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Opa binding to cellular CD66 receptors mediates the transcellular traversal of *Neisseria gonorrhoeae* across polarized T84 epithelial cell monolayers

Jun Wang,[1,2] Scott D. Gray-Owen,[1] Alexander Knorre,[1†] Thomas F. Meyer[1,2*] and Christoph Dehio[1]
[1]Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, Spemannstraße 34, D-72076 Tübingen, Germany.
[2]Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Monbijoustraße 2, D-10117 Berlin, Germany.

Summary

We have analysed the capacity of the 11 phase-variable, opacity-associated (Opa) proteins encoded by *Neisseria gonorrhoeae* MS11 to mediate traversal across polarized monolayers of the human colonic carcinoma T84 cell line. Gonococci expressing either the heparan sulphate proteoglycan (HSPG) binding Opa protein (Opa_{50}) or no Opa protein (Opa^-) did not interact with the apical pole of T84 monolayers, whereas the 10 variant Opa proteins previously shown to bind CD66 receptors were found to mediate efficient gonococcal adherence and transepithelial traversal. Consistent with this, T84 cells were shown by reverse transcriptase–polymerase chain reaction (RT–PCR) and immunoblotting to co-express CD66a (BGP), CD66c (NCA) and CD66e (CEA). The recruitment of CD66 receptors by Opa-expressing gonococci indicates their involvement in mediating adherence to the surface of T84 cells, and these bacterial interactions could be inhibited completely using polyclonal antibodies cross-reacting with all of the CD66 proteins co-expressed on T84 cells. Consistent results were obtained when Opa proteins were expressed in *Escherichia coli*, suggesting that the Opa–CD66 interaction is sufficient to mediate bacterial traversal. Transcytosis of Opa-expressing *N. gonorrhoeae* or *E. coli* did not disrupt the barrier function of infected monolayers, as indicated by a sustained transepithelial electrical resistance (TEER) throughout the course of infection, and confocal laser scanning and electron microscopy both suggest a transcellular rather than a paracellular route of traversal across the monolayers. Parallels between the results seen here and previous work done with organ cultures confirm that T84 monolayers provide a valid model for studying neisserial interactions with the mucosal surface, and suggest that CD66 receptors contribute to this process *in vivo*.

Received 10 April, 1997; revised 1 August, 1997; accepted 5 August, 1997. †Present address: Klinik für Tumorbiologie, Institut für Experimentelle Krebsforschung, D-79106 Freiburg, Germany. *For correspondence in Tübingen. E-mail sinfbio@mpib-tuebingen.mpg.de; Tel. (7071) 601 221; Fax (7071) 610 379.

Introduction

Neisseria gonorrhoeae, the aetiological agent of gonorrhoea, is a strictly human pathogen, and no animal model of gonococcal infection is yet available. Because of this, the precise mechanism of interaction between *N. gonorrhoeae* and mucosal tissues is still not fully appreciated. Previous reports have yielded morphological descriptions of the initial stages of infection on various epithelial cell lines cultured *in vitro* (van Putten and Duensing, 1997). Pili play a central role in localized neisserial infection by mediating the selective primary adherence to secretory (non-ciliated) epithelial cells (McGee *et al*., 1981; Mosleh *et al*., 1997) and possess additional binding functions, which may contribute to gonococcal colonization of mucosal surfaces (Rudel *et al*., 1992). After pilus-mediated events, the gonococci obtain a secondary tight contact with epithelial surfaces via the phase-variable, colony opacity-associated (Opa) proteins. One of these variants, Opa_{50}, mediates adherence to, and invasion into, cultured epithelial cells via binding to heparan sulphate-containing proteoglycan (HSPG) receptors (Chen *et al*., 1995; van Putten and Paul, 1995) and the extracellular matrix proteins vitronectin (Vn) (Duensing and van Putten, 1997; Gomez-Duarte *et al*., 1997; Dehio *et al*., 1998) and fibronectin (van Putten *et al*., 1998). Recently, members of the CD66 family, CD66a (biliary glycoprotein; BGP), CD66c (non-specific cross-reacting antigen; NCA) and CD66d (CEA gene family member 1; CGM1) have been identified as receptors for the other Opa protein variants, which mediate neisserial interactions with polymorphonuclear neutrophils (PMNs; Chen and Gotschlich, 1996; Virji *et al*., 1996; Gray-Owen *et al*., 1997a). CD66b (CEA gene family member 6; CGM6) is also expressed on PMNs, but it is not bound by any of the 11 *N. gonorrhoeae* MS11 Opa protein variants (Bos *et al*., 1997; Chen *et al*., 1997; Gray-Owen *et al*., 1997a, b). Individual members of the CD66 family

are also distributed differentially on various other tissue types (Berling *et al*., 1990; Thompson *et al*., 1991; Majuri *et al*., 1994). Virji *et al*. (1996) have shown that CD66a expressed on non-polarized HT29 colonic carcinoma and A549 lung carcinoma cell lines can mediate the binding and invasion of meningococci *in vitro*. The gonococcal Opa variants also mediate adherence to, and invasion into, stably transfected HeLa cell lines expressing individual CD66 receptors, including CD66e (carcinoembryonic antigen; CEA; Bos *et al*., 1997; Chen *et al*., 1997; Gray-Owen *et al*., 1997a,b), a related protein that is absent from granulocytes but is expressed on certain epithelial and endothelial tissues (Majuri *et al*., 1994; Prall *et al*., 1996). Although 10 different Opa proteins bind to CD66 receptors, differential specificities do exist (Bos *et al*., 1997; Chen *et al*., 1997; Gray-Owen *et al*., 1997b), thus implying that they may mediate distinct cellular responses depending on the pattern of CD66 proteins that are expressed. This is consistent with the fact that an enhanced oxidative burst in granulocytic cells is triggered by all Opa proteins that bind CD66a (Gray-Owen *et al*., 1997b).

The conventional semi-confluent growth of epithelial cells in standard culture dishes does not mimic the polarized arrangement of mucosal cells that exists *in situ*. The differential expression of certain receptor proteins, including the syndecan receptors (Carey, 1997) that are recognized by gonococcal Opa_{50} (van Putten and Paul, 1995), on the apical and basolateral surfaces of polarized cells does make such considerations critical for understanding the biological relevance of receptor interactions during infection. Previously, neisserial infection of fallopian tube organ cultures (FTOCs; McGee *et al*., 1981), nasopharyngeal organ cultures (NPOCs; Stephens *et al*., 1983), and primary epithelial cell cultures (Boxberger *et al*., 1994; Mosleh *et al*., 1997) have been used to represent better the cellular architecture of the mucosa that exists *in vivo*. These techniques do, however, require a source of donor tissues, and the *in vitro* manipulation of such cell cultures and tissues is difficult. Recently, polarized monolayers of the T84 cell line were used to study the effect of neisserial pilus expression on cellular binding and traversal across the epithelium (Merz *et al*., 1996; Pujol *et al*., 1997). These cells are derived from a lung metastasis of a human colonic carcinoma and can form polarized monolayers with a robust barrier function when grown on a filter support that allows them to feed from the basolateral surface (Murakami and Masui, 1980; McRoberts and Barrett, 1989; Dharmsathaphorn and Madara, 1990). Under these conditions, T84 cells can form tight junctions, generate numerous microvilli on their apical surface and generally show structural similarity to epithelial cells seen *in vivo* (Dharmsathaphorn and Madara, 1990). The T84 model system provides a valuable tool for studying primary events that occur during colonization, including the potential effects of polarized receptor distribution and cellular architecture on bacterial binding and intracellular trafficking following phagocytosis. Because of this, T84 cells have been used previously in the study of polymorphonuclear cell (PMN) migration across epithelial monolayers stimulated by *Salmonella* (McCormick *et al*., 1993; 1995) and *Shigella* (Perdomo *et al*., 1994), as well as for the analysis of adherence to, invasion into and transcytosis across epithelial monolayers by *Helicobacter pylori* (Corthesy-Theulaz *et al*., 1996) and pathogenic *E. coli* (EAEC, EPEC and EHEC; Winsor *et al*., 1992; Gabastou *et al*., 1995; Nataro *et al*., 1996; Philpott *et al*., 1996). The use of a colon-derived cell line as a model system for gonococcal infection is also justified because anorectal colonization is seen in approximately 40% of infected women and homosexual men (Handsfield, 1990).

In this study, we have analysed the capacity of distinct gonococcal Opa proteins to mediate bacterial traversal across the polarized T84 epithelial cell monolayer. A cellular basis for the observed transcytotic process was characterized with respect to the contribution of both previously defined host cell receptors and ultrastructural events. We demonstrate that the specific interaction of distinct Opa proteins with their cellular CD66 receptors leads to major rearrangements of the apical cell surface, resulting in tight bacterial adherence and, subsequently, in the transcellular passage of bacteria across the infected monolayer. Opa-mediated binding to CD66 receptors is itself sufficient to mediate these events, as *E. coli* strains expressing recombinant neisserial Opa proteins also transmigrate to the basolateral surface. The Opa/CD66-mediated cellular binding has been shown previously to mediate interactions between the pathogenic *Neisseria* and granulocytic cells (Chen and Gotschlich, 1996; Virji *et al*., 1996; Gray-Owen *et al*., 1997), epithelial cells lines (Virji *et al*., 1996; Chen *et al*., 1997) and with activated primary endothelium (Gray-Owen *et al*., 1997b). The data presented here suggest that CD66 receptors may also provide a means for the pathogen to achieve the submucosal colonization typical of localized gonococcal infection (McGee *et al*., 1981), and which may subsequently lead to disseminated gonococcal disease.

Results

CD66-specific Opa proteins mediate bacterial traversal across polarized T84 monolayers

Previously, Kupsch *et al*. (1993) generated a series of 11 isogenic *N. gonorrhoeae* and *E. coli* strains, which express individual recombinant Opa proteins cloned from the gonococcal strain MS11. The cloned *opa* genes were expressed in an MS11 derivative strain N279, which is non-piliated and contains a chromosomal deletion in the HSPG receptor-associated $opaC_{30}$ locus. To assess the role of pilus, the MS11 derivative N280, which is piliated and also contains a chromosomal deletion in the $opaC_{30}$ locus, was used to

express individual recombinant Opa proteins. Using these strains, we have analysed the influence of distinct Opa protein variants on bacterial interactions with the apical surface of polarized T84 monolayers. Monolayers were grown on polycarbonate filters with a pore size of 3 μm held in a Transwell filter support. In order to ensure that the monolayer's barrier function was intact, only those monolayers that showed a transepithelial electrical resistance (TEER) of more than 700 Ω cm^2 at the beginning and end of the experiment were used. Our early experiments indicated that, when the starting concentration of gonococci in the apical reservoir was greater than 4×10^7 bacteria ml^{-1} or that of *E. coli* was over 2×10^7 bacteria ml^{-1}, a marked decrease in the TEER of T84 monolayers would occur as early as 4–8 h after infection (data not shown). Similarly, when the interval between exchanging medium was more than 4 h for gonococcal infections or 2 h with *E. coli*, a decline in TEER was apparent. No correlation existed between epithelial barrier function as measured by TEER and neisserial traversal of the monolayers. We therefore routinely replaced half of the culture medium in the upper and all of the culture medium in the lower reservoirs at 4-h intervals for gonococci and 2-h intervals for *E. coli* throughout all infections. This prevented overgrowth by the bacteria and the accumulation of toxic bacterial products such as lipopolysaccharide (LPS), which could damage the monolayers. Using these conditions, the barrier function of polarized T84 remained intact throughout the infection.

In order to ascertain the level of gonococcal adherence to the apical surface of polarized T84 cells, monolayers were fixed after 4 h of infection and stained immunocytochemically for bacteria (red) and F-actin (green) and were then analysed by confocal laser scanning microscopy (Fig. 1). Only a few Opa_{50}-expressing gonococci (Fig. 1A) or non-opaque (Opa^-) gonococci (data not shown) were associated with the monolayer, while a high density of each of the remaining 10 Opa proteins (Opa_{51-60}; as a representative, see Opa_{52} in Fig. 1B) were found to adhere. As shown previously by Merz *et al.* (1996), piliated gonococci also adhered to T84 cells (Fig. 1C), typically existing as larger clusters than Opa-expressing bacteria.

The microscopic analysis of infected samples suggested that gonococci that express Opa proteins specific for CD66 were engulfed by T84 cells. To determine whether adherent bacteria could traverse across the polarized monolayer, the number of viable bacteria present within the lower, basolateral reservoir was determined at various time points after infection of the apical surface. The filters were also moved to fresh basolateral medium at each time point in order to reduce the contribution of bacterial growth to observed changes in bacterial density. Consistent with the fact that they do not interact with the apical surface (Fig. 1A), no significant levels of either Opa^-- or Opa_{50}-expressing gonococci were found in the basolateral reservoir even after 24 h (Fig. 2A). Expression of any one of the other 10 gonococcal Opa proteins (i.e. Opa_{51}–Opa_{60}) did, however, result in the appearance of bacteria in the lower reservoir, thus indicating that these variants can mediate efficient transmigration across T84 monolayers. The lack of transmigration by an isogenic gonococcal strain expressing Opa_{30}, the chromosomally expressed homologue of Opa_{50}, confirms that this phenotype is not caused by the inefficient or improper expression of the recombinant protein. In agreement with Merz *et al.* (1996), we did not observe significant traversal of T84 cells by piliated,

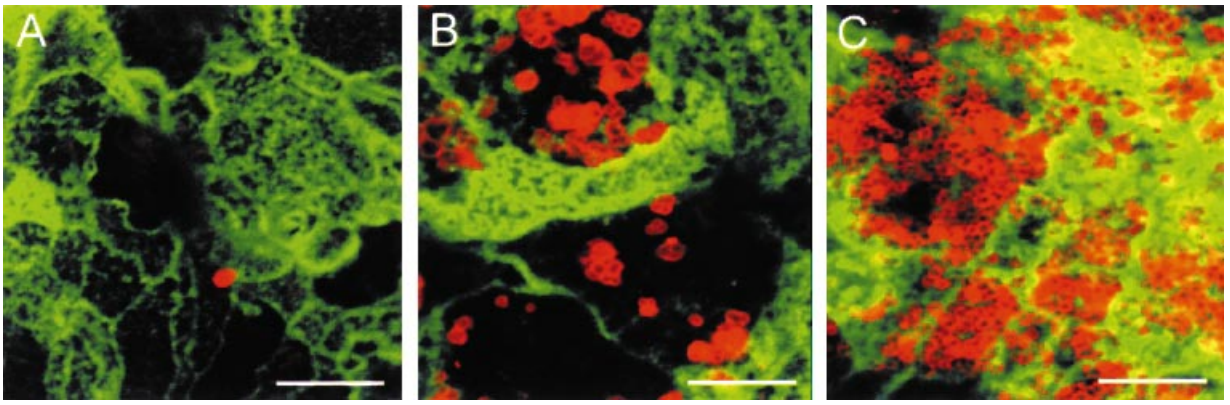
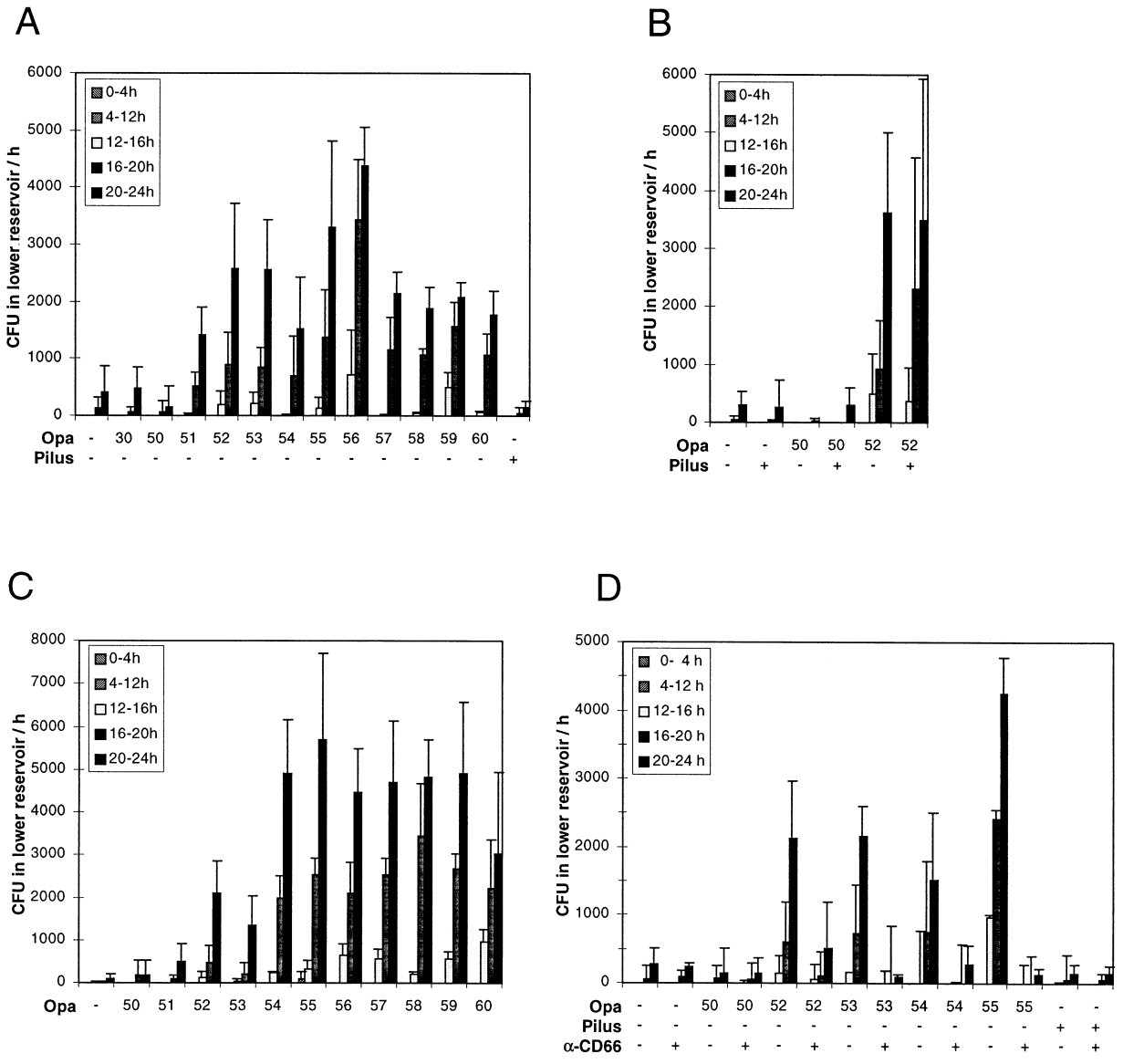


Fig. 1. Adherence of *N. gonorrhoeae* to the apical surface of polarized T84 monolayers. Polarized T84 monolayers cultured on Transwell polycarbonate filters were infected for 4 h with unpiliated (P^-) gonococci expressing Opa_{50} (A, strain N303) or Opa_{52} (B, strain N309) and gonococci expressing pili but no Opa protein (P^+ Opa^-; C, strain N496). Filters were then fixed in formaldehyde and stained for immunofluorescence by double-labelling for bacteria (red) using rat-anti MS11 antiserum (AK231) and secondary Cy3-conjugated goat anti-rat antibodies, and for F-actin (green) by FITC-labelled phalloidin as outlined in *Experimental procedures*. Specimens were analysed by confocal laser scanning microscopy, and a representative *x–y* plane taken from the apical surface of infected monolayers is presented. The scale bars represent 10 μm.

non-opaque neisserial strains within 24 h of infection. In order to determine how the co-expression of these two adhesins affects transmigration, infection assays were also performed with piliated gonococcal strains that express the recombinant Opa proteins (Fig. 2B). Within the examined period of infection (up to 24 h), the expression of pilus or Opa_{50}, either separately or together, did not influence the levels of gonococci appearing in the basolateral chamber compared with that seen with the non-piliated, non-opaque strain. It is likely that background levels of transmigration may result from the phase-variable switching of Opa phenotypes during bacterial growth in the apical reservoir. However, the existence of 11 *opa* loci in the gonococcal genome precludes the generation of a genotypically *opa*$^-$ strain to test this assumption. Opa_{52} expression clearly resulted in an increased number of gonococci appearing in the basolateral chamber, and pilus expression did not significantly influence this. Interestingly, the level of transmigration mediated by Opa_{52} in the non-piliated strain N280 background (N547) was reduced compared with that seen in the N279 background (N309) when these two strains were used in parallel experiments (data not shown); however, the reason for this difference is uncertain.

Recombinant *E. coli* DH5 strains expressing each of the Opa proteins encoded by *N. gonorrhoeae* strain MS11 were tested for their ability to traverse the T84 monolayer system to determine whether Opa protein expression in a distant bacterial species was itself sufficient to allow transmigration. Consistent with results obtained using the



gonococcal-expressed Opa proteins, Opa proteins other than Opa_{50} mediated traversal of the epithelial barrier more than did the parental *E. coli* DH5 containing the Hermes-10 expression vector alone (Fig. 2C). Of these, the *E. coli* strain expressing Opa_{51} was least efficient in mediating bacterial traversal, although bacterial levels in the lower reservoir were at least fourfold higher than that of the Opa_{50}-expressing strain.

Expression of CD66 receptor proteins by polarized T84 monolayers

The Opa proteins seen to mediate adherence to and traversal across T84 monolayers have been shown previously to recognize specifically up to four different members of the CD66 carcinoembryonic family of receptors that are differentially expressed on endothelia, epithelia and phagocytic cells (Chen and Gotschlich, 1996; Bos *et al*., 1997; Chen *et al*., 1997; Gray-Owen *et al*., 1997a,b). In order to determine the expression pattern of CD66 receptors on T84 cells, we performed an immunoblot analysis of total protein extracted from monolayers grown on filter supports and treated similar to those used in the infection assays. The CD66 cross-reactive monoclonal antibody D14HD11 recognized proteins of approximately 200 kDa, 160 kDa and 95 kDa (Fig. 3A). As indicated by their electrophoretic mobility (Fig. 3A, lane 1) in comparison with CD66 members expressed by stably transfected HeLa cell lines, this generally corresponds with the sizes of CD66e (Fig. 3A, lane 3), CD66a (Fig. 3A, lane 4) and CD66c (Fig. 3A, lane 2) respectively. The 95 kDa protein expressed by T84 cells has an apparent molecular weight slightly higher than that seen in the HeLa-NCA cell line (Fig. 3A, compare lanes 1 and 2). A replicate blot probed with the CD66c-specific monoclonal antibody 9A6 was therefore used to confirm that this protein is expressed (data not shown), as CD66b typically co-migrates with CD66c. The heterogeneous migration patterns seen in different cell types are a common feature of highly glycosylated proteins such as those of the CD66 family. Unfortunately, the lack of antibodies available that are capable of distinguishing between CD66a and CD66e prevents the conclusive identification of these proteins based on immunological characteristics alone. We therefore used a semi-quantitative reverse transcriptase (RT)–PCR approach employing oligonucleotide primers for the specific amplification of individual CD66 gene fragments (Table 1) in order to confirm the expression patterns indicated by immunoblot analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a constitutively expressed housekeeping gene (Wong *et al*., 1994), was used as an internal control in each amplification reaction. Stably transfected HeLa cell lines expressing individual CD66 receptors were used as positive controls for all primer sets. The respective RT–PCR products of uninfected T84 monolayers are shown in Fig. 3B. Using this approach, PCR products were obtained that are consistent with the expression of CD66a, CD66c and CD66e by T84 monolayers. Although the size of the CD66a RT–PCR product from T84 cells was lower than that obtained from the CD66a-expressing HeLa cell line (Fig. 3B, compare lanes 5 and 6), a repetition of this experiment with another CD66a-specific primer set confirmed the presence of CD66a mRNA expression by T84 cells (data not shown). Interestingly, the migration of CD66a RT–PCR products obtained using RNA isolated from infected T84 monolayers existed as a doublet corresponding to the

Fig. 2. Time course and CD66 dependency of the traversal of T84 monolayers by Opa-expressing *N. gonorrhoeae* and *E. coli*. The apical pole of polarized T84 monolayers cultured on Transwell polycarbonate filters was infected for 24 h with gonococcal (A, B and D) or *E. coli* (C) strains expressing defined recombinant Opa proteins and/or pili in the absence (A–C) or presence (D) of anti-CD66 antibodies as indicated. Colony-forming units (cfus) of bacteria passing into the basolateral reservoir during the indicated time intervals were determined by plating bacteria from the culture medium of the lower reservoir onto GC agar at 4 h, 12 h, 16 h, 20 h and 24 h after infection. At each of these time points, Transwell inserts with the infected monolayer were transferred to a new well containing fresh medium in the lower reservoir. Assays were performed in at least three independent experiments with two to eight filters per strain per assay. Data illustrated represent the means and standard deviations of a representative experiment performed, except in (B) where means + standard deviations were calculated instead using the three median values from two independent experiments performed in duplicate. All data were normalized by calculating cfu appearing in the lower reservoir per hour in order to simplify comparison of transmigration rates throughout the infection period.
A. Opa-dependent transcytosis of T84 monolayers. The gonococcal strains used were non-piliated (P^-) and expressing no Opa protein (Opa^-, strain N302), chromosomally encoded Opa_{30} (strain N483) or recombinant Opa_{50} (strain N303), Opa_{51} (strain N305), Opa_{52} (strain N309), Opa_{53} (strain N304), Opa_{54} (strain N311), Opa_{55} (strain N307), Opa_{56} (strain N308), Opa_{57} (strain N313), Opa_{58} (strain N312), Opa_{59} (strain N306) or Opa_{60} (strain N310) or piliated (P^+) and non-opaque (Opa^-; strain N496).
B. Influence of pilus expression on Opa-dependent transcytosis. The gonococcal strains used were non-piliated (P^-) expressing either no Opa protein (Opa^-, strain N302) or recombinant Opa_{50} (strain N303) or Opa_{52} (strain N547, phenotypically selected P^- variant) or piliated (P^+) variants expressing either no Opa protein (Opa^-, strain N280) or recombinant Opa_{50} (strain N541) or Opa_{52} (strain N547).
C. Transcytosis of Opa-expressing *E. coli*. The DH5 strains used carry the IPTG-inducible expression vector Hermes-10 as a control (Opa^-, strain H1887) or derivatives directing the expression of recombinant Opa proteins (Opa_{50}, strain H1888; Opa_{51}, strain H1903; Opa_{52}, strain H1907; Opa_{53}, strain H1902; Opa_{54}, strain H1909; Opa_{55}, strain H1905; Opa_{56}, strain H1906; Opa_{57}, strain H1911; Opa_{58}, strain H1910; Opa_{59}, strain H1904 and Opa_{60}, strain H1908).
D. Effect of antibody blockage of CD66 on Opa-dependent transcytosis. The gonococcal strains used were non-piliated (P^-) expressing either no Opa protein (Opa^-, strain N302) or recombinant Opa_{52} (strain N309), Opa_{53} (strain N304), Opa_{54} (strain N311) or Opa_{55} (strain N307) or were piliated (P^+) expressing no Opa protein (Opa^-, strain N496). Infection was performed for 24 h in the presence or absence of polyclonal anti-CD66 antibodies cross-reacting with all CD66 receptors co-expressed on T84 cells.

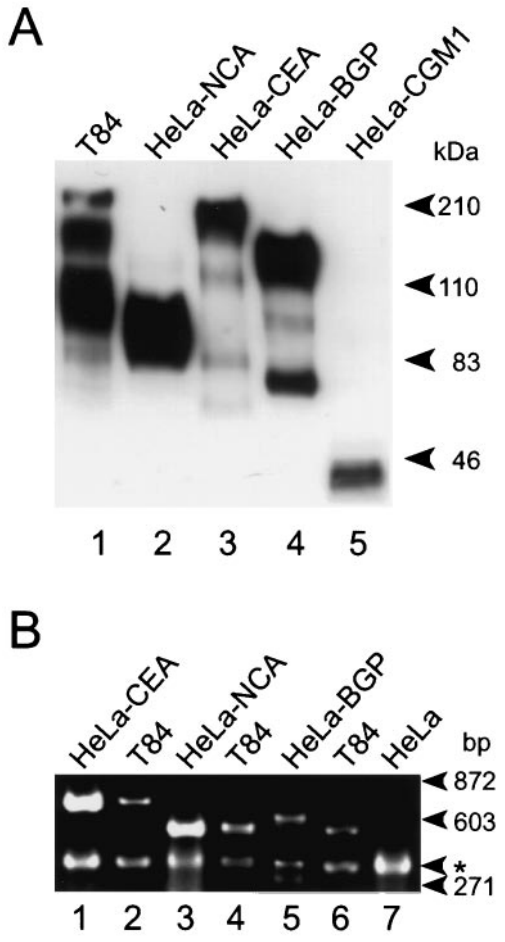


Fig. 3. Analysis of CD66 expression in polarized T84 monolayers. Polarized T84 monolayers grown on Transwell polycarbonate filters similar to those used for transepithelial traversal experiments or stably transfected HeLa cell lines expressing CD66a (HeLa-BGPa), CD66c (HeLa-NCA), CD66d (HeLa-CGM1a) or CD66e (HeLa-CEA) grown on plastic tissue culture dishes were extracted by TRIzol reagent, and the expression of CD66 family members was analysed by immunoblotting of total protein (A) or semi-quantitative RT–PCR using isolated total RNA (B).
A. Total protein prepared from TRIzol extracts was separated on 10% gels by SDS–PAGE, transferred to PVDF membranes and probed with monoclonal mouse anti-CD66 antibody (clone D14HD11) followed by horseradish peroxidase-conjugated goat anti-mouse antibodies. Immunoblots were developed by enhanced chemiluminescence (ECL). The electrophoretic mobility of molecular weight markers is indicated.
B. Total RNA prepared from TRIzol extracts was subjected to semi-quantitative duplex RT–PCR using primer pairs specific for BGPa (CD66a), NCA (CD66c) or CEA (CD66e). GAPDH-specific primers that generate a 306 bp fragment from cDNA served as an internal control (amplified GADPH fragment indicated by a star).

individual bands obtained from uninfected HeLa-CD66a and T84 cells respectively (data not shown). The reason for these differences are not clear at this time. However, the potential induction of differential splice variants (Barnett *et al.*, 1993; Thompson *et al.*, 1991) by neisserial infection is currently being explored.

Traversal of T84 monolayer requires Opa-mediated binding to CD66 receptors

Immunofluorescence staining and confocal laser scanning microscopic analysis of infected T84 monolayers was performed in order to compare gonococcal binding with the expression pattern of CD66 receptors. Co-localization of CD66 receptors with adherent Opa_{52}-expressing gonococci clearly indicates the efficient recruitment of receptor by this Opa (Fig. 4A–D), while no similar accumulation of CD66 antigens occurred in association with bound Opa_{50}-expressing bacteria (data not shown). Receptor association patterns seen when using gonococci expressing either CD66a or CD66e-specific Opa proteins (Opa_{53} and Opa_{55} respectively) were indistinguishable from those seen for Opa_{52}-expressing bacteria (data not shown), indicating that either of these receptors could also mediate bacterial binding to T84 cells. Confocal laser scanning microscopy in an *x–z* plane illustrated that CD66 receptor expression is polarized, occurring at the apical surface of monolayers (compare Fig. 4F and G). Occasionally, intracellular bacteria were seen to be associated with CD66 proteins, suggesting a continued association between Opa and CD66 during transmigration. In the majority of cases, however, intracellular bacteria did not associate with CD66 labelling (arrow in Fig. 4F–H, arrows point to the position of a traversing bacterium).

In order to correlate bacterial binding to CD66 receptors on the apical surface directly with their ability to traverse the monolayer, infection experiments were repeated in the presence and in the absence of polyclonal anti-CD66 antibodies that cross-react with all three CD66 receptors expressed on T84 cells. Non-piliated (P^-) gonococcal strains that express Opa proteins representative of the four CD66 binding specificities were used: Opa_{52} (binds CD66a, CD66c, CD66d and CD66e); Opa_{53} (CD66a specific); Opa_{55} (CD66e specific); and Opa_{54} (binds CD66a and CD66e). Non-piliated (P^-) gonococcal strains expressing Opa_{50} or no Opa (Opa^-) and a piliated, but non-opaque (P^+ Opa^-) strain were also used as non-CD66 binding controls. Confocal laser scanning microscopy of the infected samples confirmed that the antibodies competitively inhibited binding of CD66-specific, Opa-expressing strains to the T84 monolayer (compare Fig. 4I and J), but did not affect interactions seen with either Opa_{50}- or pilus-expressing strains (data not shown). Importantly, no cross-reactive binding of the CD66-specific antiserum was seen with isolated gonococci by direct immunofluorescence staining, supporting the premise that its inhibitory effects are caused by antibody binding of CD66 receptors directly (data not shown). A clear inhibition of transmigration was also seen for all strains expressing CD66-specific Opa proteins, while no obvious effect of antibody on transmigration by the other strains was seen (Fig. 2D). Consistent results

Table 1. Primer sets for specific amplification of CD66 family and control transcripts by RT–PCR.

Transcript	Primer	Sequence	$T_{annealing}$ (°C)	Fragment length	Reference
CEA (CD66e)	CEA-5′	5′-CCATGGAGTCTCCCTCG-3′	56	641 bp	Thompson *et al.* (1993)
	CEA-3′	5′-GTAGCTTGCTGTGTCATTTC-3′			
NCA (CD66c)	NCA-5′	5′-TTCTTCTACTCGCCCACAAC-3′	58	474 bp	Thompson *et al.* (1993)
	NCA-3′	5′-GTTCCTTTTGACGCTGAGTA-3′			
BGP (CD66a)	BGP2-5′	5′-ACAGTCAAGACGATCATAGT-3′	56	530 bp	R. Kammerer and S. Hahn, personal communication
	BGP2-3′	5′-ATCTTGTTAGGTGGGTCATT-3′			
GAPDH	GADPH-5′	5′-CGGAGTCAACGGATTTGGTCGTAT-3′	56–58[a]	306 bp	Wong *et al.* (1994)
	GADPH-3′	5′-AGCCTTCTCCATGGTGGTGAAGAC-3′			

a. GAPDH primers were used in duplex RT–PCR with annealing temperatures being determined by the co-amplifying of a CD66-specific primer pair.

were obtained by a similar analysis of the effect of polyclonal anti-CD66 antibodies on transmigration by the recombinant Opa-expressing *E. coli* strains (data not shown). As there is no known Opa protein that binds only CD66c, a monolayer infected with the Opa_{52}-expressing gonococcal strain was stained with the CD66c-specific monoclonal antibody 9A6 and analysed by confocal laser scanning microscopy. Similar to that shown previously when using the stably transfected HeLa cell line expressing CD66c alone (HeLa-NCA; Gray-Owen *et al.*, 1997a), an obvious recruitment of CD66c to the site of bacterial binding was observed (data not shown).

Time course of T84 cell entry and traversal by Opa expressing N. gonorrhoeae

The kinetics of gonococcal uptake into T84 cells was measured by the addition of gentamicin to apical and basolateral reservoirs after various time points and then recovering viable intracellular bacteria by saponin lysis of the T84 cell membranes. As shown in Fig. 5A, intracellular gonococci expressing Opa_{52} could already be recovered after 3 h infection, with the number of intracellular bacteria increasing with the infection time before gentamicin treatment. The time required by traversing bacteria to be internalized into the monolayer initially was determined instead by adding gentamicin only to the apical reservoir and then allowing the infection to continue (Fig. 5B). When gentamicin was added 3 h after infection, very few bacteria were seen in the lower reservoir even after 28 h. Consistent with the fact that apical uptake of bacteria is an ongoing process (Fig. 5A), postponing the gentamicin treatment until 6 h allowed low levels (20–30% of control) of transmigration to occur, and longer intervals allowed consistently more bacteria to be recovered (30–50% of control). Gentamicin treatment at later time points did not obviously reduce the level of bacteria recovered, suggesting that bacteria recovered by 24 h after infection had all penetrated the apical membrane by this time.

In order to determine whether a similar period was necessary for transepithelial traversal mediated by Opa proteins with different CD66 binding patterns, representative CD66-specific, Opa-expressing strains were used in a similar gentamicin assay in parallel to non-opaque, Opa_{50}-expressing and a non-opaque, pilus-expressing (Opa^- P^+) strain. In all cases, about 10% of control levels of bacteria were recovered by gentamicin treatment after 6 h (data not shown). As Opa_{53} and Opa_{55} are specific for CD66a and CD66e, respectively, these results indicate that the specific recruitment of two different receptors results in a similar kinetics of bacterial uptake at the apical surface.

The transepithelial traversal of N. gonorrhoeae *and* E. coli *does not disrupt the barrier function of T84 monolayers*

Scanning electron microscopic (SEM) analysis of infected monolayers displayed very few Opa_{50}-expressing (Fig. 6A) or transparent (Opa^-; data not shown) gonococci on the surface of infected cells. These bacteria typically appeared as small, loosely associated clusters without any obvious specific interaction with the apical surface, and no microvilli reorganization or internalization of bacteria was evident even after 24 h of infection. In strong contrast to this, infection by the Opa_{52}-expressing strain resulted in large numbers of individually associated bacteria initially showing a tight binding and bending of microvilli (Fig. 6B). Later, bacteria at various stages of interaction were evident (e.g. Fig 6C, the plasma membrane fusing above an internalized bacterium is indicated by an arrow). The general disruption of normal microvilli distribution within the area of Opa_{52}-mediated binding is suggestive of major rearrangements in the underlying cytoskeletal architecture.

Transmission electron microscopic (TEM) analysis indicated that primary Opa_{52}-mediated interactions involve adherence to the tip of cellular microvilli (Fig. 6D), while subsequent engulfment seemed to involve the restructuring of microvilli structures leading ultimately to an intimate contact between bacterial and cellular membranes (Fig. 6E). The quantity of engulfed and intracellular gonococci increased with time throughout the period of infection,

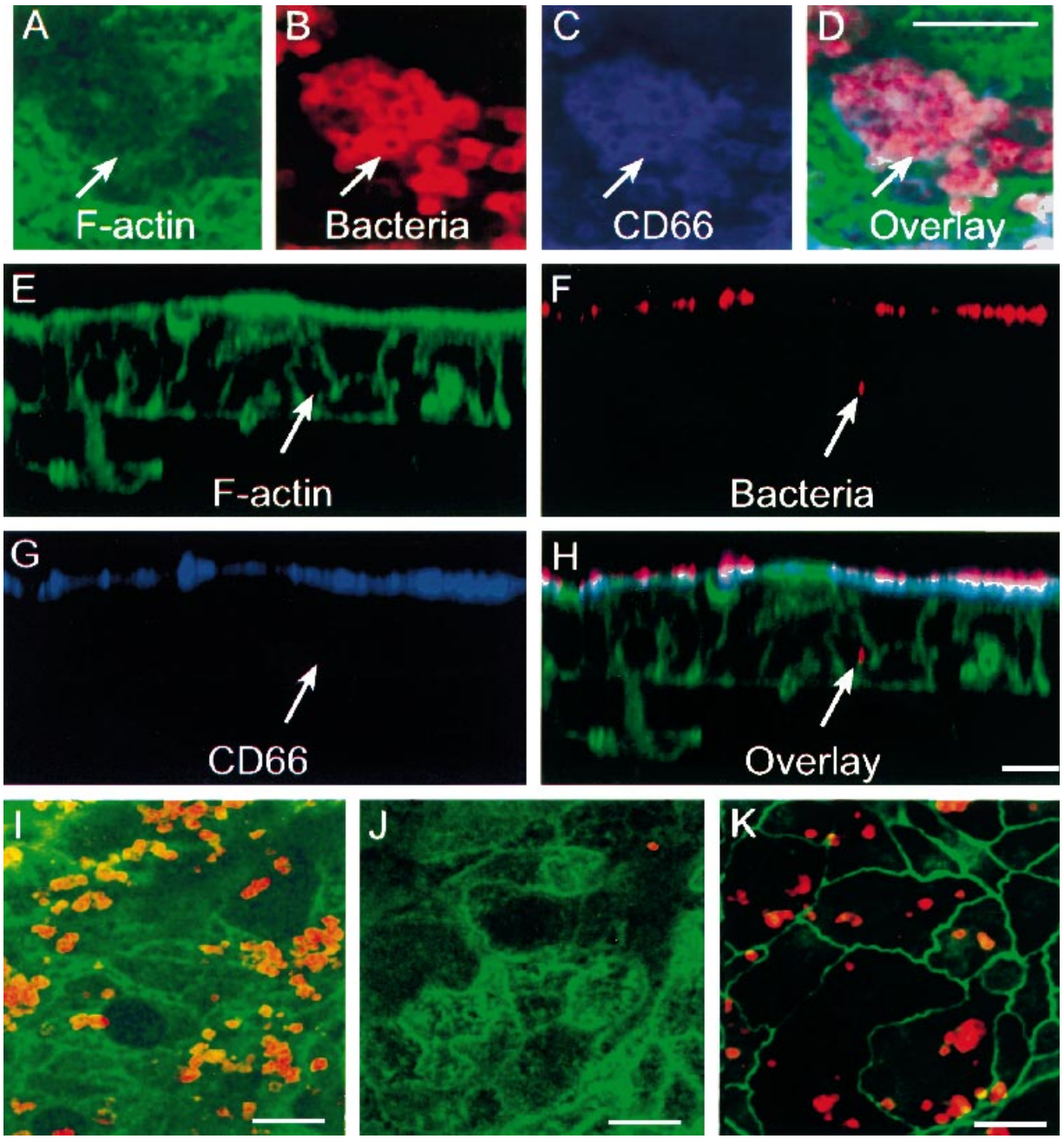


Fig. 4. Confocal microscopic analysis of T84 monolayer traversal by Opa-expressing *N. gonorrhoeae*. The apical pole of polarized T84 monolayers cultured on Transwell polycarbonate filters was infected for 24 h with non-piliated (P^-), Opa_{52}-expressing gonococci (strain N309) in the absence (A–I and K) or presence (J) of polyclonal anti-CD66 antibodies. Filters were then fixed in formaldehyde and stained for immunofluorescence.

A–H. Specimens were triple stained for F-actin using FITC–phalloidin (green in A, D, E and H), bacteria using rat anti-MS11 antiserum (AK231) and secondary Cy5-conjugated goat anti-rat antibodies (red in B, D, F and H) and CD66 using monoclonal antibody clone D14HD11 and secondary lissamine rhodamine (LRSC)-conjugated goat anti-mouse antibodies (blue in C, D, G and H).

I and J. Monolayers were double stained for bacteria (red) and F-actin (green) as described in the legend to Fig. 1.

K. The monolayer was double stained for ZO-1 (green) using rabbit anti-ZO-1 antibodies and secondary Cy5-conjugated goat anti-rabbit antibodies, and for bacteria (red) using rat anti-MS11 antiserum (AK231) and secondary Cy3-conjugated goat anti-rat antibodies.

Representative confocal *x–y* planes taken from the apical surface (A–D and I–K) or a representative *x–z* cross-section through an infected monolayer (E–H) are presented. The arrows in (A–D) point to one representative bacterium co-localizing with increased CD66 staining. The arrows in (E–H) point to the position of a bacterium traversing the monolayer. The scale bars represent 10 μm.

and all stages of internalization were apparent at later time points (data not shown). Transparent and Opa_{50}-expressing gonococci were found to be neither associated nor intracellular in the T84 monolayer, even after 24 h of infection (data not shown). Intracellular Opa_{52}-expressing bacteria appeared to move along a transcellular pathway (Fig. 6F, arrow pointing to an intracellular bacterium and an increased magnification of this shown in Fig. 6G). Bacteria seen were typically single or diplococci and continued to be enclosed by a tightly associated phagosomal membrane throughout traversal (arrow in Fig. 6G). No obvious disruption of the cellular architecture correlated with either intracellular gonococci or with the Opa-mediated transepithelial traversal seen in Fig. 2, and tight junctions were visible at the apical border of adjacent cells infected with Opa_{52}-expressing gonococci at all time points investigated (arrows in Fig. 6D and H). Confocal microscopic analysis of samples stained using the ZO-1 specific antibody also illustrates that a uniform staining of apical borders was maintained even after 24 h of infection with Opa_{52}-expressing gonococci (Fig. 4K). Consistent with this assertion, the transepithelial electrical resistance (TEER) of T84 cell monolayers typically remained above 700 $\Omega\,cm^{-2}$ during the infection by both recombinant gonococci and *E. coli* strains (data not shown), thus confirming the maintenance of barrier function throughout these assays.

Discussion

Previous studies have used various cellular and tissue models to yield a morphological description of the early stages of neisserial infection, and there is considerable evidence that the pathogenic *Neisseriae* can enter epithelial cells (Silverstein *et al*., 1977; McGee *et al*., 1983; Shaw and Falkow, 1988; Chen *et al*., 1991). For example, infection of organ cultures derived from fallopian tubes (FTOCs) or the nasopharynx (NPOCs) has demonstrated neisserial entry into epithelial cells at the apical surface, traversal into the basal region and movement into the stromal matrix (McGee *et al*., 1981; 1983; Stephens *et al*., 1983; Stephens and Farley, 1991). Clinical isolates of *N. meningitidis* have also been shown to transmigrate across polarized T84 monolayers within 12–24 h of infection (Pujol *et al*., 1997), while piliated gonococci appeared in the basolateral chamber only after 36–48 h (Merz *et al*., 1996). Non-piliated isogenic strains transmigrated at levels much below that of

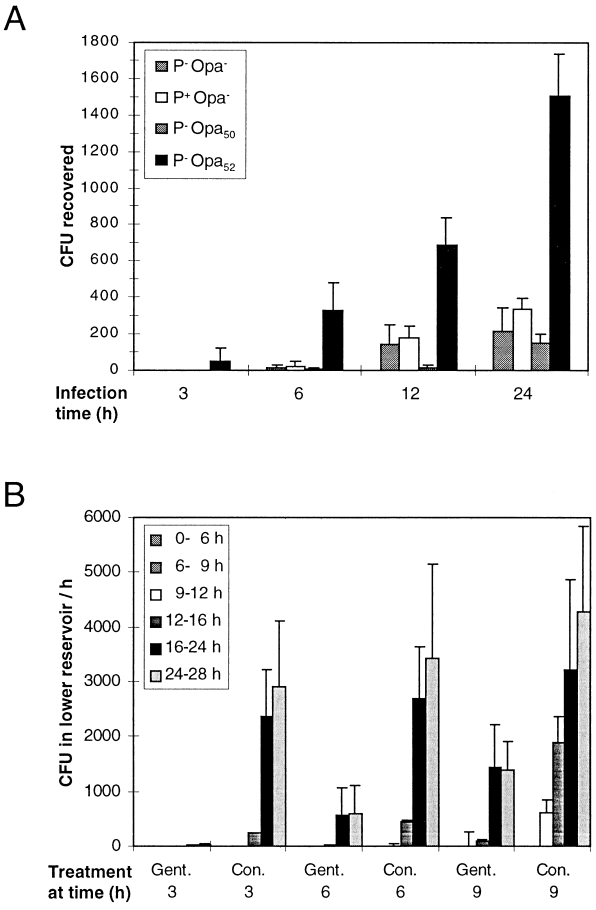


Fig. 5. Time requirement for Opa-dependent traversal of T84 monolayers by *N. gonorrhoeae*.
A. Time requirement and Opa dependency of the apical invasion of the T84 monolayer. The apical pole of polarized T84 monolayers cultured on Transwell polycarbonate filters was infected with gonococcal strains that were non-piliated expressing either no Opa protein (P^- Opa^-, strain N302) or recombinant Opa_{50} (P^- Opa_{50}, strain N303) or Opa_{52} (P^- Opa_{52}, strain N309) or piliated and non-opaque (P^+ Opa^-, strain N280). After the time period indicated (3 h, 6 h, 12 h and 24 h), gentamicin (100 $\mu g\,ml^{-1}$) was added to both reservoirs for 2 h in order to kill extracellular bacteria. Colony-forming units (cfus) of intracellular bacteria surviving gentamicin treatment were determined by saponin lysis of the monolayer and plating bacteria onto GC agar.
B. The apical pole of polarized T84 monolayers cultured on Transwell polycarbonate filters was infected with a non-piliated (P^-) gonococcal strain expressing recombinant Opa_{52} (N309) for 28 h. After the time period indicated (3 h, 6 h or 9 h), gentamicin (100 $\mu g\,ml^{-1}$, indicated as Gent) was added to the upper reservoir for 2 h in order to kill all extracellular bacteria that had not yet entered the monolayer. After this time, a lower concentration of gentamicin (20 $\mu g\,ml^{-1}$) was kept in the upper reservoir until the end of the experiment. The addition of gentamicin was omitted in otherwise identically treated filters that served as controls (indicated as Con). Colony-forming units (cfus) of bacteria traversed in the time intervals indicated (0–6 h, 6–9 h, 9–12 h, 12–16 h, 16–24 h and 24–28 h) were determined by plating bacteria from the culture medium in the lower reservoir onto GC agar at 6 h, 9 h, 12 h, 16 h, 24 h and 28 h after infection. At each of these time points, Transwell inserts with the infected monolayer were transferred to a new well with fresh medium in the lower reservoir. Assays were performed in at least three independent experiments with two to eight filters per strain and assay. Data illustrated represent the mean and standard deviations of a representative experiment and were normalized by calculating cfus appearing in the lower reservoir per hour in order to simplify comparison of transcytosis rates throughout the infection period.

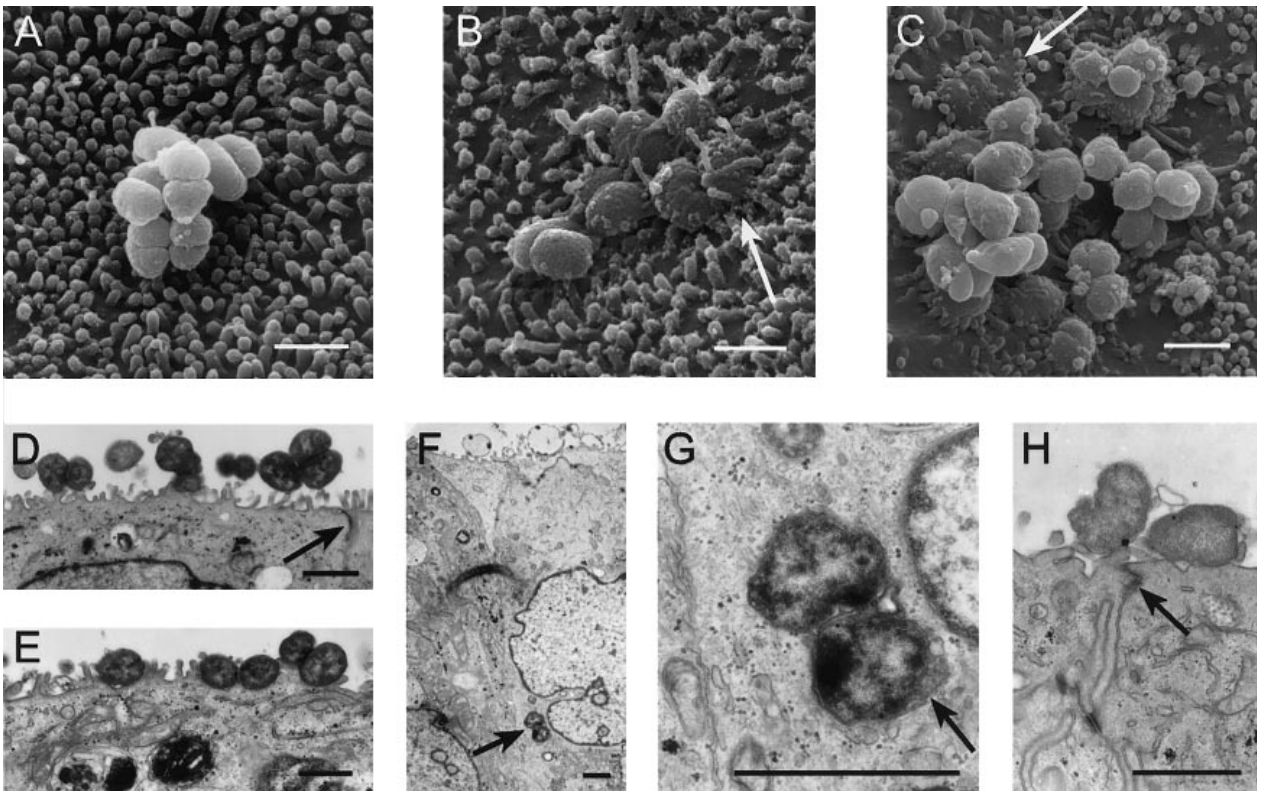


Fig. 6. Ultrastructural characterization of the Opa-dependent traversal of polarized T84 monolayers by *N. gonorrhoeae*. The apical pole of polarized T84 monolayers cultured on Transwell polycarbonate filters was infected with non-piliated gonococci expressing Opa_{50} (A) or Opa_{52} (B–H) for 4 h (D and E) or 24 h (A–C and F–H). Cells were then fixed, processed and analysed by scanning electron microscopy (SEM, A–C) or transmission electron microscopy (TEM, D–H) as outlined in *Experimental procedures*. Arrows indicate bent microvilli in close association with adherent Opa_{52}-expressing bacteria (B), the membrane protrusions fused on top of internalized bacteria (C), tight junctions (D and H), intracellular bacteria traversing the monolayer (F) or their close-fitting phagosomal membrane (G). The scale bars represent 2 μm. For further details, see *Results*.

the piliated variants (Merz *et al*., 1996; Pujol *et al*., 1997), and pilus-mediated binding facilitates an intimate association between the bacterial and apical membranes (Pujol *et al*., 1997). This situation does, however, correlate with a reduced piliation of adherent bacteria, leading the authors to speculate that the tight adherence is mediated by some other adhesin. As the piliated bacteria used in these previous studies were Opa^- (Merz *et al*., 1996; Pujol *et al*., 1997), the contribution of Opa proteins to neisserial interactions with T84 monolayers has not been explored previously. In the present study, we have demonstrated clearly that an effective traversal of polarized T84 monolayers by *N. gonorrhoeae* is mediated by distinct Opa protein variants that have previously been shown specifically to bind defined members of the CD66 epitope-containing carcinoembryonic antigen family (Opa_{51}–Opa_{60}; Bos *et al*., 1997; Chen *et al*., 1997; Gray-Owen *et al*., 1997b). The efficiency of this process is evident from the fact that viable Opa-expressing *N. gonorrhoeae* and *E. coli* begin to pass into the lower reservoir as early as 12 h after infection (Fig. 2). Consistent with this, the CD66a, CD66c and CD66e receptors were seen to be expressed by a combination of immunoblot and RT–PCR analysis (Fig. 3). A high density of CD66 proteins was also seen to co-localize with Opa_{52}-expressing bacteria adherent at the apical surface of T84 cells (Fig. 4A–D), indicating the efficient recruitment of these receptors. Ultrastructural analysis reveals that a primary interaction with the tips of uniformly arranged microvilli subsequently leads to an intimate association between bacterial and cellular membranes (Fig. 6D and E). This process appears to involve a significant reorganization of the underlying cytoskeletal framework, as microvilli adjacent to tightly adherent bacteria typically retract (compare Fig. 6A, B and C). Once engulfed, intracellular gonococci exist as single bacteria or diplococci and apparently remain surrounded by a tightly associated phagosomal membrane (Fig. 6F and G), which is occasionally seen to contain CD66 receptors. The absence of CD66 antigens in association with most intracellular bacteria may indicate that the receptor is cycled away from the phagosome after bacterial internalization. Alternatively, this observation may result from a masking of the epitope recognized by

CD66-specific antibodies used in the immunocytochemical staining of samples, either by bacterial Opa-mediated association with the receptors or as a result of the presence of high levels of proteins, which become associated with the phagolysosomal vesicle (e.g. h-lamp). Previous studies have shown that gonococci can exist as large clusters of bacteria within a single vacuole (Apicella *et al*., 1996; Mosleh *et al*., 1997). However, urethral exudates from patients with gonococcal urethritis show that bacteria are engulfed as single organisms (Apicella *et al*., 1996). Whether the subsequent appearance of phagosomes containing multiple bacteria arise from the intracellular fusion of phagosomes or via bacteria replication is not yet certain. Pujol *et al*. (1997) displayed piliated meningococci occurring within a more spacious phagosome, suggesting that the phagocytic events that follow pilus vs. Opa-mediated uptake may be distinct.

The tight junction, or zonula occludens (ZO), is a narrow belt-like structure on the plasma membrane of transporting epithelial cells that wraps each cell circumferentially and adjoins it to its neighbours. This organization provides an efficient barrier restricting flow through the paracellular pathway (Madara, 1988). Ultrastructural analyses using confocal laser scanning microscopy, SEM and TEM indicate that the transmigration of gonococci that express CD66-specific Opa proteins occurs via a transcellular rather than a paracellular route (Figs 4E–H and 6F and G). This is consistent with the fact that the monolayer's barrier function remains intact, as seen by both the continued presence of tight junctions (Figs 4K and 6D and H) and the maintenance of transepithelial electrical resistance (TEER) throughout infection experiments. This is clearly different from *Salmonella typhimurium* and the enteropathogenic *E. coli* (EPEC), which can disrupt the organization of tight junctions and barrier function of epithelial tissues to allow paracellular migration to occur (Wallis *et al*., 1986; Canil *et al*., 1993; Jepson *et al*., 1995; Philpott *et al*., 1996). Binding to the apical surface of epithelia is not strictly correlated with transmigration, however, as *H. pylori* has been shown to adhere tightly to polarized T84 monolayers in a manner that resembles the gonococcal binding seen here, but does not enter these cells (Corthesy-Theulaz *et al*., 1996).

The indistinguishable characteristics of binding, engulfment and transmigration by recombinant gonococcal and *E. coli* strains expressing Opa proteins with different CD66 specificity patterns, as exemplified by Opa_{52} (binds CD66a, CD66c, CD66d and CD66e), Opa_{53} (specific for CD66a) and Opa_{55} (specific for CD66e), suggests that either CD66a or CD66e can mediate the process of transepithelial traversal (Fig. 2). No CD66c-specific Opa protein has been identified, and so the specific contribution of this receptor in bacterial binding and engulfment cannot be discerned. Immunocytochemical analysis of samples stained with the CD66c-specific monoclonal antibody 9A6 does, however, illustrate that this receptor also co-localizes with bound Opa_{52}-expressing gonococci (data not shown), suggesting that CD66c is recruited during binding. Together with the complete abrogation of Opa-mediated transmigration by the competitive inhibition of binding by CD66-specific antibodies (Fig. 2D), these data directly implicate CD66 receptors as the mediators of these phenomena. Although microvilli are not typical of most tissues infected by the pathogenic *Neisseriae*, colorectal tissues of approximately 40% of women with uncomplicated gonorrhoea become colonized because of contamination from infected vaginal secretions (Handsfield, 1990; Knapp and Rice, 1995). The expression of CD66 receptors on the epithelium of the cervix and uterus (Prall *et al*., 1996) suggests that cellular transmigration, as described here, may also contribute to gonococcal dissemination from these sites.

The Opa_{50} protein has been described previously as the only gonococcal Opa that mediates the efficient invasion of cells grown into semi-confluent cultures *in vitro* (Makino *et al*., 1991; Kupsch *et al*., 1993; Chen *et al*., 1995a; van Putten and Paul, 1995). This observation is explained by the fact that transformed epithelial cell lines do not usually synthesize any CD66 receptors. Our results presented here, however, clearly show that gonococci expressing the HSPG-specific Opa_{50}, or its chromosomally encoded Opa_{30} homologue, do not bind to or traverse the polarized T84 cells (Figs 1A, 2 and 6A). This finding is consistent with the fact that the HSPG-containing syndecan receptors that are recognized by Opa_{50} are expressed exclusively at the basolateral surface of polarized cells (Carey, 1997). Previous studies have reported a thinning of multilayered tissue and organ cultures and detachment of epithelial cells from the urethral lumen during neisserial infection (Stephens and Farley, 1991; Apicella *et al*., 1996; Mosleh *et al*., 1997). Such a breakdown of the polarized arrangement of a mucosal barrier would allow access to otherwise basolaterally localized receptors, thus suggesting that pilus-mediated binding to the apical surface would be a prerequisite for Opa_{50}-mediated invasion.

Interestingly, confocal laser scanning microscopic analysis of T84 monolayers in the x–z plane illustrated that CD66 proteins are expressed predominantly on the apical surface (Fig. 4E–H). This organization of neisserial receptors supports the premise that distinct adhesins of the gonococcal repertoire contribute to tissue interactions at different stages of the infectious process. Pilus is necessary for primary colonization of the male urethra in human infections (Seifert *et al*., 1994) and also mediates gonococcal binding to the apical surface of polarized cells *in vitro* (Fig. 1C; Merz *et al*., 1996; Pujol *et al*., 1997), suggesting that the initial adherence to mucosal epithelial tissues may be mediated by pilus. Subsequent tight association at the apical surface may occur via the CD66

receptor-specific Opa proteins, leading to the engulfment and transcellular migration of gonococci across the intact polarized barrier. Colonization of the basolateral surface may then be mediated by the HSPG-specific Opa protein either via the cellularly expressed syndecan receptors (Chen *et al*., 1995; van Putten and Paul, 1995) or after anchorage to the extracellular matrix via vitronectin (Duensing and van Putten, 1997; Gomez-Duarte *et al*., 1997). Ligation of syndecan and the α_v integrin-containing vitronectin receptor can enhance the uptake of Opa_{50}-expressing gonococci into epithelial cells (Dehio *et al*., 1998). It is intriguing to speculate that this provides a mechanism by which the gonococci can recolonize the apical surface, thereby continually reseeding the urethral lumen to make transmission to a new host possible. This model is consistent with the fact that there is a period after gonococcal inoculation during which bacteria cannot be recovered, while after this time, they return to levels seen at inoculation (Schneider *et al*., 1995). Adherence to CD66 receptors expressed on granulocytes (Chen and Gotschlich, 1996; Virji *et al*., 1996; Gray-Owen *et al*., 1997a) and activated endothelial cells (Gray-Owen *et al*., 1997b) may also contribute to interactions that occur during an inflammatory response to neisserial infection and during disseminated disease respectively.

Experimental procedures

Bacterial strains and cell lines

Recombinant neisserial (N302–N313; for further details, see Fig. 2) and *E. coli* (H1887, H1888, H1902–H1911; for further details, see Fig. 2) strains expressing the 11 genetically defined Opa proteins from *N. gonorrhoeae* MS11 were constructed by Kupsch *et al.* (1993). *N. gonorrhoeae* MS11-B2.1 strain N483 (P^- Opa_{30}; Makino *et al*., 1991) and MS11-F3 strain N496 (P^+ Opa^-; Haas *et al*., 1987) have also been described previously. N280 (P^+ Opa^-) is a derivative of MS11-F3 carrying a chromosomal mutation of the *opaC*$_{30}$ locus. N541 and N547 are derivatives of N280 expressing recombinant Opa_{50} and Opa_{52} respectively.

The culture of T84 cells was essentially as described previously (Dharmsathaphorn and Madara, 1990; Madara *et al*., 1992; Merz *et al*., 1996). T84 cells from the American Type Culture Collection (kindly provided by Dr Paul Hofman, Nice, France) were grown between passages 46 and 52 in T84 medium, a 1:1 mix of Dulbecco's modified Eagle medium (DMEM) and Ham's F-12 medium (Life Technologies) supplemented with 15 mM HEPES buffer (pH 7.4), 14 mM $NaHCO_3$ and 6% heat-inactivated fetal calf serum (56°C for 30 min; PAA Laboratories). Cells recovered from frozen stocks were cultured in T84 medium to a confluency of 70–80% before seeding for subsequent experiments. For adherence and traversal assays, harvested cells were seeded in 100 μl of T84 medium containing 4×10^4 cells onto collagen I (Collaborative Biotech)-coated Transwell filters (3421; 0.33 cm^2 suspended polycarbonate filters; Costar), and 500 μl of T84 medium was added in the lower reservoir. For immunoblot and RT–PCR assays, harvested cells were seeded in 500 μl containing 2×10^5 cells onto collagen I-coated Transwell filters (3414; 4.7 cm^2 suspended polycarbonate filters; Costar), and 2 ml of T84 medium was added to the lower reservoir. Growth medium was changed daily, and the confluence and integrity of monolayers were assessed by measuring transepithelial electrical resistance (TEER) using a dual-voltage Ohmmeter clamp (Millicell-ERS; Millipore) inserted into the upper and lower reservoirs simultaneously (Perdomo *et al*., 1994). The TEER of most filters used for infection experiments typically exceeded 700 Ω cm^2 per 3421 filter and 800 Ω cm^2 per 3414 filter within 6–10 days after cell seeding.

Immunoblot and RT–PCR analysis of CD66 receptor expression

After washing with phosphate-buffered saline (PBS), all liquid was removed from the apical wells of uninfected T84 monolayer grown on 3414 Transwell filters, and total protein and RNA were extracted using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. Mouse monoclonal antibody D14HD11, cross-reactive against CD66a, CD66c, CD66d and CD66e, and the CD66c-specific 9A6 used for immunoblot analysis of T84 cells were generously provided by Dr Fritz Grunert (University of Freiburg, Germany). Blots were developed after incubation with a peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) using the ECL enhanced chemiluminescence substrate (Amersham).

Preparation of cDNA and subsequent PCR were performed as described previously (Thompson *et al*., 1993; Naumann *et al*., 1997). Primer sets used in this study are listed in Table 1. CD66 receptor-specific primers were used at 20–40 pmol per reaction, with 0.5–1 pmol of GAPDH primers also included as an internal control in each reaction.

Bacterial infection and transmigration assays

Polarized T84 monolayers grown on 3421 Transwell filters and displaying a TEER of more than 700 Ω cm^2 were rinsed with T84 medium and placed in fresh 24-well plates containing 0.5 ml of T84 medium. Aliquots of 100 μl of medium containing either 2×10^6 *N. gonorrhoeae* or 1×10^6 *E. coli* and indicated supplements were carefully added to the upper reservoir. Infected cells were incubated at 37°C with 5% CO_2. One half of the medium in the apical reservoir was replaced at 4-h (gonococcal infections) or 2-h (*E. coli* infections) intervals, and the medium in the lower (basolateral) reservoir was also changed by moving the filters to a new well containing fresh T84 medium. Dilutions of medium recovered from the basal reservoir were plated onto GC agar and incubated overnight at 37°C with 5% CO_2 in order to quantify bacterial transmigration (expressed as colony-forming units, cfu). Each strain was assayed in three to seven independent experiments with two to eight filters per strain per assay, unless otherwise stated. At the end of the assay (24 h after infection), each filter was assayed for TEER to verify that the barrier function had remained intact (> 700 Ω cm^{-2}).

For antibody inhibition assays, polyclonal antiserum reactive against CD66a, CD66c, CD66d and CD66e (anti-CEA; Dako) was added to obtain a final concentration of 100 μg

ml^{-1} in the culture medium of the upper reservoir. Before use, the azide-containing antibody storage buffer was replaced by T84 medium without FCS using a Centricon-30 concentrator (Amicon), as outlined by the manufacturer. The lack of this antibody's reactivity with *N. gonorrhoeae* was confirmed by direct immunofluorescence staining to assure that the effects seen resulted from its binding to CD66 receptors directly (data not shown).

For killing extracellular bacteria, gentamicin (100 $\mu g\,ml^{-1}$) was added to both reservoirs for 2 h. Protected intracellular bacteria were released by washing the filters three times in medium without gentamicin and lysis of the monolayer in 1% saponin/PBS for 15 min, followed by rigorous pipetting of the liquid up and down to break cellular clusters further. cfu were determined by plating dilutions on GC agar. The time required by traversing bacteria for initial internalization into the monolayer was determined by adding gentamicin (100 $\mu g\,ml^{-1}$) for 2 h only to the apical reservoir and then allowing the infection to continue in the continuous presence of a lower concentration of gentamicin (20 $\mu g\,ml^{-1}$) in the upper reservoir. The number of cfu appearing in the lower reservoir was determined as described above.

Immunofluorescence staining and confocal laser scanning

After infection, filters were washed three times with T84 medium for 5 min and then fixed using 3.7% (w/v) paraformaldehyde (PFA) at room temperature for 20 min. Filters were submerged in PFA and stored at 4°C until further use. Immunofluorescence staining was based on the method described previously (Dehio *et al*., 1997). PFA-fixed filters were washed three times in PBS for 5 min before and after each incubation step. To stain intracellular bacteria, fixed cells were incubated with 0.1% Triton X-100 (v/v) in PBS for 1 h, followed by 0.2% BSA (w/v) in PBS for 10 min to block non-specific binding. Fixed monolayers were incubated sequentially with rat anti-*N. gonorrhoeae* MS11 B2.1 antiserum (AK231; diluted 1:300 in the blocking buffer), and then either Cy5-conjugated goat anti-rat IgG conjugate or Cy3-conjugated goat anti-rat IgG antibody (diluted 1:100 in blocking buffer), as indicated. Antibody conjugates were both from Jackson ImmunoResearch. For triple stainings, this was followed by either the CD66 cross-specific monoclonal antibody D14HD11 (diluted 1:400 in blocking buffer), CD66c-specific 9A6 (diluted 1:200 in blocking buffer) or rabbit anti-ZO-1 antibody (diluted 1:75 in blocking buffer; ADE Laborbedarf), as indicated, and then a mixture of either lissamine rhodamine (LRSC)-conjugated goat anti-mouse IgG (H + L; diluted 1:100 in blocking buffer) or Cy5-conjugated goat anti-rabbit antibody, as indicated, and fluorescein isothiocyanate (FITC)–phalloidin (1:25 in blocking buffer; Sigma). All incubation steps were performed for 1 h. After staining, filters were cut out of their Transwell supports and then mounted apical side up under coverslips in 100 mM Tris-HCl, pH 8.5, 25% glycerol (w/v) and 10% Moviol 4-88 (w/v; Hoechst) for 30 min. The specimens were sealed using nail polish and then stored at 4°C until analysis using a Leica TCS NT confocal laser scanning microscope (Leica Lasertechnik) equipped with an argon–krypton mixed gas laser. In triple stainings, the three channels were recorded simultaneously. Corresponding images were processed digitally using Photoshop 4.0 (Adobe Systems).

Electron microscopy (SEM and TEM)

After infection, filters were washed three times for 5 min with T84 medium without FCS, fixed with 2% glutaraldehyde (GA) in 0.2 M HEPES, pH 7.4, at room temperature for 20 min, and then submerged in GA at 4°C for storage. Electron microscopy was performed essentially as described previously (Dehio *et al*., 1997; Mosleh *et al*., 1997). Briefly, GA-fixed filters were immersed in 1% osmium tetroxide in PBS (OsO_4; Science Services) for 2 h, dehydrated in ethanol and critical point dried using liquid CO_2. The filters were cut out of their Transwell supports, and the apical side was sputter coated with 1 nm chromium. The samples were then examined with a 10 kV accelerating voltage using a Hitachi S-800 field emission scanning electron microscope equipped with a detector for back-scattered electrons (BSEs) of the YAG type. Photographs were taken using Agfa APX25 film (Agfa-Gevaert). For transmission electron microscopy (TEM), GA-fixed filters were immersed in 1% OsO_4 for 1 h and, after dehydration through a graded series of ethanols, ultrathin sections were stained using 1% aqueous uranyl acetate and lead citrate. Stained filters were cut out, laid apical side up and then embedded in Epon. Samples were cut with a Diatome diamond knife (Reicher-Jung) using an ultramicrotome (LKB) and viewed using a Philips CM10 electron microscope. TEM micrographs were printed on Agfa Scientia film (Agfa-Gevaert).

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