

# Experimental Infection of Native Human Ureteral Tissue with *Neisseria gonorrhoeae*: Adhesion, Invasion, Intracellular Fate, Exocytosis, and Passage through a Stratified Epithelium

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Received 16 December 1996/Returned for modification 10 February 1997/Accepted 28 May 1997

**The exact mechanisms by which *Neisseria gonorrhoeae* invades the mucosal lining to cause local and disseminated infections are still not fully understood. The ability of gonococci to infect the human ureter and the mechanism of gonococcal infection in a stratified epithelium were investigated by using distal ureters excised from healthy adult kidney donors. In morphological terms, this tissue closely resembles parts of the urethral proximal epithelium, a site of natural gonococcal infection. Using piliated and nonpiliated variants of *N. gonorrhoeae* MS11, we demonstrated the importance of pili in the attachment of gonococci to native epithelial cells as well as their association with epithelial damage. By electron microscopy we elucidated the different mechanisms of colonization and invasion of a stratified epithelium, including adherence to surface cells, invasion and eventual release from infected cells, disintegration of intercellular connections followed by paracellular tissue infiltration, invasion of deeper cells, and initiation of cellular destruction and exfoliation resulting in thinning of the mucosa.**

Gonorrhea, the sexually transmitted disease caused by *Neisseria gonorrhoeae*, is still a major public health problem. In the United States, for example, about 400,000 cases are reported yearly to the Centers for Disease Control and Prevention (33). The exact mechanisms by which *N. gonorrhoeae* invades the mucosal lining to cause local and disseminated infection are still not fully understood. Pili are known to play a central role in localized neisserial infection by mediating the initial contact between the bacterium and the surfaces of epithelial cells (25). Recent studies indicate a function of the PilC protein in pilus-mediated adherence (29, 32, 44). However, pili possess additional binding functions which may contribute to the colonization of mucosal surfaces by gonococci (31). Following the pilus-mediated events, the bacteria contact the epithelial surface tightly via their phase-variable colony opacity-associated (Opa) proteins. However, only distinct variant forms of Opa proteins are capable of conferring contact to epithelial cells (21, 23), which is mediated by a heparan sulfate-containing proteoglycan receptor (6, 40). The binding to this receptor is essential for the invasion of epithelial cells by gonococci, but it is unclear whether this receptor is directly involved in the signaling events required for the engulfment of adherent gonococci (13). Recently, members of the CD66 receptor family have been identified as receptors for several Opa proteins which mediate neisserial interactions with phagocytic cells (7, 14, 43). One member of this family, the carcinoembryonic antigen, is expressed on transformed or embryonic epithelial cells (38). Whether this or other CD66 molecules play a role in the invasion of normal mucosal surfaces by *N. gonorrhoeae* is currently not known.

Electron microscopic studies by a number of investigators found ingested gonococci most often embedded in the mem-

branes of mucosal cells and, less frequently, inside epithelial cells (1, 8, 17, 38). In reports of studies with the human fallopian tube in organ cultures as a model for gonococcal infection, McGee and coworkers (25, 26) suggested that ingested gonococci are transported in a membrane-bound vacuole to the basolateral surfaces of the cells and released into the subepithelial tissue (transcytosis). In contrast, Ward et al. (46), using the same model, showed that gonococci are discharged into deeper tissues through exfoliation and lysis of infected cells.

In the present work, we used human distal ureters excised from healthy adult kidney donors as a model to study the interaction of gonococci with a native complex epithelium susceptible to in vitro infection. Despite significant morphological differences, the complex morphology of the ureteral epithelium resembles that of several natural sites of gonococcal infection, such as the urethra, cervix, and conjunctiva. This electron microscopical investigation elucidated the mechanisms employed by gonococci to interact with and traverse such a stratified epithelium.

## MATERIALS AND METHODS

**Bacterial strains and variants.** Strains used in this study were derivatives of *N. gonorrhoeae* MS11 (28), i.e., the piliated Opa30-expressing variant N137 (E1) and the nonpiliated Opa30 variant N263 (B2.1) (23). Expression of pili and Opa was confirmed by Western blotting with pilin- and Opa-specific antibodies. The strains were stored on glass beads at  $-70^{\circ}\text{C}$  and plated out overnight on a GC agar base containing a vitamin supplement (Becton Dickinson) at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$ .

**Preparation of ureters.** Distal human ureters were prepared as previously described (5). Briefly, pieces of the distal ureter were obtained from four healthy adult kidney donors whose ages ranged between 20 and 50 years and were transported to the laboratory in serum-free wash medium supplemented with antibiotics (4). The ureteral samples were rinsed several times with fresh wash medium and clipped free of loose adventitial connective tissue. They were then opened, cut into 0.25- to 0.5-cm<sup>2</sup> fragments, immersed for 4 to 6 h in the wash medium, and then left overnight in antibiotic-free complete medium. The contents of the media used here have been described elsewhere (4).

**Infection experiments.** The day after preparation, fragments from each of the four ureter pieces were infected with the test neisserial strains by incubating

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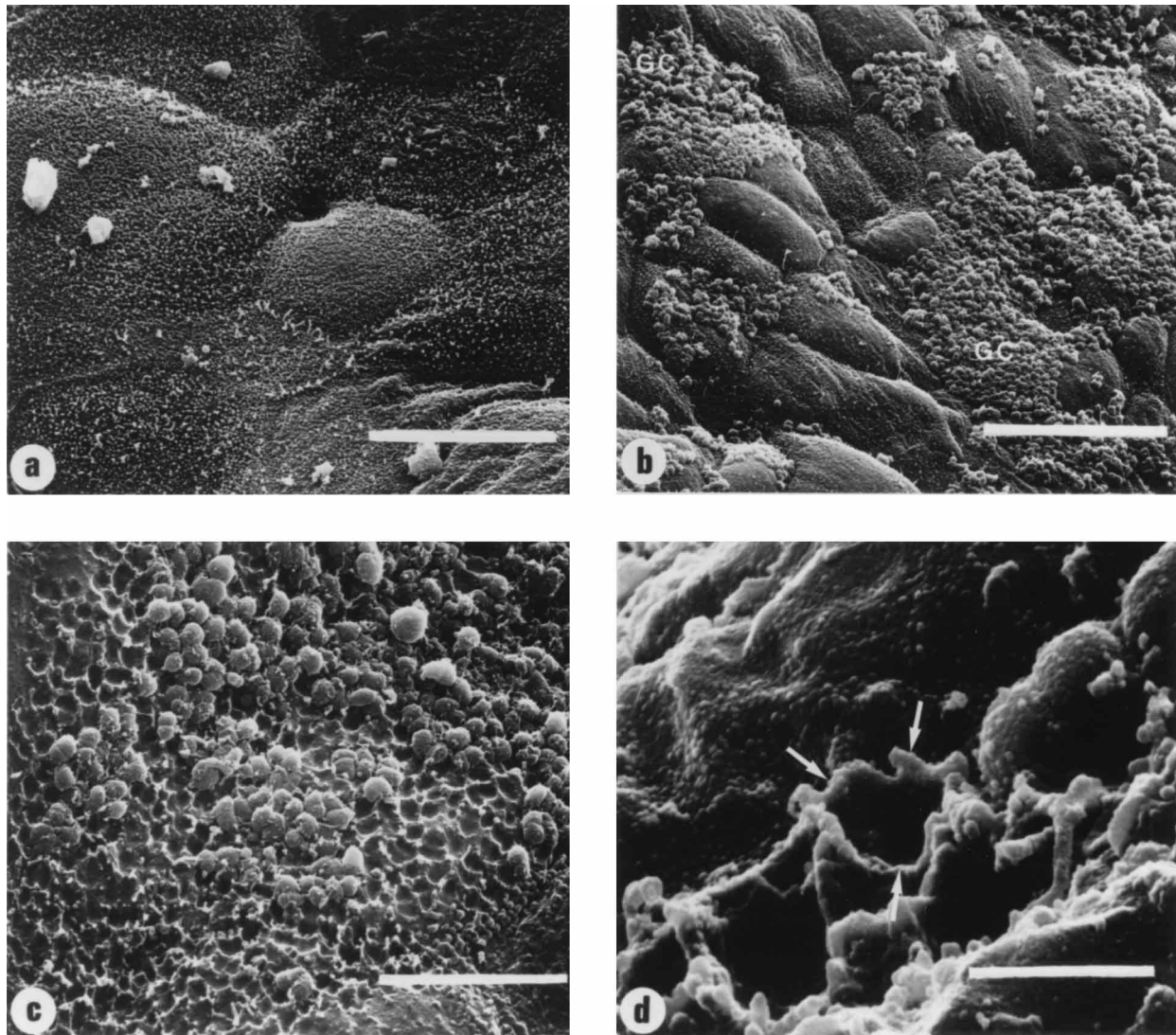


FIG. 1. SEM of control (a) and strain E1-infected (b to d) ureteral epithelium. (b) SEM of massive clumps of adherent gonococci (GC) occupying large areas of the epithelium 12 h after infection; (c) fingerprintlike impressions left by gonococci broken off and formed during SEM preparation (12 h postinfection); (d) higher magnification of a portion of panel c showing cytoplasmic protrusions (arrows) indicating a stage during the process of engulfment of a gonococcus. Bars, 13.6 (a), 20.0 (b), 6.0 (c), and 1.0 (d)  $\mu\text{m}$ .

them with their epithelial sides directed upward in a 2-ml suspension of gonococci ( $40 \times 10^6$  cell/ml of antibiotic-free complete medium). The medium was changed at 2-h intervals during the infection period. After 4, 6, 9, and 12 h, the pieces were thoroughly washed five times with 2 ml of phosphate-buffered saline (pH 7.2) and once with Hanks' balanced salt solution to remove nonadherent gonococci. The infected pieces and noninfected control sample were then fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.2) containing 5 mM  $\text{CaCl}_2$  either for 1 h at room temperature or overnight at 4°C.

**SEM.** Glutaraldehyde-fixed pieces of ureters were processed for scanning electron microscopy (SEM) according to a modification of König (20). In brief, a small amount of nonwatersoluble glue (UHU, Bühl, Germany) was spread on cellulose acetate coverslips (Plano) that were subsequently submerged in a watch glass containing ice-cold sodium cacodylate buffer with 5 mM  $\text{CaCl}_2$ . After being washed (three times for 10 min) in cacodylate buffer, ureter pieces were held carefully with a forceps and fixed on the glue. Samples on coverslips were afterwards immersed in 1%  $\text{OsO}_4$  (Science Services, Munich, Germany) for 2 h. They were then washed with cacodylate buffer (three times for 10 min), dehydrated in a graded series of ethanol, and dried at the critical point with liquid  $\text{CO}_2$ . Finally, the samples were coated with Au-Pd and examined with a Hitachi model S-5000 scanning electron microscope. Photographs were taken on Agfa APX25 film (Agfa-Gevaert, Leverkusen, Germany).

**TEM.** Glutaraldehyde-treated specimens were post-fixed in 1%  $\text{OsO}_4$ , dehydrated stepwise in ethanol, and embedded in Durcupan (Fluka). Ultrathin sec-

tions were cut with glass knives and a Diatome diamond knife (Reichert-Jung, Vienna, Austria) on an ultramicrotome (LKB, Stockholm, Sweden) at areas occupied by adherent gonococci after light microscopical examinations of semithin sections. Transmission electron microscopy (TEM) studies were carried out with a Zeiss EM109, and micrographs were reproduced on Agfa Scientia film (Agfa-Gevaert).

## RESULTS

**Adherence of *N. gonorrhoeae* to ureteral epithelial cells.** To determine how gonococci interact with stratified primary epithelial tissue, native ureteral pieces were challenged with two variants of *N. gonorrhoeae* MS11 and investigated by SEM and TEM. Figure 1a shows an SEM micrograph of the epithelium of a noninfected human ureter control. Infection of ureteral pieces with the wild type, strain E1, revealed the ability of the piliated gonococcus to adhere to this epithelium. The degree of adhesion increased gradually with time; at 12 h after infection, clumps of the piliated gonococci were seen covering most of the cells over the entire epithelium of pieces taken from the

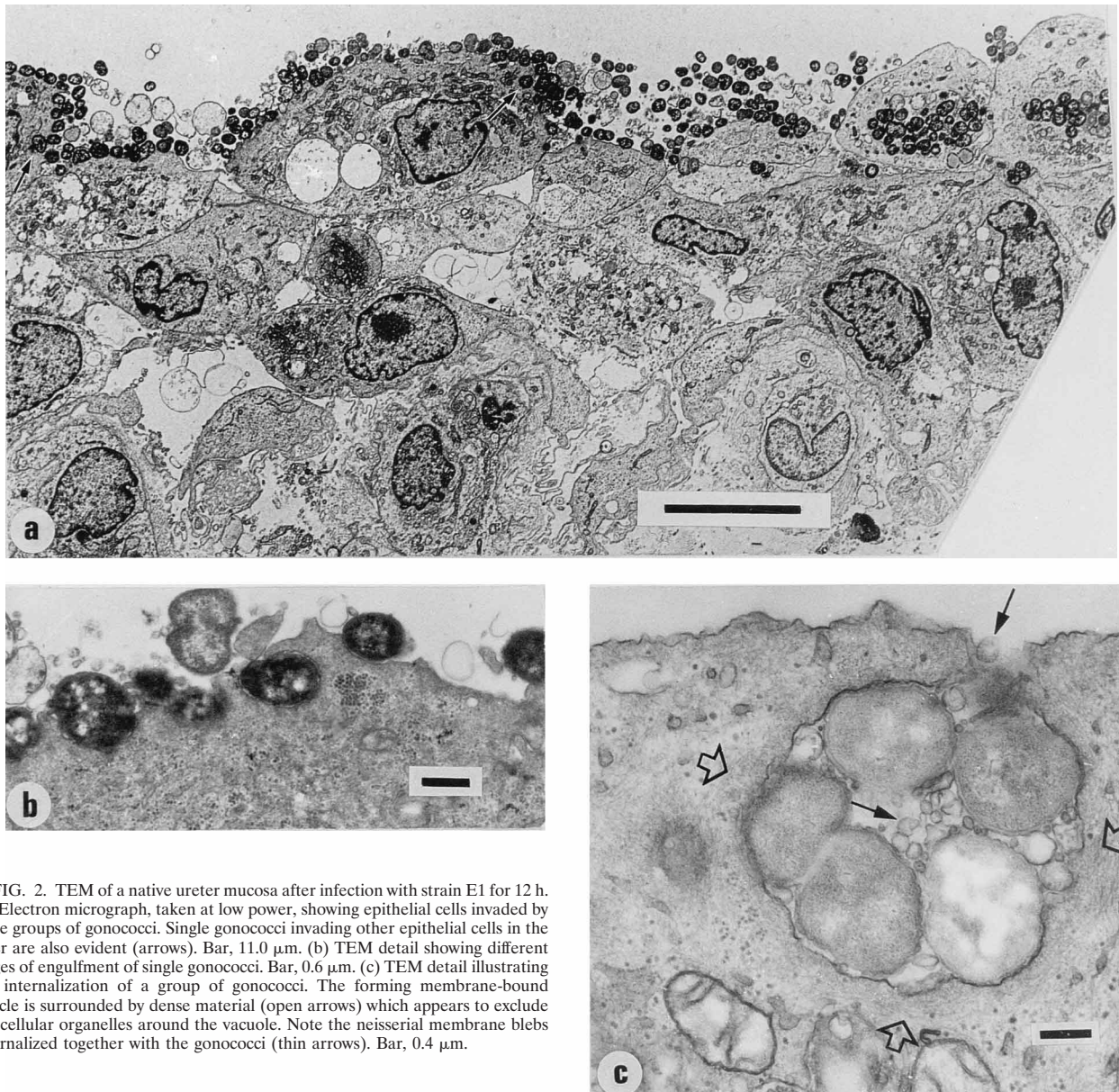


FIG. 2. TEM of a native ureter mucosa after infection with strain E1 for 12 h. (a) Electron micrograph, taken at low power, showing epithelial cells invaded by large groups of gonococci. Single gonococci invading other epithelial cells in the layer are also evident (arrows). Bar, 11.0  $\mu\text{m}$ . (b) TEM detail showing different stages of engulfment of single gonococci. Bar, 0.6  $\mu\text{m}$ . (c) TEM detail illustrating the internalization of a group of gonococci. The forming membrane-bound vesicle is surrounded by dense material (open arrows) which appears to exclude the cellular organelles around the vacuole. Note the neisserial membrane blebs internalized together with the gonococci (thin arrows). Bar, 0.4  $\mu\text{m}$ .

four individuals (for example, see Fig. 1b). By 4 h, a lesser degree of adhesion was observed in three of four specimens taken from different kidney donors (data not shown). In the remaining specimen, only a very few gonococci were evident. However, when other pieces taken from the same individual were incubated for 9 or 12 h, the same degree of adhesion as in Fig. 1b was seen. In contrast, when pieces of ureter were infected with the nonpiliated strain expressing Opa30, no adherent gonococci were observed (similar to the findings depicted in Fig. 1a), even after longer periods of incubation (9 and 12 h).

The fingerprintlike impressions left by gonococci broken off and formed during SEM preparation (Fig. 1c) show that most of the adherent gonococci were partially embedded in a cushionlike structure. Engulfment of adherent gonococci is also indicated by the membrane of the host cell being pushed up around the diplococcus which resided there (Fig. 1d).

**Gonococcal invasion and release.** TEM analysis confirmed the internalization of the piliated strain (E1) into the ureteral

mucosal tissue. In two ureters infected for 4 and 6 h, gonococci were first seen after 6 h of infection. Gonococcal invasion increased with time over the period of infection. At 12 h, large numbers of intracellular gonococci were observed in sections cut from different parts of the ureteral pieces taken from the four individuals (for example, see Fig. 2a). At the investigated areas, the degree of invasion differed from one sample to another and from an area to another in the same ureter. Likewise, some intracellular events, such as release of gonococci from invaded cells, were seen in one or two samples and not in the others. Therefore, the results presented here are collective data for samples from the four individuals. In areas with no or with a small number of invaded cells, large numbers of adherent gonococci were seen either embedded in or sitting on the plasma membrane. Internalization of both single and groups of gonococci was observed (Fig. 2b and c). The forming vacuole may be mobilized by the dense material which appears, excluding the cellular organelles around the vacuole (Fig. 2c). Morphologically, this material can be identified as cytoskeletal

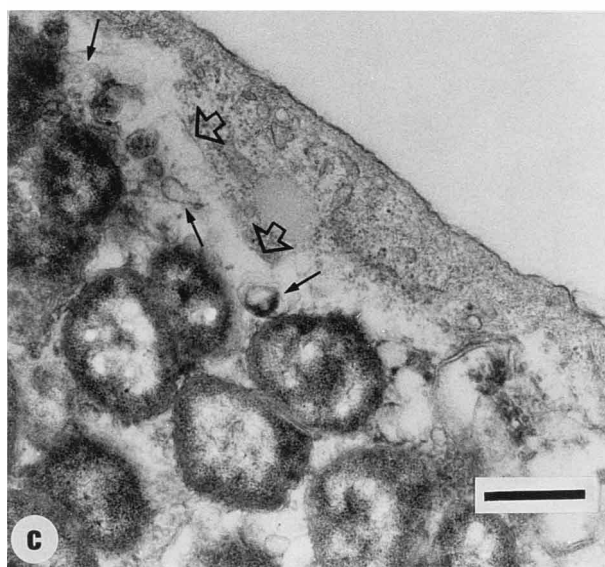
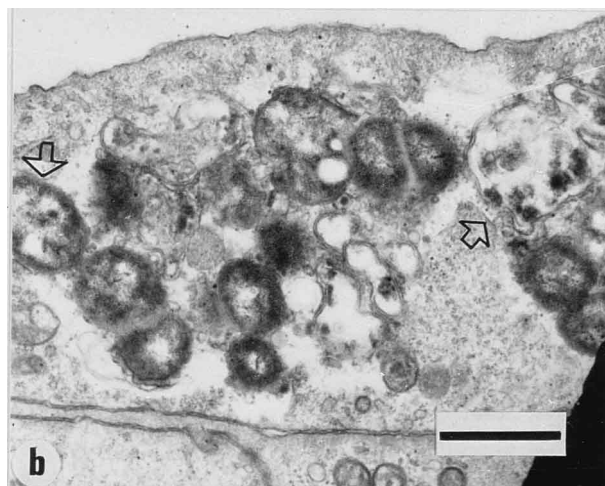
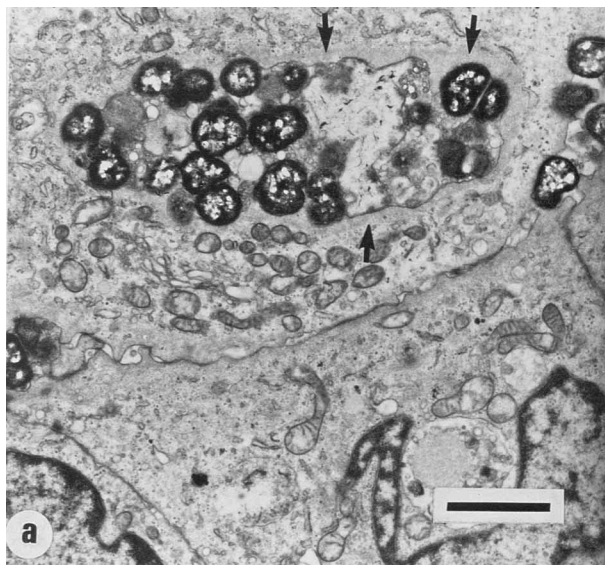


FIG. 3. TEM micrographs at 12 h after infection with strain E1 illustrating intracellular gonococci located in an intact membrane-bound vacuole (a) and another cluster of gonococci within an epithelial cell lacking the vacuolar membrane (b). (c) Higher magnification illustrating a cluster of intracellular gonococci lacking the vacuolar membrane. Remnants of the fragmented vacuolar membrane are still evident in panels b and c (open arrows), and the vesicular structures indicated by the thin arrows in panel c may be formed after lysis of the membrane. Note the dense material surrounding the intact vacuole in panel a (arrows). Bars, 2.2 (a), 1.2 (b), and 0.8 (c)  $\mu\text{m}$ .

elements. Gonococcal blebs are also incorporated together with the bacterium (Fig. 2c). After internalization, gonococci were mostly seen as clusters in large, distinct, and intact membrane-bound vacuoles (Fig. 3a). In some sections, however, a vacuolar membrane around the bacteria was not distinguishable (data not shown). On rare occasions, intracellular gonococci were seen not surrounded by a membrane and remnants of a ruptured vacuolar membrane were seen around the clusters of gonococci (Fig. 3b and c). Ongoing disruption of the surrounding membrane was not observed. At locations deep in the cytoplasm, the vacuoles were often surrounded with dense material (Fig. 3a).

By a process similar to exocytosis, intracellular gonococci were released from the cells having intact vacuole. Fusion of the vacuolar membrane with the basolateral plasma membrane of the infected cell is illustrated in Fig. 4a and b. Exocytosis also seemed to be promoted by the dense material around the vacuoles (Fig. 4b). The fate of the cells after delivery of their bacterial contents could not be determined. In Fig. 4a, as seen on the right side, the cell maintained the intercellular junction with the adjacent cell during exocytosis. Bacteria in cells with ruptured vacuoles, on the other hand, were apparently liberated by lysis of these cells, which was often seen in TEM sections (Fig. 4c). The released bacteria soon afterwards had the opportunity to interact with adjacent cells (Fig. 4). Gonococci were seen to invade cells belonging to deeper epithelial layers, as shown in Fig. 5a. Although these cells seem to have been invaded by an intercellular route, another cycle of invasion of new cells by released bacteria can also occur. Figure 4 shows released gonococci interacting with adjacent cells belonging to the same layer of the epithelium (Fig. 4a) and with cells belonging to deeper layers (Fig. 4b and c).

**Epithelial tissue integrity.** At many areas along the epithelial surface, piliated gonococci migrated between epithelial cells and traveled in a lateral direction, disrupting the integrity

of the tissue (Fig. 5a). Other areas along the pieces of ureters were also occupied by adherent gonococci but appeared to be still integrated. In the diseased areas, TEM revealed that infected cells which lost their intercellular connections tended to exfoliate. Exfoliation was not necessarily associated with gonococcal invasion of these cells, since cells with adherent gonococci tended also to exfoliate from the epithelial layer (data not shown). Together, cell lysis and exfoliation could explain the thinning of the ureteral epithelium observed in Fig. 5b, making it possible for the gonococci to interact with the basal mucosal cells. In contrast to the ureteral epithelia infected with the piliated strain E1, tissues challenged with B2.1 appeared to be intact during the course of infection (data not shown).

## DISCUSSION

In this study, human ureters taken from healthy adult kidney donors were used as a model to study the interaction of gonococci with native mucosal tissue. Ureters from patients suffering from renal cell carcinoma have previously been used by Fujita and coworkers (9–11) to study the adhesion of *Escherichia coli* and *Staphylococcus* species to epithelial cells. Here, we demonstrate the adherence to and invasion of the native

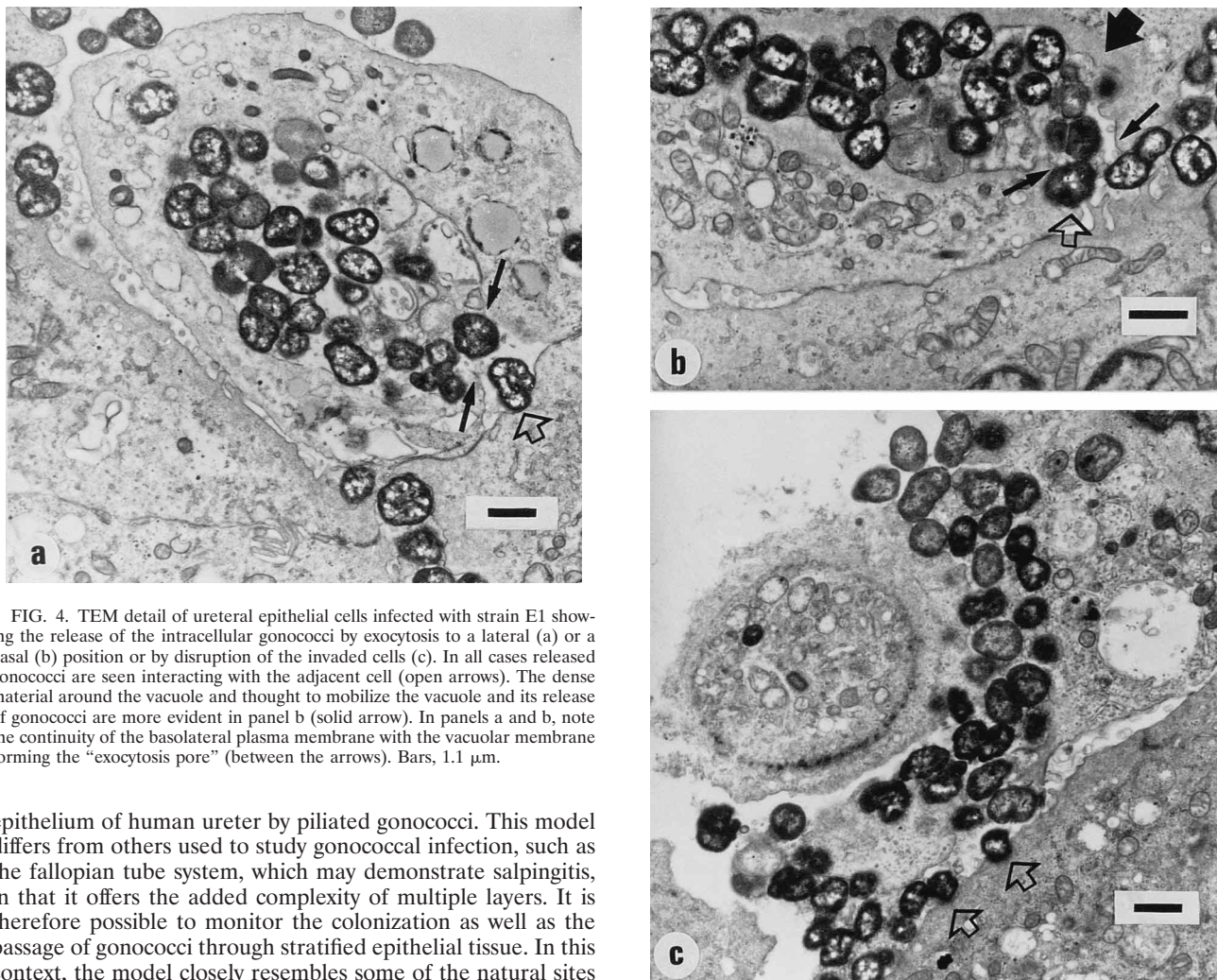


FIG. 4. TEM detail of ureteral epithelial cells infected with strain E1 showing the release of the intracellular gonococci by exocytosis to a lateral (a) or a basal (b) position or by disruption of the invaded cells (c). In all cases released gonococci are seen interacting with the adjacent cell (open arrows). The dense material around the vacuole and thought to mobilize the vacuole and its release of gonococci are more evident in panel b (solid arrow). In panels a and b, note the continuity of the basolateral plasma membrane with the vacuolar membrane forming the "exocytosis pore" (between the arrows). Bars, 1.1  $\mu\text{m}$ .

epithelium of human ureter by piliated gonococci. This model differs from others used to study gonococcal infection, such as the fallopian tube system, which may demonstrate salpingitis, in that it offers the added complexity of multiple layers. It is therefore possible to monitor the colonization as well as the passage of gonococci through stratified epithelial tissue. In this context, the model closely resembles some of the natural sites of gonococcal infection, including urethral, cervical, and conjunctival epithelial tissues. Although the ureter is an extremely rare natural site of gonococcal infection (17), probably due to the anatomic barrier provided by the urinary bladder, it may be an appropriate model to delineate the mechanisms of urethral infection. The two organs are developmentally and, in part, also functionally related. Morphological differences between these tissues do exist, since ureteral-lining cells are transitional epithelial cells while urethral cells are either stratified squamous or pseudostratified columnar cells. The ureter model does, however, compensate for the difficulty in obtaining representative clinical samples from infected tissues, since it is available and easy to prepare. A model which offers the same stratification is the corneal organ culture (39). Unlike human ureters, however, corneas are not generally available and are difficult to cultivate.

SEM and TEM studies showed that pili are absolutely essential to mediate attachment of strain MS11 to the ureteral mucosa and that this attachment is the precursor of invasion. This is consistent with natural neisserial infection, in which pili were found to be an absolute requirement for the initiation of an infection (19). Studies involving organ culture models also indicate that pili are required for attachment (25, 36, 39). In the fallopian tube model, however, nonpiliated gonococci did adhere, although to a lesser extent than those expressing pili (22, 25). Opa proteins are believed to be the surface proteins which mediate this adhesion. In contrast, in the present study,

Opa30, the protein which confers tropism of *N. gonorrhoeae* MS11 for epithelial cells (21), facilitated neither adhesion to nor invasion of the ureteral mucosa, even after longer periods of incubation of the nonpiliated variant B2.1 with the ureteral tissue. It remains, however, to be determined whether distinct types of Opa protein expressed by strains other than MS11 can mediate adhesion to ureteral mucosae. Opa proteins A to E, expressed by strain FA1090 and shown to initiate attachment and damage to fallopian tube mucosae and HEC-1B cells (22), are candidates for further investigations.

TEM investigations confirmed the internalization of the piliated gonococci observed by SEM. Single as well as groups of gonococci were found in intracellular locations, which confirms their ability to invade the ureteral mucosa. The presence of large numbers of intracellular gonococci is a common phenomenon of gonococcal pathogenesis in natural urethral infection. Apicella et al. (1) reported epithelial cells with large numbers of bacteria in each of the patients with gonococcal urethritis tested in their study. Incorporation of membrane blebs may be an important event for the benefit of gonococci. The significance of this phenomenon is not clear, but a possible eventual fusion of these bacterial structures with the vacuolar membrane may modify the intracellular processing of the vacuole containing the gonococci. Entry into mucosal cells has been

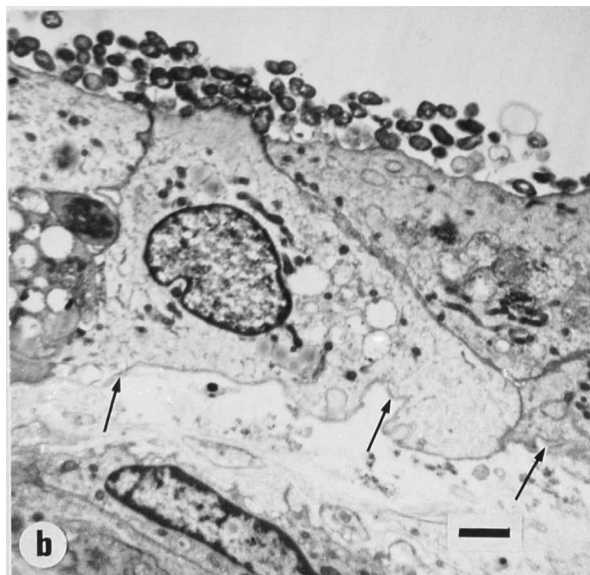
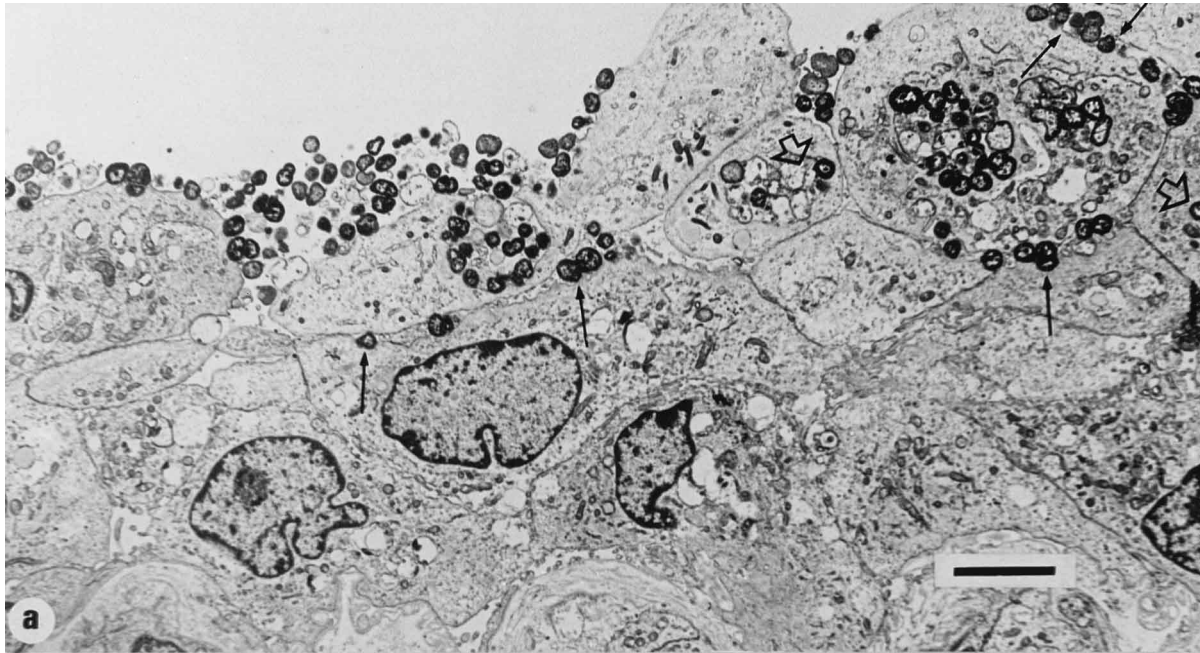


FIG. 5. TEM of a native ureter mucosa infected with strain E1 for 12 h. (a) Electron micrograph, taken at low power, showing the gonococci migrating between the epithelial cells (thin arrows) after disrupting the intercellular integrity. Note the gonococci within cells belonging to the second layer of the epithelium (open arrows). Bar, 7.0  $\mu\text{m}$ . (b) Electron micrograph, taken at low power, showing gonococci adherent to the basal epithelial cells of the ureter mucosa. Note the basal lamina, indicated by arrows. Bar, 1.7  $\mu\text{m}$ .

suggested as a way for gonococci to ultimately invade subepithelial tissues (26, 46). Interestingly, bacterial internalization in ureteral cells was not the dead end. Gonococci were found to be released by exocytosis from cells loaded with groups of bacteria into an intercellular position, apparently to further infect neighboring and deeper cells. The release takes place after fusion of the vacuolar membrane with the basal or the lateral plasma membrane. Gonococci are also released after rupturing the infected cells; the latter event may follow the vacuolar membrane destruction observed in cells with intracellular gonococci not surrounded with a membrane. Either manner of release indicates that gonococci survive the intracellular processing of ureteral epithelial cells and proceed to further invasions. Intracellular gonococci free of a surrounding membrane have been very recently demonstrated in a natural infection (1) where intracytoplasmic gonococci were present in 3 of 12 exudates from men with gonococcal urethritis. Gonococci

free in the cytoplasm have been also demonstrated in HEC-1B cells (34). How gonococci might lyse their vacuoles is currently not known. Candidate lysins include the neisserial porin which is believed to translocate into the vacuolar membrane (3, 47, 48). An accessory factor for the lysis of phagosomes may be the neisserial immunoglobulin A protease, which was observed to cleave the luminal domain of h-lamp-1 (18). This lysosome-associated membrane protein is thought to protect and stabilize membranes of the endocytic pathway by forming a carbohydrate coat on the inner surface of the vacuolar membrane (12), and its disruption may therefore make these membranes more susceptible to other bacterial and/or endocytic components.

TEM investigations demonstrated the presence of dense material around the vacuoles containing gonococci during formation, intracellular transport, and exocytosis of the intravacuolar gonococci. We believe that this material is cytoskeletal in nature and functions in phagosomal mobilization. Experiments with epithelial cell lines suggest that both actin microfilaments (2, 23, 34) and microtubules (30) are required for gonococcal entry. Grassmé et al. (13), who used Chang cells to investigate Opa protein-dependent uptake, have shown that gonococci are internalized via microfilament-dependent phagocytosis.

Unlike the nonpiliated variant B2.1, the piliated strain E1 caused a marked disintegrity of the ureteral mucosa, thus suggesting that attachment is related to tissue damage. The inability of the nonpiliated variant B2.1 to cause damage to the ureteral mucosa seems to correlate directly with its failure to adhere to these cells. A similar finding was obtained with the cornea organ culture model, where nonpiliated gonococci that do not adhere to corneal epithelium caused no harm (39). With Chang epithelial cells, Virji and Everson (41) found that variants of gonococci expressing either pili or Opa protein

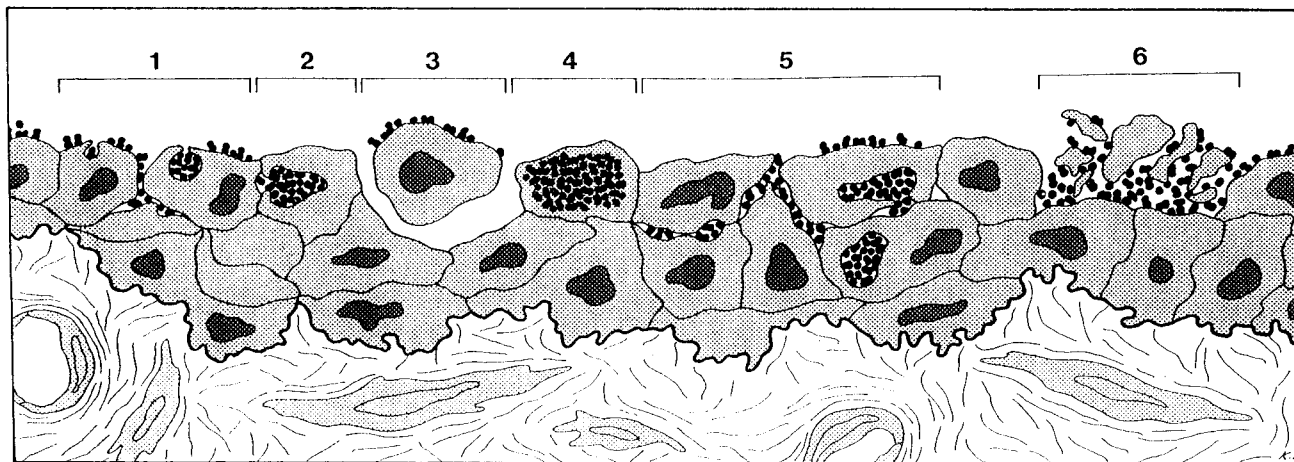


FIG. 6. Diagram summarizing the mechanisms employed by *N. gonorrhoeae* to interact and pass through a complex epithelium: adhesion to surface cells and invasion of these cells by single and groups of gonococci (area 1), exocytosis of gonococci residing in membrane-bound vacuoles to lateral (area 2) and basal (area 5) locations, intercellular penetration (areas 1 and 5), destruction of the vacuolar membrane (area 4), initiation of cellular destruction (area 6) and exfoliation (area 3) resulting in a thinning of the complex epithelium, interaction with deeper cells through intracellular (area 5) and intercellular (areas 1 and 5) routes and after cellular destruction (area 6), and invasion of deeper cells (area 5).

were cytotoxic, whereas gonococci that lack both adhesins were not. Pili and Opa protein are known to mediate attachment to this cell line. Infection with pathogenic *Neisseria* was reported to produce cytotoxic effects after infection (35, 41), and such cytopathic effects were also observed with endothelial cells (42). In the fallopian tube model, the nonpiliated gonococci that adhere to the fallopian mucosa caused a marked toxic effect to the epithelium, possibly through the release of lipopolysaccharide and cell wall structures (15, 16, 24, 27). Our results and the above-mentioned studies suggest that an intimate contact between the gonococci and the epithelial cells, achieved by an adhesin such as pili or Opa protein, may be a prerequisite for the initiation of a potent toxic effect. It is not clear whether an increased delivery of gonococcal toxins to the host cells after intimate contact or whether the interaction of adhesins themselves with the plasma membrane causes this damage. Contact between the gonococci and the cell was, however, not a prerequisite for damaging the ciliary activity of the fallopian tube and the nasopharynx (37).

Invasion of epithelial cells belonging to deeper layers of the ureteral mucosa demonstrates the susceptibility of less mature cells to neisserial infection. This invasion, which may be the result of gonococci penetrating by an intercellular route and/or of released intracellular bacteria, is a further important step on the way for the gonococcus to complete a successful passage through a complex mucosal lining towards the deeper tissues. The localization of adherent gonococci on the apical sides of basal epithelial cells indicates their ability to colonize and penetrate a complex epithelial tissue. To achieve this, gonococci employ different mechanisms. These include, in summary and as illustrated in Fig. 6, adherence to surface cells, invasion of and eventual release from these cells, disintegration of intercellular connections followed by paracellular tissue invasion, invasion of deeper cells, and initiation of cellular destruction and exfoliation resulting in a thinning of the mucosa. Very recently, Apicella et al. (1) found infected and noninfected epithelial cells in exudate samples from patients suffering from gonococcal urethritis, which may indicate that a thinning of the epithelium covering the urethra is an essential event in the pathogenesis of gonococci. Gonococcal infection of human cornea organ culture caused also thinning of the epithelium

covering the explants (39). It is worth mentioning here that many of the events observed during the course of ureteral infection are similar to the histopathology of the urethral gonococcal infection described by Harkness (17).

#### ACKNOWLEDGMENTS

We thank S. Gray-Owen for critical comments on the manuscript and H. Schwarz for helpful suggestions. We also thank K. Lamberty (artwork), Carmen Müller (photography), Jürgen Berger (SEM), and Donna Devine (manuscript preparation) for their skillful assistance.

I.M. is a recipient of a Ph.D. fellowship from the Deutscher Akademischer Austauschdienst (DAAD). This work was supported by the Fonds der Chemischen Industrie (to T.F.M.).

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