME-180 cells appeared 
to be different. In contrast, Covaspheres coated with strain 
N556 pili or with isolated PilC2His6 protein bound well to ME-180 cells. However, the same Covaspheres coated with N556 pili bound to ME-180 but not MDCK cells when supplemented with 400 ng of purified PilC protein (Fig. 5g and h). In a control experiment, Covaspheres coated with other proteins, such as fetuin, a glycoprotein purified from FCS, showed no binding to any of the cell lines (Fig. 5a and b).

To strengthen the evidence obtained for the adherence of 
PilC to epithelial cells, purified N. gonorrhoeae F3 pili pro-
duced in P. aeruginosa X91 (11) were tested in the same assay 
and compared to a control pilus preparation from nonrecom-
binant P. aeruginosa X90. N. gonorrhoeae F3 pili from X91 
as well as pili from X90 coated on beads were unable to bind to 
ME-180 cells. However, when PilC protein was added to these 
beads, X91 pili but not X90 pili adhered strongly to ME-180 
cells (Table 3). These experiments suggested that variant pili 
derived from either N556 or X91 are not able to interact with 
epithelial cells in the absence of PilC protein. Rather, in this

**TABLE 3. Binding of fluorescent particles coated with purified 
components to ME-180 cells**

<table>
<thead>
<tr>
<th>Purified protein + supplement</th>
<th>Binding to ME-180 cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>N137 pili</td>
<td>+</td>
</tr>
<tr>
<td>N556 pili</td>
<td>-</td>
</tr>
<tr>
<td>X91 × N138 pili</td>
<td>-</td>
</tr>
<tr>
<td>X90 pili</td>
<td>-</td>
</tr>
<tr>
<td>N556 pili + PilC2_His6 protein</td>
<td>+</td>
</tr>
<tr>
<td>X91 + PilC2_His6 protein</td>
<td>-</td>
</tr>
<tr>
<td>X90 + PilC2_His6 protein</td>
<td>-</td>
</tr>
<tr>
<td>PilC2_His6 protein</td>
<td>+</td>
</tr>
<tr>
<td>Fetuin</td>
<td>-</td>
</tr>
<tr>
<td>Fetuin + PilC2_His6 Protein</td>
<td>-</td>
</tr>
</tbody>
</table>

a 1+, very strong binding; +, strong binding; -, no binding.
FIG. 5. Adherence of Covaspheres fluorescent particles to ME-180 cells as seen by fluorescent microscopy (left panels) and light microscopy (right panels). Particles were coated with fetuin (a and b), purified PilC2\textsubscript{exo} protein (c and d), pili purified from *N. gonorrhoeae* N137 (e and f), and pili from the pilC double mutant N556 (g and h). (i and k) Restoration of binding of Covasphere particles coated with pili from N556 after preincubation of the particles with 400 ng of purified PilC2\textsubscript{exo} protein ml\textsuperscript{-1}.
novel in vitro binding assay, PilC protein is an essential component for the binding of pilus to human epithelial cells.

**PilE is an adhesin for human erythrocytes.** Besides epithelial cell-specific adherence, gonococcal pili are known to cause agglutination of human erythrocytes (3, 15, 38). Since PilC-deficient mutants still agglutinate human erythrocytes, this type of binding might be independent of PilC protein. To elucidate the pili adhesin specific for erythrocytes, several strains, purified pili, and pure PilC2His6 protein were analyzed in hemagglutination experiments. Recombinant *P. aeruginosa* X91 expressing *N. gonorrhoeae* MS11 F3 pili (11) as well as preparations of *N. gonorrhoeae* F3 pili produced in *P. aeruginosa* were included in the hemagglutination assay. All pilated *N. gonorrhoeae* strains as well as the purified pili caused a strong hemagglutination reaction, independent of the particular variant PilE and of the presence or absence of PilC protein (Table 4). Also, the recombinant *P. aeruginosa* strain producing *N. gonorrhoeae* F3 pili and purified pili from the recombinant *P. aeruginosa* strain agglutinated human erythrocytes, whereas the parental *P. aeruginosa* strain or pilus preparations from this strain did not agglutinate erythrocytes. The addition of pure PilC2His6 protein to human erythrocytes did not result in agglutination (Table 4). Nor could agglutination of erythrocytes by purified pili be inhibited by preincubation of the erythrocytes with PilC2His6 protein purified from *N. gonorrhoeae* or *E. coli*.

In agreement with the results of these experiments, immunofluorescence studies with antisera raised against purified PilC2His6 protein (AK217) confirmed that the PilC protein did not bind to human erythrocytes (data not shown). This result suggests that the two different binding specificities of the gonococcal pili are elicited by two different pilus proteins: PilC functions as an adhesin for pilus-mediated binding to epithelial and endothelial cells, and PilE probably functions as a hemagglutinin. These biochemical data are thus consistent with our previous genetic data (38).

**DISCUSSION**

The type 4 pili of *N. gonorrhoeae* and *N. meningitidis* constitute key determinants for the adherence of these pathogens to epithelial and endothelial cells and for the agglutination of human erythrocytes (27, 38, 45, 48, 49). Under natural conditions, pili represent the only means for capsulated *N. meningitidis* to adhere to human mucosal surfaces (45, 49); pili were demonstrated to be essential for the establishment of experimental infections by *N. gonorrhoeae* (20, 43, 51).

The characterization of the pilus adhesins is complicated by the extreme structural variability of the major pilus subunit PilE (for a review, see references 23 and 42), which influences pili binding to epithelial cells (18, 27, 38, 45) and, in a different way, also to endothelial cells (45, 48, 49) but not to human erythrocytes (38). One or more of these binding specificities could be due to adhesive domains located in PilE, as already suggested (34, 41, 47). Furthermore, both the *N. gonorrhoeae* and the *N. meningitidis* pili were recently found to carry unique glycosylation sites (30, 50), which could exert adhesive properties or modulate the adherence of neisserial pili.

Other experimental approaches indicated that the gonococcal pilus was not a homogeneous structure consisting of a single repeated subunit but rather contained minor proteins (25, 29). One of these pilus-associated proteins, PilC, was cloned and characterized as a pilus assembly factor found in the bacterial outer membrane (12). However, it was possible to assemble pili in the absence of PilC, and such pili had lost the ability to adhere to human epithelial cells (35, 38). Furthermore, in a *N. meningitidis* strain, pili assembled in the presence of two variant PilC proteins differed dramatically in their binding to human epithelial as well as endothelial cells (26). Only recently were gonococcal PilC proteins shown to represent type 4 pilus tip-located adhesins capable of competing for the pilus receptor on human epithelial cells (37). This observation agrees with the notion that neisserial pili, perhaps in contrast to other known type 4 pili, require a relatively conserved adhesin because of the extreme and unique variability of the major subunit PilE.

The aims of this study were to further assess the role of PilC (besides its accessory function in pilus assembly and natural transformation competence) as a pilus adhesin in the gonococcal pilus and to evaluate its specificity for different targets encountered during neisserial infections in the human host. The production of PilC2His6 protein in recombinant *E. coli* definitely ruled out the activity of any other neisserial factors in the preparation, such as lipopolysaccharide or other proteins. Attempts to purify a functional PilC2 fusion protein from *E. coli* inclusion bodies have failed so far (33, 36a), probably because the proper three-dimensional structure of PilC is essential for receptor recognition. PilC proteins contain several cysteine residues which are oxidized in the native molecule to form disulfide bonds. This fact is clearly apparent in the different migrations of PilC1 and PilC2 in SDS-polyacrylamide gels under reducing versus nonreducing conditions (36a). It is therefore not surprising that purified PilC fusion proteins are not functional as adhesins because folding during membrane transport may be an essential step. Consistent with this assumption is that *E. coli*-derived PilC2His6 purified from membranes exhibited the same specificity for human epithelial cells as did PilC2His6 purified from the *N. gonorrhoeae* overproducing strain.

As targets for *E. coli* PilC2His6, human primary cornea epithelial cells and permanent epithelial cell lines were identified, but primary cornea epithelial cells and permanent epithelial cell lines from nonhuman animals and human erythrocytes were not targets. Thus, independent of the bacterial background in which the PilC2His6 protein was produced, it exhibited the same strong species and cell type specificities. Furthermore, the binding affinities of PilC2His6 protein purified from *N. gonorrhoeae* and *E. coli* were probably identical, since about the same amounts of PilC2His6 protein were needed in order to competitively inhibit pilus-mediated binding of several different neisserial strains.

The novel in vitro binding assay allowed us to study the binding of cell-free pilus preparations. Cowasphere fluorescent particles coated with wild-type pili or purified PilC2His6 protein

<table>
<thead>
<tr>
<th>Strain or purified protein</th>
<th>Agglutination of erythrocytes by strain/purified protein</th>
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</thead>
<tbody>
<tr>
<td>N137 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>N138 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>N140 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>N200 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>N300 cells</td>
<td>+/+</td>
</tr>
<tr>
<td>N556 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>X91 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>X90 cells or pili</td>
<td>+/+</td>
</tr>
<tr>
<td>N560 PilC2His6 protein</td>
<td>−</td>
</tr>
<tr>
<td>H2627 PilC2His6 protein</td>
<td>−</td>
</tr>
</tbody>
</table>

* +, positive reaction; −, negative reaction.
adhered strongly to epithelial cells, whereas coating of the same particles with PilC-free pili purified from strain N556 or recombinant \emph{P. aeruginosa} X91 did not result in significant binding. In contrast, at least two variant forms of PilE, PilEF\textsubscript{N556} and PilEF\textsubscript{X91}, were able to result in adherent pili when produced in PilC\textsuperscript{+} gonococci (35, 38). The in vitro binding behavior of pilus preparations was thus consistent with the binding of piliated PilC-producing \emph{N. gonorrhoeae} strains and the inability of piliated PilC-deficient mutants to bind to epithelial cells (37, 38).

The addition of pure PilC\textsubscript{246}p was sufficient to convert the Covasphere particles coated with PilC-deficient pili produced by \emph{N. gonorrhoeae} or \emph{P. aeruginosa} from no binding to strong and specific binding. This result might indicate that PilC directly interacts with purified PilE but not with pili from non-recombinant \emph{P. aeruginosa} or with fetuin. We observed, however, slightly weaker binding of PilC-complemented X91 pili than of N556 pili. This result might well have depended on different binding efficiencies of variant pilins. We cannot, however, exclude the possibility that additional factors in the gonococcal pili facilitated the proper presentation of adhesive PilC.

In this context, it is also of interest that slightly different binding patterns were observed depending on whether the Covasphere particles were coated with PilC alone or in the context of purified pili (i.e., PilE). This result may suggest that PilE somehow modulates or contributes to the binding of \emph{N. gonorrhoeae} pili to epithelial cells, perhaps by recognizing a secondary receptor. This secondary receptor may be related to the postulated PilE-specific receptor on human erythrocytes. The receptor for PilC on target cells has not been identified yet. However, the membrane cofactor protein (MCP or CD46) was recently shown to function as a cellular receptor for the pili of both pathogenic Neisseria species (14). Consistent with the phase-variable binding patterns of piliated strains, MCP is expressed on almost every human cell type, with the exception of erythrocytes. This interesting distribution of MCP\textsubscript{P} correlates well with the binding specificity of the PilC adhesin, making MCP\textsubscript{P} a candidate receptor for PilC.

The same pilus preparations which displayed PilC-dependent binding to epithelial cells efficiently agglutinated human erythrocytes independent of the presence of PilE in vitro. The PilC-independent agglutination of human erythrocytes has been described before, leading to the hypothesis of two different binding properties associated with different binding domains of gonococcal pilus pili (38). Strong evidence for PilE as the hemagglutinin was provided by an analysis of the gonococcal pilus formed by a recombinant \emph{P. aeruginosa} strain. Whereas neither intact wild-type \emph{P. aeruginosa} nor wild-type pili preparations agglutinated erythrocytes, a clonal gonococcal PilE protein expressed in the recombinant strain and pilus preparations from the recombinant strain strongly agglutinated erythrocytes. Therefore, the hemagglutinin is located in PilE and likely includes the relatively conserved regions already suggested to be involved in receptor recognition (34, 41).

As already described for pili of \emph{Enterobacteriaceae}, gonococcal pili, belonging to the type 4 pilus class, represent a further example of pili exhibiting multiple binding specificities conferred by different pilus proteins. For instance, Pap pili of the F7 type contain, in addition to the \(\alpha\)-Gal-(1-4)-(Gal)-specific adhesin FsoG, the pilus-associated FsoE and FsoF proteins, which bind to fibronectin (52). Similarly, the tip adhesin SfaS of \emph{E. coli} binds to receptors containing neuraminic acid, whereas the major subunit confers binding to brain sulfate glycolipids, which lack any neuraminic acid (32).

The intriguing question of how PilC-dependent binding to epithelial and endothelial cells is modulated by variant PilE remains to be answered. Virji et al. (45) found several meningococcal PilE variants which bound more strongly to endothelial cells than to epithelial cells. Since we and others (26) were able to demonstrate the involvement of PilC in pilus adhesion to both cell types, PilE or other pilus proteins may influence the recognition of the receptor of PilC. A similar phenomenon has been described for the neuraminic acid-specific adhesin SfaS, which is able to cause agglutination of human erythrocytes. The SfaS adhesin is able to acquire two conformations, depending on expressed SfaA. A change in the conformation of the SfaA subunit leads to a change in the conformation of the SfaS adhesin and results in altered pilus receptor specificities (7, 31). Certain domains of PilE may be masked or may be presented differently, depending on the context of the variant pilus proteins. These characteristics may influence the recognition of similar but not identical receptors on epithelial and endothelial cells.

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