# In Situ Expression and Localization of *Neisseria gonorrhoeae* Opacity Proteins in Infected Epithelial Cells: Apparent Role of Opa Proteins in Cellular Invasion

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## Summary

During natural infection, gonococcal opacity proteins (Opa) undergo rapid phase variation, but how this phenomenon contributes to the virulence of the bacteria is not well understood. In the present immunomorphological study we examined the actual Opa status of individual gonococci during various stages of gonococcal infection of Chang epithelial cells, by probing ultrathin sections of infected specimens with Opa-specific monoclonal antibodies. Our results demonstrate a heterogeneous Opa expression during the initial interaction of the bacteria, but an almost 100% expression of one of the probed Opas during their secondary attachment and entry into the host cells, suggesting a role for distinct Opas in cellular penetration. The association between Opa expression, tight attachment, and bacterial invasion into the host cells could be confirmed with isogenic variants that expressed different Opa proteins. Once inside the epithelial cells, both morphologically intact, Opa positive and morphologically disintegrated, Opa negative bacteria were observed. The loss of Opa immunoreactivity in intracellular gonococci could not be related to the presence of a particular Opa protein, but could be mimicked by incubating the organisms with extracts of sonicated uninfected epithelial cells, suggesting that it was caused by host cell proteolytic activity. Taken together, our data suggest that Opa phase transitions confer a functional adaptation of the bacteria enabling host cell penetration.

*Neisseria gonorrhoeae*, the causitive agent of the sexually transmitted disease gonorrhea, is characterized by a broad structural and antigenic diversity of its surface antigens (1–5). One family of proteins that shows inter- and intrastrain variation are the surface exposed opacity proteins (Opas)[1] (6–9). A single gonococcus may contain up to twelve different Opa genes (5), and the corresponding Opas, which have an apparent mol wt of 24–30 kD (8), are variably expressed at the surface of the bacterium (10, 11). The switching in Opa production, which occurs in vitro with a frequency of about $10^{-2}$–$10^{-3}$ per cell generation (12), can be explained by ribosomal frame shifting due to slipped-strand mispairing in a repetitive DNA sequence (13–15).

Several lines of functional evidence indicate that Opa phase variation is an important virulence determinant of the gonococcus. Apart from the variation of surface exposed epitopes between the different Opas, a phenomenon which may contribute to gonococcal evasion of the host immune defense (5, 16), Opa expression has been related to an increased bacterial resistance to killing by normal human serum (6, 17), to an increased killing by polymorphonuclear cells (18, 19) and to the development of intergonococcal adhesions (7, 20), which might favor bacterial colonization of the mucosa. The most extensively studied functional aspect of Opas however, has been their role in establishing gonococcal attachment to eukaryotic cells. Gonococci expressing certain Opas show a tissue specific increase in host cell adherence (18, 21, 22). The Opa variability might contribute to the tissue tropism of the infection by providing specificity in the recognition of postulated tissue specific host cell receptors (5, 23). Whether the Opa status is of importance for the gonococcal invasion of host cells or in the intracellular processing of the bacteria, is unknown.

Further understanding of the role of distinct Opas in the infection process may be achieved by constitutive expression (24) or mutagenesis of each of the proteins. An alternative

[1] *Abbreviations used in this paper:* LOS, lipooligosaccharides; Opa, opacity protein; PFA, paraformaldehyde.

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approach, chosen in the present study, is to determine Opa expression in situ, using immunoelectron microscopy. This approach, which has been shown to be appropriate for the detection of lipooligosaccharide (LOS) epitope variation in infected specimens (25, 26), has the advantage of determining the actual Opas expressed by individual bacteria at various stages of the infection, without either the selection of viable bacteria or the risk of phase transitions that exists on subculturing the bacteria. Our results demonstrate that starting from a gonococcal population with a heterogeneous Opa expression, only bacteria that express a distinct Opa strongly attach to and invade cultured Chang epithelial cells. In addition, a phase transition-independent loss of Opa immunoreactivity during intraepithelial disintegration of the bacteria was observed.

## Materials and Methods

*Bacterial Strain.* Gonococcus strain 830563 (serotype P.IA, $Opa^+$, $pili^-$) was a clinical isolate from a patient with systemic gonorrhea. The bacteria were grown on GC medium base (Difco Laboratories, Inc., Detroit, MI) containing 1% Vitox at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Light microscopy on the agar plates revealed a variety of different colony morphologies, indicative for heterogeneity in the state of piliation and opacity protein expression within the clinical isolate. When desired, colonies were selected for piliation and opacity with the help of a Leitz plate microscope.

*SDS-PAGE and Western Blot Analysis.* Gonococcal outer membranes, obtained by 1% sodium lauryl sarcosinate treatment of the bacteria (1 h, 20°C) followed by brief sonication and differential centrifugation (12,000 *g*, for 30 min to remove unbroken cells and 100,000 *g* for 60 min to pellet the outer membranes), were resuspended in 50 mM Tris-HCl buffer (pH 7.4) and electrophoresed under nondenaturing and denaturing conditions by using the discontinuous buffer system of Laemmli (27). The separated proteins were then either stained with Coomassie brilliant blue R-250 or transferred onto a nitrocellulose sheet at 1 mA/cm² for 2 h by using a semi-dry blot system (Biotec Fischer Company, Reiskirchen, FRG). Opa proteins were detected by incubating filters (saturated with 1% BSA) with Opa specific mAbs (undiluted culture supernatant) for 30 min (20°C) and thereafter with alkaline phosphatase conjugated anti-mouse immunoglobulin (Sigma Chemical Co., St. Louis, MO) (1/2000 diluted in PBS, 30 min, 20°C). The bands were visualized by addition of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl-phosphate in conjunction with Nitro Blue Tetrazolium (Sigma Chemical Co.).

*Culture of the Chang Epithelial Cell Line.* The Chang epithelial cells were grown as previously described (28).

*Infection Experiments.* Infection experiments were principally performed as previously described (28), except that the inoculum was obtained from picking multiple colonies that differed in colony morphology to increase the heterogeneity in expression of opacity proteins. Briefly, a suspension of gonococci (strain 830563) was added to confluent Chang epithelial cells at a ratio of 15:1. After 3 h of incubation, the nonadherent bacteria were removed and fresh medium was added. At various intervals postinfection, the cells were extensively washed with PBS and fixed (30 min, 20°C) in PBS containing 2% paraformaldehyde (PFA). When the 11CB8 containing Opas were probed, 0.1 M Lysine and 0.075 M sodium-meta-periodate was added to the fixative (PLP fixation) to prevent antigen damage (29). After fixation, the cells were either prepared for light microscopic quantification of the number of intracellular bacteria or collected, embedded in gelatin (2% [w/v] final concentration) and stored at 4°C in fixation solution until further processing for electron microscopy. The number of intracellular bacteria was estimated by using the recently described immunogold-silverstaining assay which enables discrimination between adherent and intracellular gonococci by conventional light microscopy (30).

*Polyclonal and Monoclonal Antibodies.* The opacity protein specific polyclonal antiserum AK-10 was generously provided by Dr. T. F. Meyer. The serum was raised by immunizing a rabbit with polyacrylamide gel extracts containing opacity protein from *N. gonorrhoeae* strain MS11. The serum shows in immunoblotting a broad crossreactivity with both gonococcal and meningococcal opacity proteins, indicating that it recognizes conserved opacity protein domains. For raising mAbs, Balb/c mice were immunized at days 1, 8, and 15 with lithium acetate-extracted outer membrane fractions (20 μg protein per immunization) prepared from gonococcus strain 830563. On day 18, spleen cells were fused with NS-1 myeloma cells according to Tam (31) and allowed to grow for 10 d. The antibody producing hybridomas were screened by ELISA using microtiter plates coated with outer membrane fractions (0.5 μg protein/well) or purified lipooligosaccharide (1.5 μg/well). The Opa specificity of the mAbs was confirmed by Western blot analysis: mAb 7D11E recognized an Opa with an apparent Mr of 27.5 kD and mAb 11CB8 recognized two different Opa proteins (Mr: 28 kD and 29.5 kD, respectively) (see Fig. 1). The LOS-specific mAb 7B1E has been previously described (25).

*Immunoelectron Microscopy.* To investigate Opa expression in native bacteria, Formvar-coated grids were inverted on a drop of gonococcal suspension (15 min) to allow binding of the bacteria. The grids were then incubated for 30 min with an appropriate dilution of antibody and thereafter with gold-conjugated protein A (10 nm gold particles, prepared according to Slot and Geuze [32]). After air drying, the grids were viewed in a Philips EM 201 at 60 kV. For post-embedding immunoelectron microscopy, the fixed and gelatin-embedded specimens were frozen and cryosectioned as previously described (28). The ultrathin sections were mounted on Formvar-coated grids and incubated with anti-Opa specific antibodies (undiluted culture supernatant, 30 min) and, after washing with PBS, with gold-conjugated protein A (30 min). In double-labeling experiments, the first immunolabeling (10 nm gold particles) was followed by an incubation with unconjugated protein A (10 μg/ml, 30 min) to saturate the binding sites. The second antibody was then applied and marked using 5 nm protein A-conjugated gold particles. All incubations were performed at 20°C. After washing the grids with PBS and distilled water, the sections were adsorption stained (33) and viewed in the electron microscope.

*Incubation of Gonococci with Bacterial and Host Cell Extracts.* Epithelial cell extracts were obtained as described (34). Briefly, Chang cells grown to confluence were washed in ice-cold PBS, collected with a rubber policeman and pelleted in an Eppendorf centrifuge (10 s, 10,000 *g*). The cells were then resuspended in 50 mM potassium phosphate buffer (pH 6.5) containing 0.1% (v/v) Triton X-100, and briefly sonicated. The homogenate was centrifuged for 10 min at 10,000 *g* and the supernatant was collected and tested for proteolytic enzyme activity. Gonococcal extracts were obtained by sonication of bacteria (Branson sonifier, position 2, 50% duty cycle, 3 min at 4°C) (Branson Sonic Power Co., Danbury, CT), followed by centrifugation (5 min, 10,000 g, 4°C) to remove the unbroken cells.

The effect of the extracts on gonococcal antigens was determined

by incubating bacteria that had been mounted onto Formvar coated grids, with various concentrations of the cell extracts for several time periods (1, 2, 4, and 6 h) in the presence or absence of 1 mM PMSF at 37°C. The grids were then washed (4 × 5 min) with ice-cold PBS containing 0.1% BSA, and incubated with anti-Opa mAbs 7D11E or 11CB8 (undiluted culture supernatant, 30 min, 20°C) or anti-LOS mAb 7B1E (1/100 diluted culture supernatant, 30 min, 20°C) and gold-conjugated protein-A. After air-drying, the bacteria were viewed in the electron microscope.

## Results

*Variability in Gonococcal Opa Expression at the Start of the Infection as Determined by Immunoblotting and Immunoelectron Microscopy.* To establish the biological significance of Opa phase variation in the gonococcal infection of epithelial cells, we infected cultured Chang conjunctiva cells with a mixed population of non-piliated bacteria expressing multiple Opas, as was judged from colonial morphology and confirmed in SDS-gels and immunoblotting (Fig. 1). The diversity in Opa expression in this population was further explored by immunoelectron microscopy using Opa-specific monoclonal antibodies. When native gonococci (strain 830563), that had been incubated with the anti-Opa mAbs 7D11E and 11CB8 and subsequently with gold-conjugated protein A, were viewed in the electron microscope, about 15% of the bacteria stained positively for the probed 7D11E epitope, 35% for the probed 11CB8 epitope and 40% for both the 7D11E and the 11CB8 epitope. 10% of the bacteria did not express either of the probed epitopes (Fig. 2). This distribution of labeling confirmed that the corresponding Opas were heterogeneously expressed among the bacteria and demonstrated that individual bacteria can express more than one type of Opa at the same time.

Incubation of ultrathin sections of the same bacteria with the Opa specific antibodies, a technique required for determination of the Opa expression of both extra- and intracellular bacteria, resulted in extensive gold labeling of the 7D11E epitope, but the 11CB8 epitope could not be detected. This unexpected observation was found to result from the destruction of the 11CB8 epitope by the PFA fixation. This damage to the antigen, which might have led to a misinterpretation of Opa expression, was prevented by adding sodium-metaperiodate and lysine to the fixative (PLP fixation). These compounds primarily stabilize carbohydrate moieties, thus diminishing PFA crosslinking damage (24). Although LOS epitopes were lost by this procedure probably because of oxidation of sugar residues, both Opa epitopes could be visualized in the electron microscope. They were found strictly localized at the gonococcal membranes. The bacterial cytoplasm was always free of gold spheres. The specificity of the labeling was demonstrated by the absence of gold particles after incubating the bacteria with a mAb directed against the gonococcal pore protein P.IB (strain 830563 expresses the P.IA phenotype) (data not shown).

*Expression and Localization of Opas During Initial Bacteria-Host Cell Contact.* The successful localization of different Opas in cryosections enabled us to investigate Opa expression at various stages of a gonococcal infection of Chang epithelial cells. Electron microscopy of ultrathin cryosections of 1.5 h infected cells that had been incubated with the Opa-specific monoclonal antibodies and the gold conjugate, revealed that about 60% of the cell-associated bacteria expressed the Opa protein containing the 7D11E epitope (Fig. 3 *A*). Approximately 75% of the bacteria were positive for the 11CB8 containing Opas. This distribution in labeling among the bound bacteria resembled that in the inoculum, and suggested that there was no preference for any of the different Opa expressing bacteria in the primary interaction with the epithelial cells. At this stage of the infection, most of the cell-associated bacteria could still be removed from the cell surface by vigorous washing of the monolayer, suggesting that the primary attachment was not a strong interaction with the host cells. Morphologically, the gonococci were found primarily attached to microvilli (Fig. 3 *A*). With respect to the localization of the gold particles, they were specifically located at the gonococcal membranes and there was no difference in gold labeling between the bacteria that were directly in contact with the host cells, and those connected via neigh-

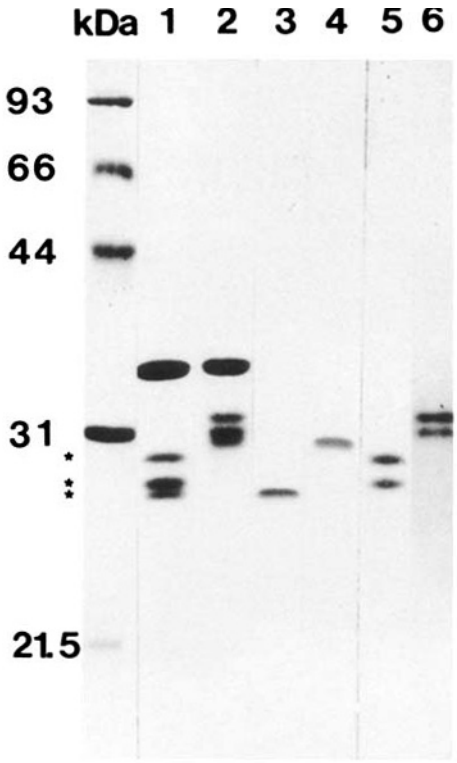


**Figure 1.** SDS-PAGE and immunoblotting of gonococci (*N. gonorrhoeae*, strain 830563) used as an inoculum in the infection experiments. Sarkosyl extracted outer membranes were electrophoresed (12% gels), and immunoblotted using the Opa specific mAbs 7D11E and 11CB8. (Lanes *1* and *2*) SDS-PAGE of membranes solubilized at 37°C and 100°C, respectively (Coomassie staining). Note the characteristic heat-modifiability of the Opa proteins, that are marked by the asterisks. (Lanes *3* and *4*) blot of the same samples incubated with MAb 7D11E. An Opa with an apparent Mr 27.5 kD is recognized. (Lanes *5* and *6*) blot incubated with mAb 11CB8. The mAb recognizes two Opa proteins with an apparent Mr of 28 kD and 29.5 kD, respectively.

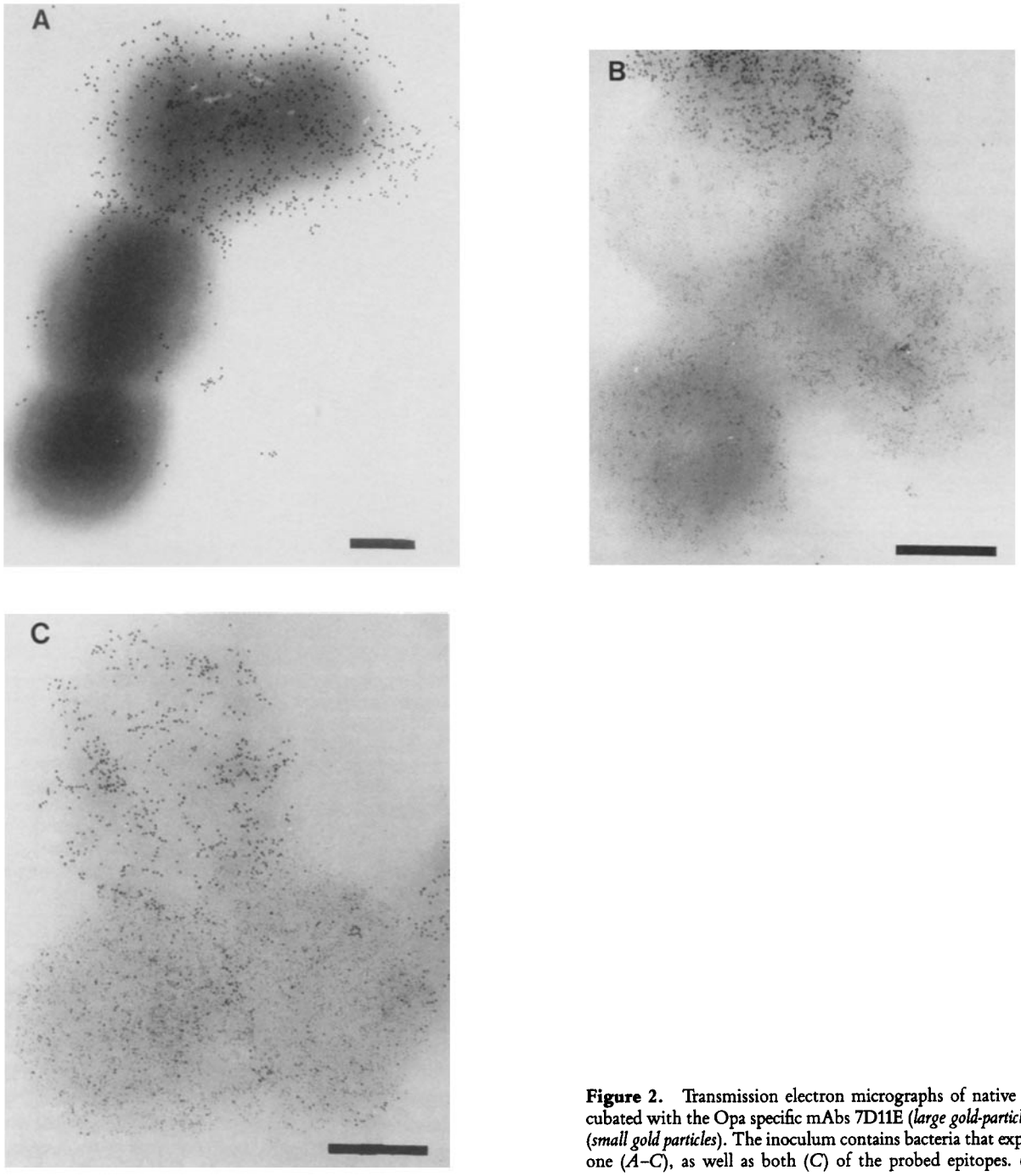


**Figure 2.** Transmission electron micrographs of native gonococci incubated with the Opa specific mAbs 7D11E (*large gold-particles*) and 11CB8 (*small gold particles*). The inoculum contains bacteria that express none (*A*), one (*A–C*), as well as both (*C*) of the probed epitopes. (*Bar*) 0.7 μm.

boring bacteria. Host-cell microvilli, that have previously been found to contain LOS molecules (25), did not react with Opa-specific antibodies (Fig. 3 *B*).

*Preferential Secondary Attachment and Entry of 7D11E Positive Bacteria.* In 4 h infected cells, the above described primary interaction of the bacteria was often followed by the formation of localized regions of intimate contact between the bacterial outer membrane and the plasma membrane of the host cells (secondary attachment; Fig. 4). The zonal contact was clearly observed where the bacteria were slightly retracted from the eukaryotic cell surface because of handling of the specimen and was even more evident when bacteria were being internalized by the host cells (Fig. 5). Interestingly, nearly all attached bacteria (99% of the several hundreds of bacteria counted) that demonstrated this intimate contact with the epithelial cell surface, and those being internalized by the host epithelial cells, expressed the 7D11E epitope. Such homogeneity in Opa expression was not observed for the

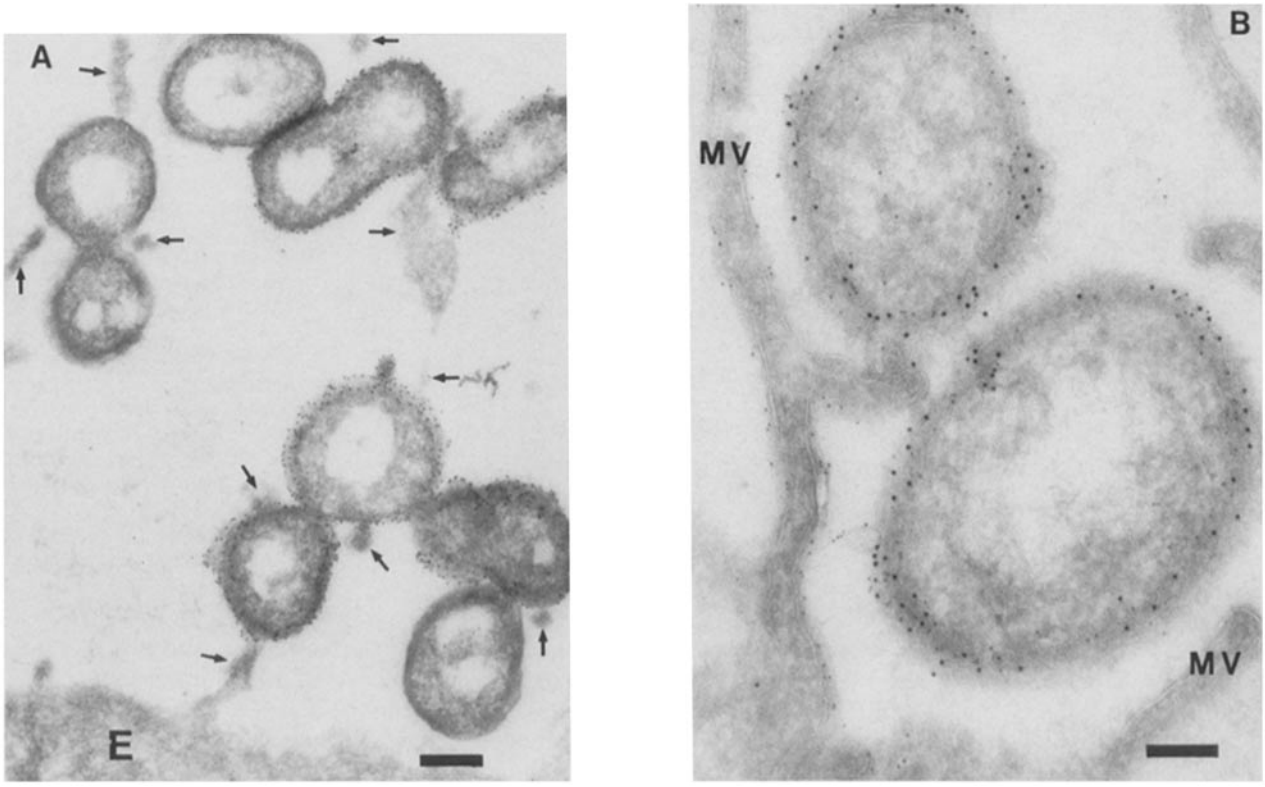


**Figure 3.** Transmission electron micrographs of immunolabeled cryosections of 90 min infected epithelial cells. (*A*) Most of the gonococci are bound to microvilli (*arrows*) protruding from the epithelial cell (*E*). The probed 7D11E Opa epitope is expressed by about 60% of the attached bacteria. (*B*) In contrast to the restricted localizations of Opas at the bacterial membranes (*10 nm gold particles*), LOS molecules (*5 nm gold particles*) are also located at epithelial cell microvilli (MV), known to elongate during the early stages of the attachment process. (*Bar A*) 0.5 μm, (*Bar B*) 0.15 μm.

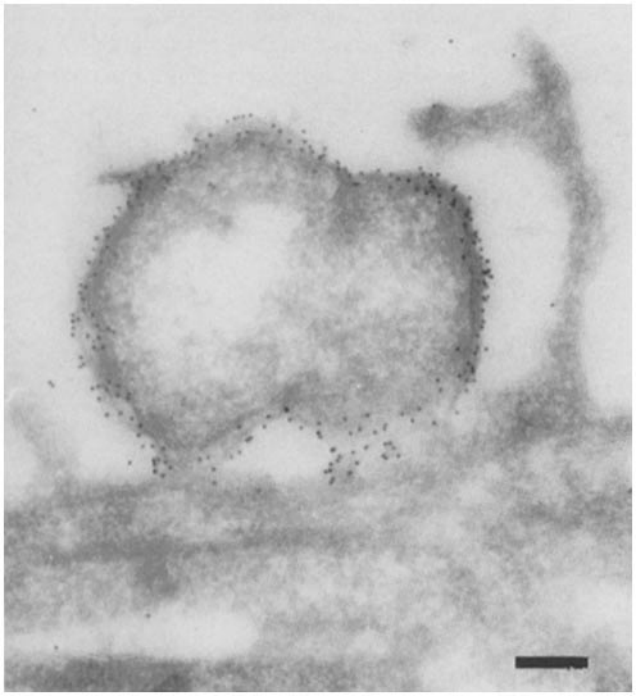

**Figure 4.** Transmission electron micrograph illustrating zones of intimate contact between a 7D11E positive gonococcus and the host cell membrane. (*Bar*) 0.2 μm.

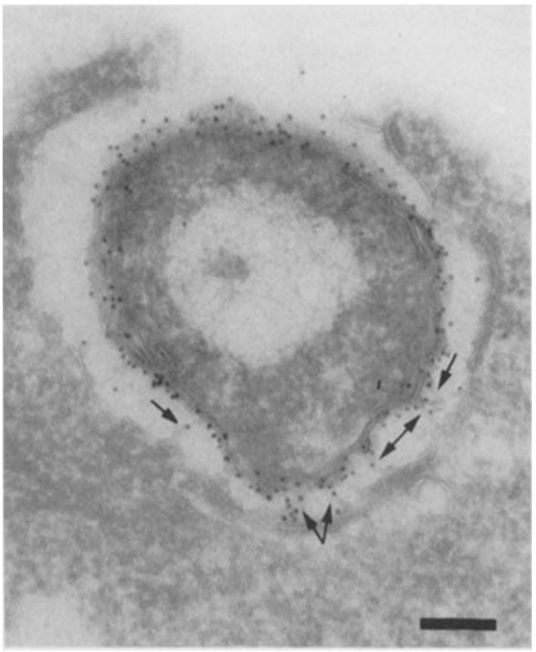

**Figure 5.** Transmission electron micrograph of a gonococcus in the process of being internalized. The gold particles, representing the 7D11E containing Opa are randomly distributed over the bacterial membranes and membraneous structures intercalating between the bacterium and the host cell membrane (*arrows*). (*Bar*) 0.2 μm.

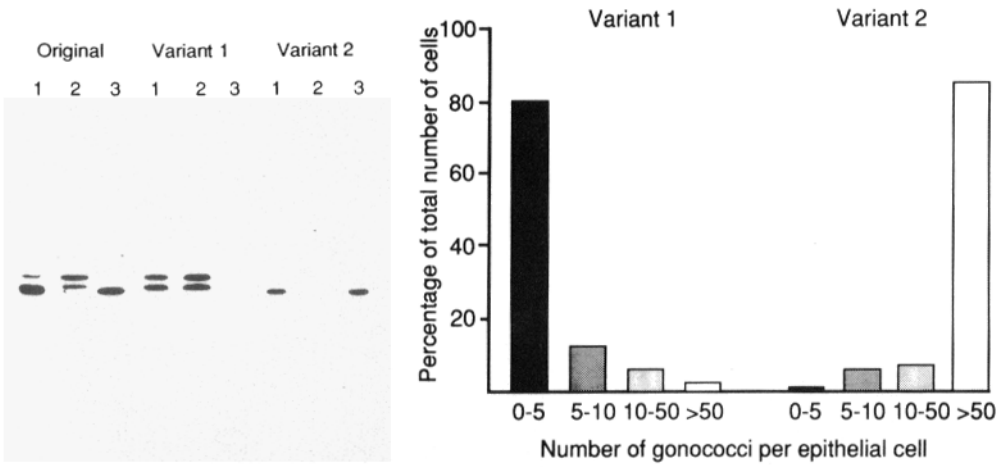


**Figure 6.** Opa profiles and invasiveness of selected Opa variants which differ in the expression of the 7D11E containing Opa. The immunoblot (*left*) shows the Opa profiles of the original inoculum and of two variants expressing either the Opa proteins containing the 11CB8 epitope (variant 1) or the 7D11E containing Opa (variant 2). The Opa proteins were identified with the polyclonal serum AK-10 (Lane *1*), mAb 11CB8 (lane *2*) and mAb 7D11E (lane *3*). The right panel shows the invasiveness of the variants after 6 h of infection, as determined by light microscopic counting of at least 100 eukaryotic cells. The 7D11E positive gonococci (variant 2) are much more invasive than the 7D11E negative, 11CB8 positive bacteria (variant 1). Data are from one of four experiments.

11CB8 epitope. 70% of the 7D11E positive bacteria counted labeled for this epitope, which was the same percentage as was found in the inoculum. These observations suggest a preferential secondary attachment and entry of 7D11E positive bacteria. This important finding was confirmed in infection experiments with isogenic 7D11E positive and negative gonococcal variants that were isolated from the infection system and that only differed in Opa profile as judged from immunoblotting experiments (Fig. 6). The variant expressing the 7D11E containing Opa was about 100-fold more invasive than the variant that lacked this protein but that still expressed the other two Opa proteins (Fig. 6).

A second interesting observation at this stage of infection was that the gold particles were not only found randomly distributed over the bacterial cell surface, but that they were also located on the membraneous structures that intercalated between the bacteria and the host cells (Fig. 5; see also Fig. 7). This finding suggests that these structures, which may function in anchoring the bacteria to the host cell membranes during bacterial entry, consist of outer membrane material.

*Intracellular Alterations in Opa Labeling.* At 12–14 h postinfection many gonococci had been internalized by the host cells. Most cells contained 15–20 gonococci in vacuoles and, occasionally, an aggregate of 2–6 bacteria was seen within one vacuole. The majority of these bacteria were morphologically intact and more than 90% of them labeled for the 7D11E and about 75% for the 11CB8 epitope, indicating unchanged Opa expression. Many bacteria still seemed connected

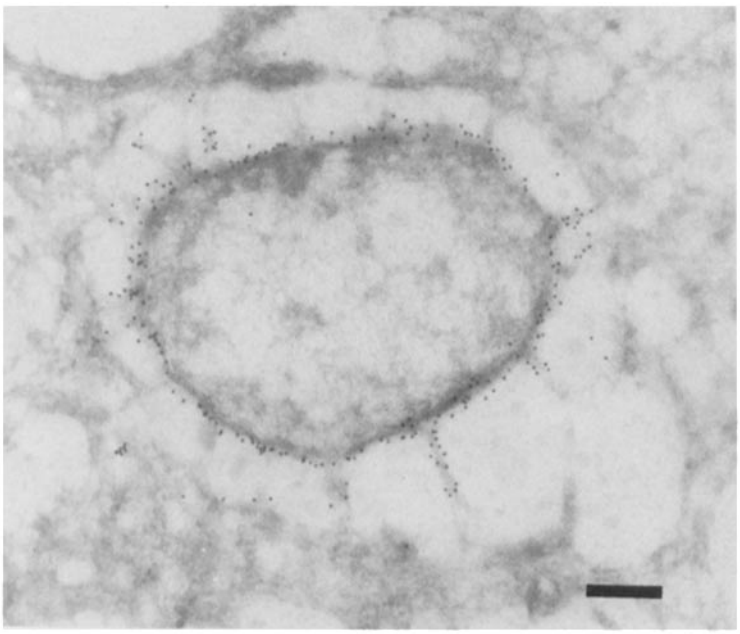

**Figure 7.** Transmission electron micrograph of a morphologically intact gonococcus lying within a membrane bound vacuole. The bacterium still expresses many 7D11E containing Opas and is tightly attached to the vacuole membrane by intercalating structures that also label for the probed 7D11E epitopes (*Bar*) 0.25 $\mu$m.

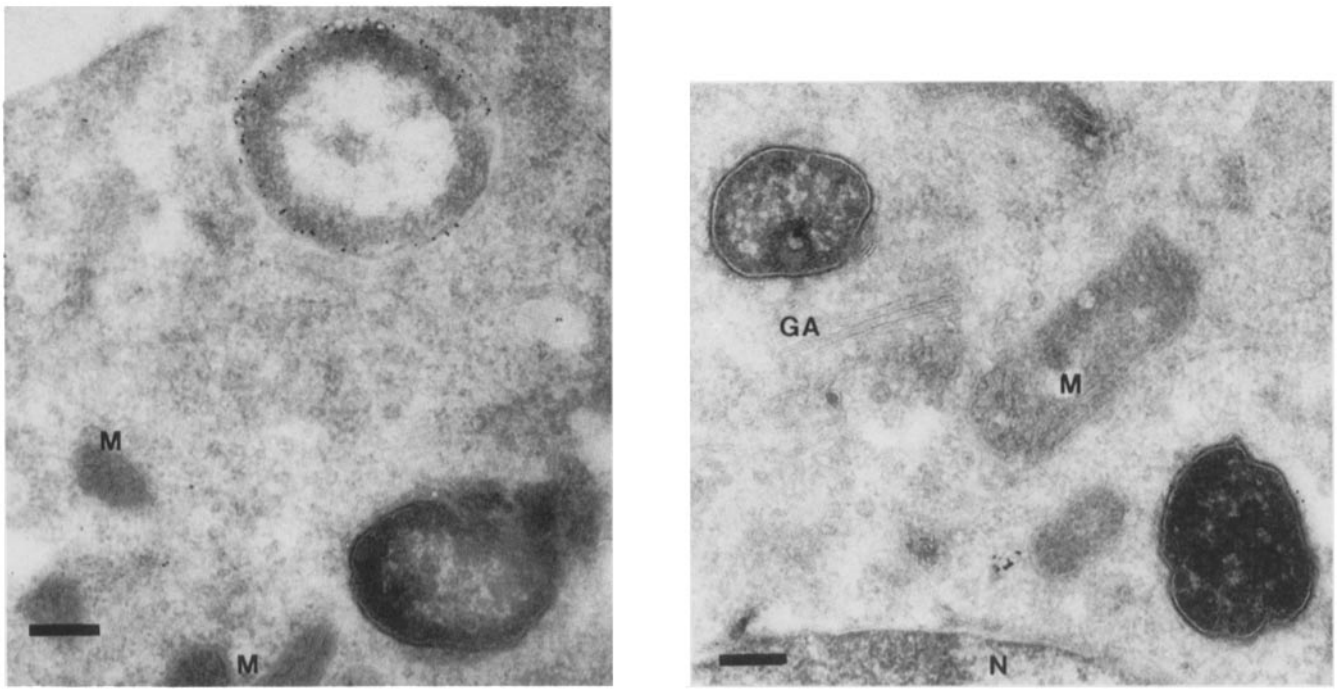


**Figure 8.** Transmission electron micrographs of epithelial cells containing gonococci that are either immunomorphologically intact (*left*) or at different stages of disintegration (*left* and *right*). All bacteria are lying within membrane bound vacuoles. The morphologically disintegrated bacteria, which are smaller in size and show a much more intense staining of their cytoplasm (25, 28, 52), are no longer labeled for the probed Opa epitope (7D11E). N-nucleus, M-mitochondrion, GA-golgi apparatus. (*Bars*) 0.375 μm.

to the vacuole membrane by Opa containing membraneous structures (Fig. 7). A minority of the intracellular bacteria however, had more or less lost the classical gonococcal appearance of a three layered membrane and a translucent cytoplasm. They showed morphological signs of disintegration with ruffling and shedding of the outer membrane and an increase in the electron density of the cytoplasm (Fig. 8). The morphological disintegration was accompanied by a gradual loss of both 7D11E and 11CB8 labeling, indicating that it were Opa positive cells that were degraded. In totally disintegrated bacteria hardly any Opa labeling could be detected (Fig. 8). The absence of a relationship between the Opa status of the bacteria and their subsequent morphological degradation suggests that the observed loss in Opa immunoreactivity results from a breakdown of epitopes rather than from switching or downregulation of Opa synthesis.

*Sensitivity of the Probed Opas to Host Cell Proteolytic Enzyme Activity.* The apparent immunomorphological degradation of certain intracellular bacteria was further investigated by incubating Opa positive gonococci with extracts of sonicated uninfected Chang epithelial cells. Exposure of the bacteria to the cell extracts (1–6 h) resulted in a drastic reduction of both 7D11E and 11CB8 labeling, but not of the probed LOS epitope (Fig. 9, *A* and *B*). This effect could be partially prevented by the addition of the protease inhibitor PMSF (1 mM) to the cell extract (not shown). Exposure of the bacteria to a gonococcal cell extract did not influence the Opa labeling (Fig. 9 *C*). These data demonstrate that the probed Opas are sensitive to proteolytic digestion and suggest that the intracellular loss in Opa immunolabeling is caused by host cell proteolytic enzyme activity rather than by endogenous gonococcal enzymes that may be released during the disintegration of the bacteria.

## Discussion

Subculturing of gonococci from a human volunteer infected with an Opa negative strain yields bacteria that express single or, after prolonged infection, multiple Opas (35). This finding along with those of others who isolated different Opa variants from various anatomical sites (36, 37), or at different times during the menstrual cycle (38), suggest that Opa expression varies with environmental conditions and that these proteins might play an important role in gonococcal pathogenesis. The present study further explored this topic by investigating, for the first time, the in situ expression and localization of Opas in infected cells, using immunoelectron microscopy. Our data demonstrate that in contrast to the initial interaction of gonococci with epithelial cells, Opa phase variation influences the formation of intense bacterial-host cell membrane contact and the ability of the bacteria to invade eukaryotic cells. Furthermore, intracellularly, a loss in Opa immunoreactivity was observed in bacteria that showed morphological signs of disintegration. This phenomenon however, was found to result from host cell proteolytic activity rather than from phase variation of the probed epitopes.

The novel strategy of determining the antigen expression in situ with the electron microscope has the advantage of

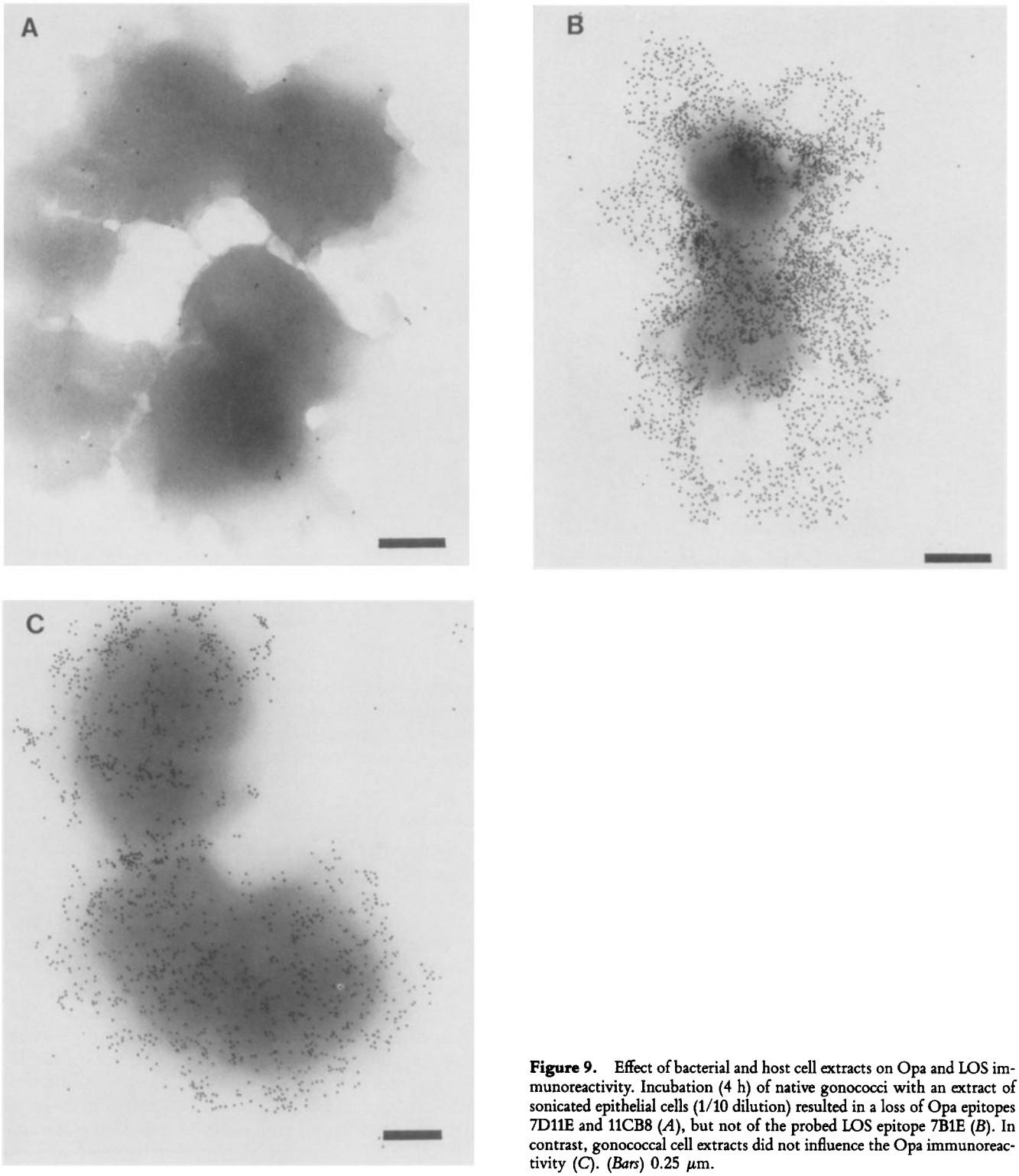


**Figure 9.** Effect of bacterial and host cell extracts on Opa and LOS immunoreactivity. Incubation (4 h) of native gonococci with an extract of sonicated epithelial cells (1/10 dilution) resulted in a loss of Opa epitopes 7D11E and 11CB8 (*A*), but not of the probed LOS epitope 7B1E (*B*). In contrast, gonococcal cell extracts did not influence the Opa immunoreactivity (*C*). (*Bars*) 0.25 μm.

providing us with information about the Opa expression at a single level, without the need of subculturing for the bacteria. The virtue of this technique was already demonstrated with the characterization of the bacteria used as an inoculum in the infection experiments. SDS-PAGE analyses and immunoblotting of these bacteria indicated simultaneous expression of three different Opas. Two of the proteins (Mr of 28 and 29.5 kD, respectively) harbored the 11CB8 epitope and a third protein (Mr of 27.5 kD) was specifically recognized by the mAb 7D11E. Immunoelectron microscopy of both

native and appropriately fixed and cryosectioned bacteria however, demonstrated that the bacterial population actually consisted of organisms that expressed none, one, and distinct Opas. This heterogeneity in Opa expression among individual bacteria, which can be explained with the high frequency of Opa phase transitions (13), may have been missed or lost with subculturing of the bacteria and could be critical in the interaction with the host cells. The observation that a single gonococcus can express more than one Opa at once, is in agreement with the finding that each Opa gene has its own functional promoter (13), and confirms previous observations with another strain (39).

The diversity in Opa expression in the inoculum was highly advantageous in relating Opa expression to the events that follow infection of epithelial cells. Gonococcal infection of Chang epithelial cells involves attachment of the bacteria to the cell surface, invasion into the host cells and, morphologically, a differential intracellular processing of the bacteria resulting in either apparent survival or degradation of the intracellular organisms (25, 28). Our immunomorphological data demonstrate that the heterogeneity in Opa expression in the inoculum continues to exist during the initial bacteria-host cell contact. This supports the view that Opas are not required for primary attachment (40, 41). The factors that are responsible for the observed relatively weak binding of the nonpiliated bacteria to the host cells are presently unknown. Recently, several additional gonococcal adhesins have been identified which might function in this way (42, 43). Our data indicate that the primary site of bacteria-host cell interaction are the host cell microvilli. It would be interesting to test whether the glycolipid receptors for these novel adhesins (42, 43), are specifically located at these cell protrusions.

In contrast to the initial bacteria-host cell interaction, when 50–60% of the bacteria expressed the 7D11E epitope containing Opa, we found a preferential secondary attachment and bacterial entry into the epithelial cells of 7D11E positive gonococci. This selective interaction, which was confirmed in infection experiments with isogenic variants that differed in the expression of the 7D11E containing Opa, suggests that a distinct Opa promotes the tight attachment and cellular penetration. Variability in Opa expression has previously been associated with a strong, irreversible attachment of gonococci to eukaryotic cells (18, 21, 44). This was suggested from experiments with isogenic Opa variants and from subculturing of adherent gonococci. Our in situ expression data confirm these results and furthermore demonstrate that the tight attachment involves a zonal interaction between the bacteria and the host cell plasma membrane. The zones of adhesion, that continued to exist during the entry of the bacteria, show a remarkable resemblance to the type of contact that can exist between bacteria (7, 45). These intergonococcal adhesions are supposed to involve the specific recognition by Opas of the sugar moiety of LOS molecules on adjacent bacteria (46). Whether tight bacteria-host cell contact involves the binding of Opa to the LOS that can be found at the host cell membrane early in the infection (Fig. 3 *B*; reference 25) or to LOS cross-reactive carbohydrate moieties on the eukaryotic plasma membrane (47), awaits further identification of Opa receptors. In polymorphonuclear cells, the Opa recognition site seems to contain carbohydrates (48). Identification of a specific Opa receptor on epithelial cells has until now been unsuccessful (23). The fact that we found one of three Opas to be associated with increased attachment to epithelial cells, and similar findings reported by others with polymorphonuclear cells (18, 49), indeed suggests that different cell types might express different Opa specific receptors.

The exciting results demonstrating a relationship between a distinct Opa and cellular invasion is the first step in unravelling the so far unknown mechanism of gonococcal entry into eukaryotic cells. The exact role of Opas in the entry process is yet to be defined. At this time, we cannot exclude that distinct Opas are able to initiate the gonococcal entry process in a similar manner to that described for the invasion of *Yersinia pseudotuberculosis* (50). On the other hand, it is possible that distinct Opa(s) promote cellular invasion by anchoring the bacteria to the host cell surface and allowing additional bacterial components to initiate the internalization process. One factor that might mediate such a process is the major outer membrane protein of the gonococcus, protein I. This protein can, when in contact with host cell membranes, translocate into these membranes causing an alteration in the plasma membrane potential (51). Definite proof that Opa phase transitions may function as an adaptive mechanism enabling cellular invasion can be obtained by mutagenesis of the invasion-associated Opa or by specific blocking of the Opa host-cell interaction. Such experiments are underway.

The current knowledge about the intracellular fate of gonococci in epithelial cells is limited and based on morphological data showing endocytic vacuoles that contain either apparently surviving or disintegrating bacteria (25, 28, 52). Our results demonstrating both morphologically intact, Opa positive and degraded, Opa negative organisms in 12–14 h infected cells extend these observations and are a good basis to acquire information about the mechanism(s) behind the apparent different intracellular fate of the bacteria. The invariable expression of the 7D11E epitope carrying Opa by gonococci that had no signs of morphological disintegration suggests that, if indeed these bacteria are still metabolically active, the intracellular environment does not induce a switching or down regulation of Opa synthesis. The observed morphological disintegration of these Opa positive bacteria inside the host cells on the other hand, indicates that the Opa expression by itself is not sufficient (if necessary at all) to prevent intracellular degradation of the organisms. These results suggest that Opas are not important determinants of the intracellular fate of the bacteria.

A second interesting observation regarding the different intracellular fate of the bacteria was the difference in sensitivity of the probed Opa epitopes for proteolysis by bacterial and host cell extracts. This finding suggests that the loss in Opa immunoreactivity that was observed intracellularly for bacteria that showed morphological signs of disintegration, results from host cell proteolytic activity rather than from endogenous gonococcal enzyme activity. This finding implies that in the infection a part of the bacterial population

is processed into a cellular compartment with proteolytic enzyme activity while the immunomorphologically intact bacteria are apparently able to escape the host cell defense machinery. This possible explanation for the observed different intracellular fate of the bacteria is currently further investigated by immunoelectron microscopic probing of other protease-sensitive bacterial epitopes and by characterization of the respective host cell compartments.

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