

STUDIES ON GONOCOCCUS INFECTION

IV. PILI: THEIR ROLE IN ATTACHMENT OF GONOCOCCI TO TISSUE CULTURE CELLS*

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Recent studies on *Neisseria gonorrhoeae* have yielded suggestions that the surfaces of these organisms play vital roles as pathogenetic determiners in gonococcal infections. Virulence has been correlated with specific gonococcal colony forms by Kellogg et al. (1, 2). This finding was established through inoculation of human volunteers with organisms derived from the various colony types that could be morphologically differentiated. Colony types 1 and 2 produced typical acute venereal infection, whereas colony types 3 and 4 produced only mild, transient symptoms without establishment of infection in the genital tract. This colony type-virulence correlation was extended by the studies of Swanson et al. (3) and of Jephcott et al. (4) who demonstrated pili on the gonococci of types 1 and 2 and the absence of pili or organisms from types 3 and 4 colonies. These studies suggest a correlation between virulence and the presence of pili on gonococci.

In the study correlating presence of pili with colony morphology of gonococci it was suggested that these cell wall appendages might play a role in virulence by promoting attachment of the organisms to cells of the potentially infected host (3). This hypothesis is central to the presently described studies in which the presence of pili is found to be associated with enhanced attachment of gonococci to human amnion cells in vitro. Light and electron microscope observations support this finding and the latter provide partial visualization of the attachment of gonococci mediated by their pili.

Materials and Methods

Gonococci.—The strains used were freshly isolated from patient material at the Mount Sinai Hospital, New York. Isolation, identification, and propagation have been described previously in detail (3). Gonococci used in experiments were grown for 18 h on GC agar base supplemented with IsoVitalX (Baltimore Biological Laboratories, Baltimore, Md.) and were suspended in Earle's basic salt solution (Grand Island Biological Co., Grand Island, N. Y.) for determination of optical opacity and suitable dilution before their addition to tissue

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culture cells. Dilution and plating of organisms for enumeration of colony-forming units were carried out in Medium 199 (Grand Island Biological Co.) and on Mueller-Hinton agar (Difco Laboratories, Inc., Detroit, Mich.) plates, respectively. The inoculated plates were incubated overnight in either candle extinction jars or in an incubator with 5% CO₂ atmosphere.

Tissue Culture Cells.—Human amnion cells were obtained from Dr. Joseph Sonnabend (Mount Sinai School of Medicine) and were maintained in either minimal essential medium or Medium 199 (M-199) (Grand Island Biological Co.) with Earle's salts, L-glutamine, and 10% heat-inactivated (at 56°C for 30 min) fetal calf serum (Grand Island Biological Co.). Penicillin and streptomycin were included in the medium for propagation of the tissue cultures but were removed by repeated washing of the monolayers with antibiotic-free medium before use of the cells for attachment experiments.

Incubation of Gonococci with Amnion Cells.—

With monolayers: Monolayers of human amnion cells, propagated as described above, were utilized when they were about 75–80% confluent on 9-cm diameter tissue culture dishes. The monolayers were repeatedly washed with antibiotic-free medium to remove penicillin and streptomycin and then were overlaid with 2 ml of a suspension of gonococci (approx. 1×10^8 colony-forming units/ml) in M-199 containing 2% bovine serum albumin (BSA)¹ (Schwarz-Mann, Orangeburg, N. Y.). Incubation in a 5% CO₂ atmosphere at 37°C proceeded for 1–2 h after which the monolayers were vigorously washed with fresh M-199, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate, and stained with 1% Giemsa stain for 30 min. These incubations were carried out with both colony types 2 and 4 (T-2 and T-4) in parallel studies. Evaluation of comparative results on attachment of gonococci to cells of the monolayers was carried out with a Zeiss photomicroscope (Carl Zeiss, Inc., New York).

With cells in suspension: Amnion cells that had been removed from monolayer cultures with 0.004% ethylenediaminetetraacetic acid disodium and 0.1% trypsin were centrifuged and washed with M-199. After enumeration the cells were diluted in M-199 with 2% BSA to a final concentration of $4-6 \times 10^6$ /ml. 2 ml of this amnion cell suspension was mixed with an equal volume of the gonococcal suspension containing approx. $1 \times 10^4-1 \times 10^5$ colony-forming units/ml in M-199 with 2% BSA. The total volume of the incubation mixture was 4 ml and this was rotated at 30 rpm in a polypropylene tube (12 x 75 mm) at 37°C. 1-ml aliquots were removed immediately after mixing bacteria and amnion cells and after 50- and 100-min rotation-incubations. These were centrifuged (500 rpm for 5 min) and the supernatant was removed for dilution and enumeration of colony-forming units present. The number of organisms enumerated by dilution and plating of this supernatant represents the organisms not associated with amnion cells. The sediment containing amnion cells and cell-associated gonococci was washed with 1 ml of M-199, resedimented (500 rpm for 5 min), diluted in 1 ml of fresh M-199, placed in a sonicating bath for 5 min, diluted, and plated for number of gonococci present. Similar conditions were used for suspensions of gonococci that were rotated-incubated in the absence of amnion cells to determine the organisms that appear in each fraction in the absence of cells.

Critical Point Drying.—Amnion cells were propagated on glass microscope slides cut to fit the critical point drying (CPD) apparatus specimen chamber, cleaned, autoclaved, and placed in a 9 cm Petri dish. After washing to remove antibiotics the tissue culture cells were exposed to a suspension of T-2 gonococci and were incubated for 1 h at 37°C in a CO₂ atmosphere. The slides were then thoroughly washed to remove bacteria not attached to amnion cells, immersed in 2% glutaraldehyde in 0.1 M sodium cacodylate for 5 min, washed in 0.1 M cacodylate, and immersed for 5 min in 1% osmium tetroxide in 0.9% sodium chloride. The specimen slide was passed through graded ethanol and amyl acetate solutions and subjected to critical point drying with CO₂ in a CPD-1 apparatus (Denton Vacuum, Inc., Cherry Hill,

¹ Abbreviations used in this paper: BSA, bovine serum albumin; CPD, critical point drying.

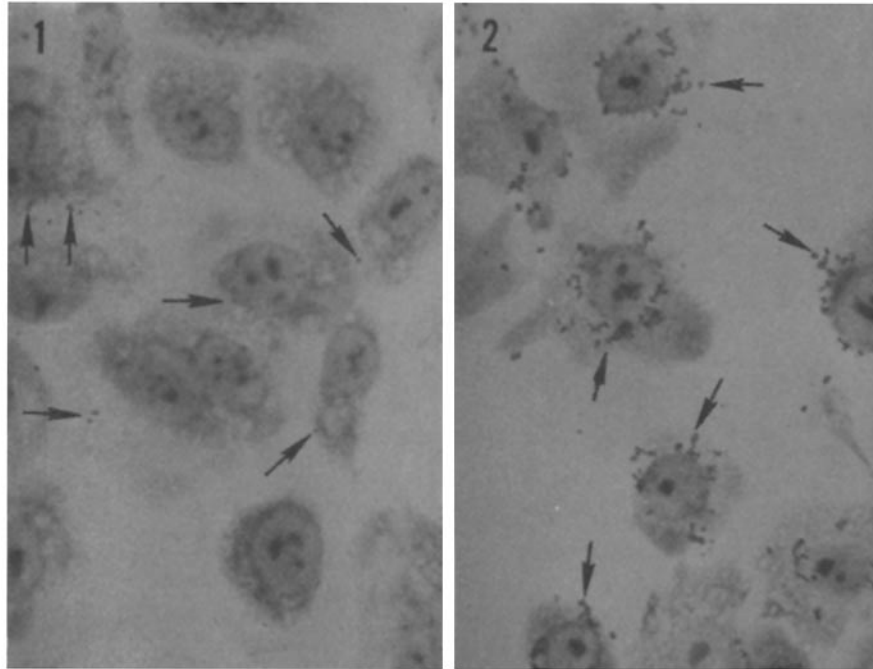
N. J.) as described in the operating brochure supplied with the equipment (5). The only modification utilized was that final venting time was 2 h after which specimens were removed and placed in a Balzers 360 M apparatus (Balzers High Vacuum Corp., Santa Ana, Calif.) for platinum-carbon evaporation. The platinum-shadowed carbon-stabilized specimen was dipped in 0.2% Formvar in ethylene dichloride, briefly dried, and immersed in Clorox. Fragments of the replica released from the slide after 12-36 h immersion in Clorox were washed several hours in distilled water and were placed on naked copper grids for electron microscope examination. All photographs were printed without reversal.

Electron Microscopy.—Amnion cells for thin sectioning were either fixed *in situ* as monolayers overlaid with gonococci or were sedimented from rotation-incubation with gonococci and fixed as a suspension of cells. Fixation with 2% glutaraldehyde in 0.1 M sodium cacodylate pH 7.0 was followed by osmication, *en bloc* staining with 1% uranyl acetate, dehydration, and embedding as previously described (3). Electron micrographs were taken either with an AEI EM801 microscope (AEI Scientific Apparatus, Inc., Elmsford, N. Y.) or with a Siemens Elmiskop IA (Siemens Corp., Iselin, N. J.).

RESULTS

Exposure of Amnion Cell Monolayers to Gonococci (Light Microscopy).—Incubation of T-4 gonococci with amnion cell monolayers results in little association between bacteria and amnion cells (Fig. 1). Occasional gonococci appear to adhere to the plastic substrate of the tissue culture dish between cells of the monolayer, but few gonococci are actually in contact with the tissue culture cells. This is in marked contrast to the picture obtainable after incubation of amnion cell monolayers with T-2 gonococci equivalent in number to those used in the T-4 experiments. T-2 gonococci exhibit marked adherence to the amnion cells (Fig. 2). Not only are the organisms clumped in their association with the eukaryotic cells, but also the numbers of clumps are abundant. Numerous gonococci appear to adhere in the perinuclear region of the amnion cells. This may be related to the relatively flat contour of the peripheral cytoplasm, the raised convex contour of the nucleus, and the concave profile of the perinuclear zone. This variable thickness of different portions of the tissue culture cell makes differentiation of extracellular from the intracellular gonococci impossible because gonococci may be seen at a level, through fine focusing of the light microscope, below that at which the top of the nucleus is in focus.

Rotation-Incubation of Amnion Cells with Gonococci.—To determine whether differences in attachment of gonococci to amnion cells could be correlated with colony type, studies were carried out utilizing T-2 and T-4 gonococci exposed to suspensions of amnion cells in parallel experiments. Association with or attachment to amnion cells is defined as appearance of gonococci in the sediment as opposed to the supernatant, which contained gonococci that did not centrifugally separate with amnion cells and, therefore, are not cell associated. Controls that did not contain amnion cells were included to determine the number of gonococci that appeared in the sediment but did not represent cell-associated organisms. The data are tabulated either as number of colony-forming units (Table I) or as the percentage of gonococci recoverable at a given time point in



FIGS. 1 and 2. Amnion cells in monolayer culture have been exposed to gonococci, washed, fixed, and stained for light microscopy with Giemsa stain. In Fig. 1 only a few nonpilated, T-4 gonococci (arrows) are found in association with the amnion cells. This is in contrast to amnion cells that have been exposed to pilated, T-2 gonococci (Fig. 2) and that have numerous, clumped organisms on their surfaces (arrows), particularly in the perinuclear regions. $\times 1,400$.

TABLE I
Distribution of Gonococci after Rotation-Incubation with Amnion Cells

| Cells added | Rotation-incubation | | | | |
|-----------------------|---------------------|-------------------|-------------------|-------------------|-------------------|
| | 0 time (total) | 50 min | | 100 min | |
| | | Supernate | Sediment | Supernate | Sediment |
| T-2 alone | 2×10^6 | 1.3×10^6 | 7.5×10^5 | 1×10^6 | 3×10^5 |
| T-2 + amnion cells | 2×10^6 | 5.3×10^5 | 2.8×10^6 | 7.7×10^4 | 1.8×10^5 |
| T-4 alone | 3×10^6 | 9×10^5 | 2.3×10^5 | 4.5×10^5 | 2.3×10^4 |
| T-4 + amnion cells | 3×10^6 | 1.5×10^6 | 5.6×10^5 | 1.4×10^5 | 2.8×10^4 |

T-2 and T-4 gonococci were incubated either alone or with amnion cells as described in Materials and Methods. The number of gonococci noted (0 time [total]) were rotated with 6×10^6 amnion cells where designated. After variable times of incubation at 37°C aliquots were removed, centrifuged, and the number of gonococcal colony-forming units determined in the supernate and sediment fractions. T-2 alone and T-4 alone are specimens not containing amnion cells that were included to determine the distribution of gonococci that is independent of the presence of amnion cells.

the sediment fraction as compared with the total number of gonococci recovered in the specimen (sediment plus supernate) (Fig. 4).

Little difference is found in the total recovery of T-2 and T-4 gonococci from incubation with amnion cells. Both colony types exhibit a decrease in the total colony-forming units recovered after 50- or 90-min incubations with amnion cells (Fig. 3). No change in colonial morphology was found by compar-

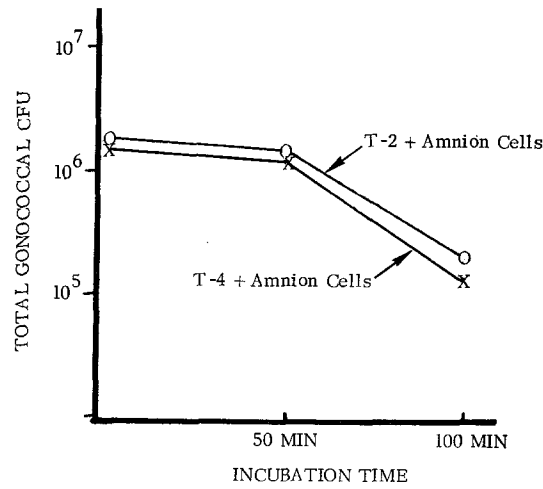


FIG. 3. The total recovery of gonococci from mixtures of T-2 or T-4 organisms with amnion cells appears to be independent of colony type. Amnion cells and gonococci were mixed as described in Materials and Methods and the total number of colony-forming units (CFU) recovered in both the sediment and supernate fractions determined by dilution and plating of aliquots after 50- and 100-min incubations.

ing input organisms with those recovered after exposure to amnion cells, i.e., there did not seem to be a selective process for one or another colony type during the short incubations of these experiments.

Segregation of gonococci into cell-associated (sediment) and non-cell-associated (supernate) fractions is strikingly dependent on the colony type of gonococci used. T-2 gonococci consistently distribute predominantly in the cell-associated fraction, whereas T-4 gonococci show preferential distribution in the non-cell-associated or supernate fraction. A typical experiment demonstrating this difference is shown in Table I in which the number of colony-forming units recovered is given from each fraction. In the amnion cell-free controls (T-2 alone and T-4 alone, Table I) as well as in the mixture of T-4 gonococci and amnion cells (T-4 + amnion cells), the supernate contains two to fourfold more colony-forming units than are found in the sediment. By contrast, after either 50- or 100-min incubations with amnion cells (T-2 + amnion cells), the number of T-2 gonococci in the cell-associated fraction is at

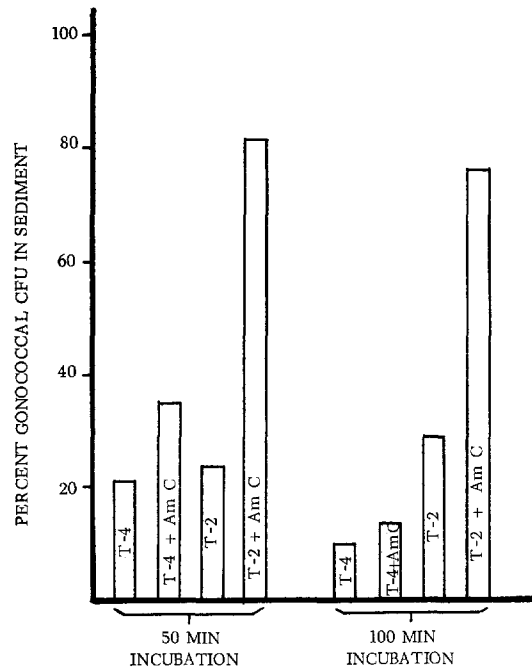


FIG. 4. The distribution of gonococci in the sediment, cell-associated fraction is quite different depending on whether nonpilated T-4 or pilated T-2 gonococci are incubated with amnion cells. Controls which do not contain amnion cells (T-4 and T-2) were utilized to determine the number of gonococci that appear in the sediment fraction but which are not associated with amnion cells. These probably represent organisms that are clumped in suspension to the extent that they sediment under the centrifugation conditions noted in Materials and Methods. In these experiments the number of colony-forming units (CFU) present in the sediment fraction was compared with the total CFU after 50- and 100-min incubations alone (T-4 and T-2) and with amnion cells (T-4 + Am C and T-2 + Am C).

least twice that found in the supernate. This preferential distribution is seen perhaps more clearly in the composite data derived from four experiments carried out on different days (Fig. 4). In this figure the amnion cell-free control values (T-2 alone and T-4 alone) are similar. The percentage of colony-forming units found in the cell-associated fraction (sediment) is markedly increased with T-2 gonococci as compared with T-4 organisms (T-2 + amnion cells vs. T-4 + amnion cells).

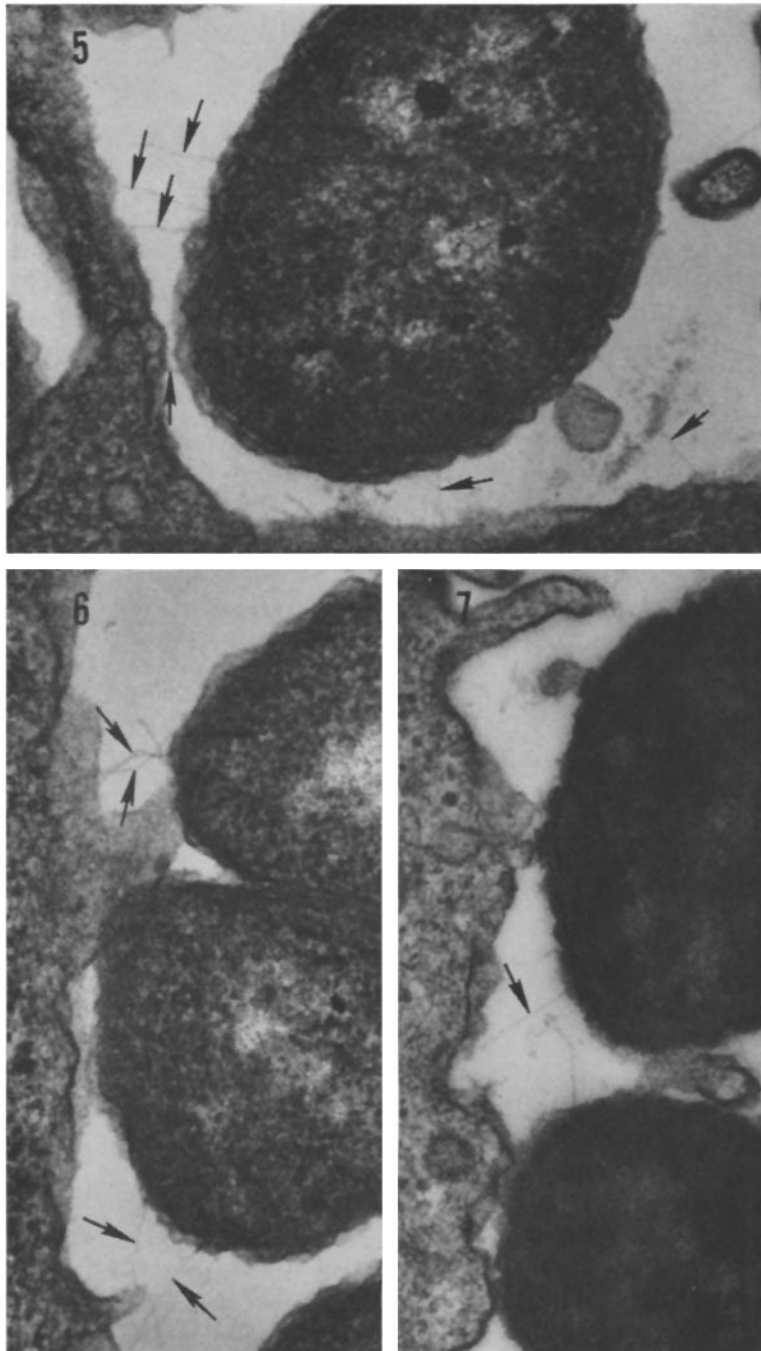
Electron Microscopy of Gonococci-Amnion Cell Mixtures.—Adequate evaluation of the association between gonococci and amnion cells requires electron microscope examination of both thick and ultrathin “thin” sections as well as critical point dried specimens. The mere difference in section thickness is instrumental in visualization of two apparently distinct morphological types of association between the prokaryotic and the eukaryotic cell in this system. In

thicker sections (dark-to-medium gold interference colors) pili are visualized for considerable distances along their lengths even if they do not course precisely parallel to the plane of section. In ultrathin sections (silver to gray interference colors) only short segments of the pili usually are visible due to the improbability of extensive portions of pili lying in a plane parallel to the very thin section. Thus, pili are seen in the thick sections as electron-opaque 80-Å thick fibrils extending radially from the gonococcus' surface (Figs. 5-7). In numerous instances multiple pili from a single bacterium appear to contact different points of an amnion cell (Figs. 5 and 6). The actual point of contact for each pilus with the plasma membrane is not visible; therefore, one can not determine whether the tip or a lateral surface of the pilus contacts the amnion cell's membrane. Pili are not seen in similar thick sections of T-4 gonococci and amnion cells as expected from the absence of pili on these organisms demonstrated by negative staining (3) and freeze-etching (6).

Participation of pili radiating from the gonococcal surface in attachment of organisms to amnion cells can be assessed also through critical point drying. This relatively little-used technique (7, 8), although somewhat limiting resolution, has a distinct advantage over other methods of specimen preparation for observing this type of association. Negative staining delineates structure of radiating pili but does not allow adequate visualization of bacterial or amnion cell surface topology. Freeze-fracture, freeze-etching adequately exhibits either amnion cell or gonococcal surface structure, but definition of the former necessitates use of glycerol, which precludes visualization of the freeze-etch exposed exterior surfaces of bacteria. Further, pili radiating from gonococci are poorly seen by freeze-fracture, freeze-etch techniques. Thin sections offer little appreciation of the third dimension in spatial relationships between gonococci and amnion cells. Simple air drying and subsequent heavy metal shadowing yield serious artifacts of shrinkage with poor visualization of cell surface topology. Critical point drying (CPD) has the advantage that volume and configuration artifacts are minimized such that relationships between gonococcal and amnion cell surfaces can be seen, as in Figs. 8-12.

Gonococci prepared by CPD have the appearance shown in Figs. 8-12 and seen best in Fig. 8. The convoluted gonococcal exterior has adherent pili (analogous to the freeze-fracture, freeze-etch appearance) as well as pili radiating from its exterior. Radiating pili lying on the substrate have diameters similar to those obtained by other methods (3, 6), but if pili are elevated above the substrate level, their diameters are increased due to the accumulated thicknesses of carbon, platinum, and Formvar of the replica. This enhanced thickness, as well as the position of the shadow derived from each pilus, is helpful in identifying those surface appendages oriented in space above the plane of the substrate on which the cells are supported.

After CPD preparation, amnion cells of monolayers have gonococci attached via their pili as shown in Figs. 9-12. The majority of gonococcal pilus-amnion



FIGS. 5-7. Pilated T-2 gonococci are numerous in association with amnion cells both after exposure to the cells in suspensions (Fig. 5) or as monolayers (Figs. 6 and 7). Pili can be seen extending from the gonococci (arrows) toward the plasma membranes of amnion cells in these thick sections. In some instances (Figs. 5 and 6) several pili course from each organism to the amnion cell surface. $\times 80,000$ (Figs. 5 and 6) and $\times 60,000$ (Fig. 7).

cell connections appear to involve fingerlike surface projections of the amnion cells. These projections are found on most parts of amnion cells after CPD and are especially numerous centrad from the peripheral, apronlike leading edge of cytoplasm. Striking examples of pilus-cytoplasmic projection attachments involve bacterial and amnion cell structures raised above the plane of the supporting substrate as ascertained by heavy metal shadows derived from these structures (Figs. 10-12). As noted in thick sections of gonococci and amnion cells, several pili from a single bacterium may communicate with a single amnion cell (Fig. 9).

Not all gonococci associated with amnion cells have visible pilus-mediated connections to the cells. In many instances (Fig. 11) gonococci appear to rest on the amnion cell exterior in contact with fingerlike cell projections, but pili radiating from the bacterium are not apparent. This probably represents a second type of association found between gonococci and amnion cells as described below.

The second type of association, between plasma membrane of amnion cells and the cell wall of gonococci, is seen clearly only in ultrathin sections. Overlapping segments of gonococcal cell wall and amnion cell cytoplasm preclude clear visualization of this type of interaction in thick sections. In the ultrathin sections, however, segments of the gonococcal cell wall outer membrane lie in close apposition to areas of the amnion cell plasma membrane that have contours similar to those of the gonococcal exteriors (Figs. 13-16). An electron-lucent space 70-90 Å wide is consistently seen between the external-most aspect of the gonococcal cell wall and the exterior limit of the amnion cell plasma membrane. An analogous space is not present in the region of apposition between T-4 gonococci and amnion cells. The cell walls of T-4 gonococci appear to impinge directly on the plasma membranes of amnion cells (Figs. 17-20) without the intervening space described with T-2 gonococci (Figs. 13-16). Only a few T-4 organisms have been found closely applied to amnion cells in numerous sections examined, whereas numerous T-2 gonococci are available for morphological study because of the differing avidity with which these colony types adhere to the eukaryotic cells. Nevertheless, the differing morphologies of associations between the amnion cells and the two colony types of gonococci seem consistent.

Occasional amnion cells, either after exposure to T-2 or to T-4 gonococci, contain intracellular gonococci (Figs. 21-23). The bacteria are enclosed within membrane-limited vesicles, appear morphologically intact, and are often seen in the perinuclear region where membrane-enclosed gonococci appear to impinge closely on the perinuclear cistern (Fig. 23).

DISCUSSION

Gonococci capable of becoming established in the male urethra and producing classical signs and symptoms of acute gonorrhoeal venereal disease have distinc-

tive characteristics. This was shown by Kellogg et al. (1, 2) who not only defined the colony forms (types 1 and 2) that result from growth of virulent gonococci but also showed that other organisms (colony types 3 and 4), which are *Neisseria gonorrhoeae* by the usual biochemical and light microscope criteria, are not associated with production of gonorrhea in experimental subjects. These findings provided a model system that could be used to study virulence factors through comparison of the pathogenic and the nonpathogenic forms of gonococci. Pili are demonstrable appendages of the cell walls of virulent colony type gonococci but are absent from the gonococcal surfaces of the avirulent colony types (3, 4).

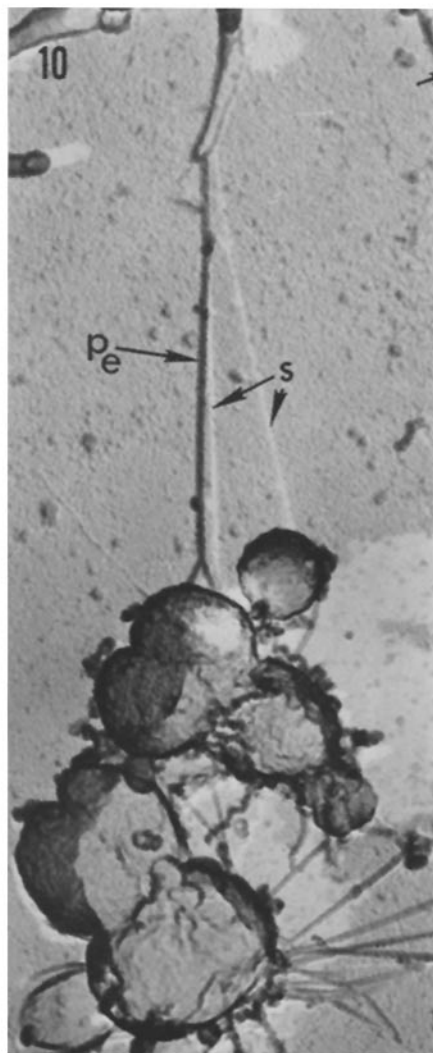
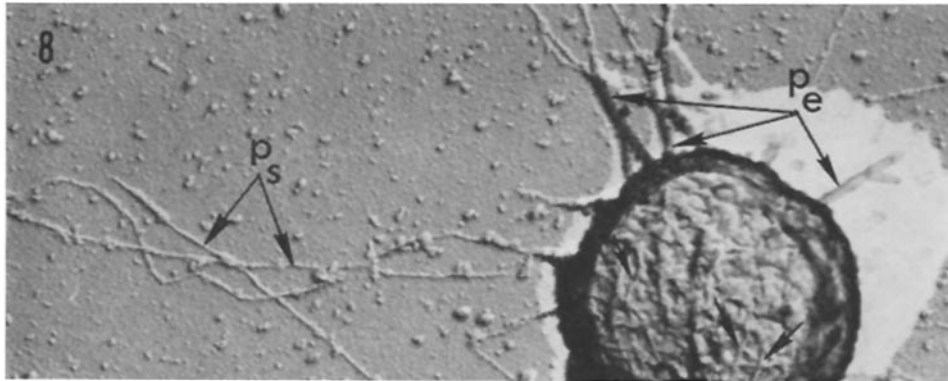
The present study provides evidence for a mechanism through which pili may play a role in determining virulence of *Neisseria gonorrhoeae*. The presence of pili is associated with enhanced attachment of gonococci to amnion cells in vitro. Whether pili have a similar function in vivo in promoting attachment of gonococci to cells of the urogenital tract, anorectal mucosa, or nasopharyngeal tract awaits further study. Such an attachment hypothesis with regard to gonococcal pili has been suggested previously (3) and is attractive because of the "flushing" by secretions, excretions, etc., of the cell-lined surfaces susceptible to gonorrheal infections. Enhancement in gonococcal attachment to these surfaces could conceivably account for virulence of pilus-bearing organisms in contrast to the avirulence of gonococci lacking pili.

Attachment of gonococci to amnion cells seems to have two morphologically different components. The first component or type of attachment is mediated through pili that radiate from the gonococcus' surface and are seen in sections or in critical point dried specimens. The diameter of gonococcus bearing radially oriented pili is much greater than that of a gonococcus devoid of pili. Further, bacterial pili often display a "sticky" nature relative to mammalian cells (9). These attributes of pili are possibly influential in determining attachment of gonococci to cells of host target tissues.

The second morphological type of attachment of gonococci to amnion cells appears to be more intimate and involves segments of the organism's cell wall with corresponding portions of the amnion cell's plasmalemma. One question concerning this type of attachment arises because of the demonstrable difference between T-2 and T-4 gonococci in their apposition to amnion cells. In the former, T-2, pilus-bearing organisms there is a consistent space separating the

FIG. 8. Gonococci prepared by critical point drying exhibit detail on their surfaces including pili adherent to the exterior of the organism (arrows). Pili that lie on the substrate (P_s) have adherent globular material but have diameters similar to those obtained by negative staining. Pili that are elevated above the substrate surface (P_e) have thicker profiles by virtue of accumulation of metal and Formvar. $\times 45,000$.

FIGS. 9 and 10. Pilated gonococci appear attached to projections of amnion cells by pili whose profiles (P_e) and shadows (s) demonstrate their position as being above the plane of the substrate. The double shadows (s) of the pilus in Fig. 10 reflect platinum and carbon evaporation both of which produced shadows beneath the pilus. $\times 18,000$ (Fig. 9) and $\times 27,000$ (Fig. 10).

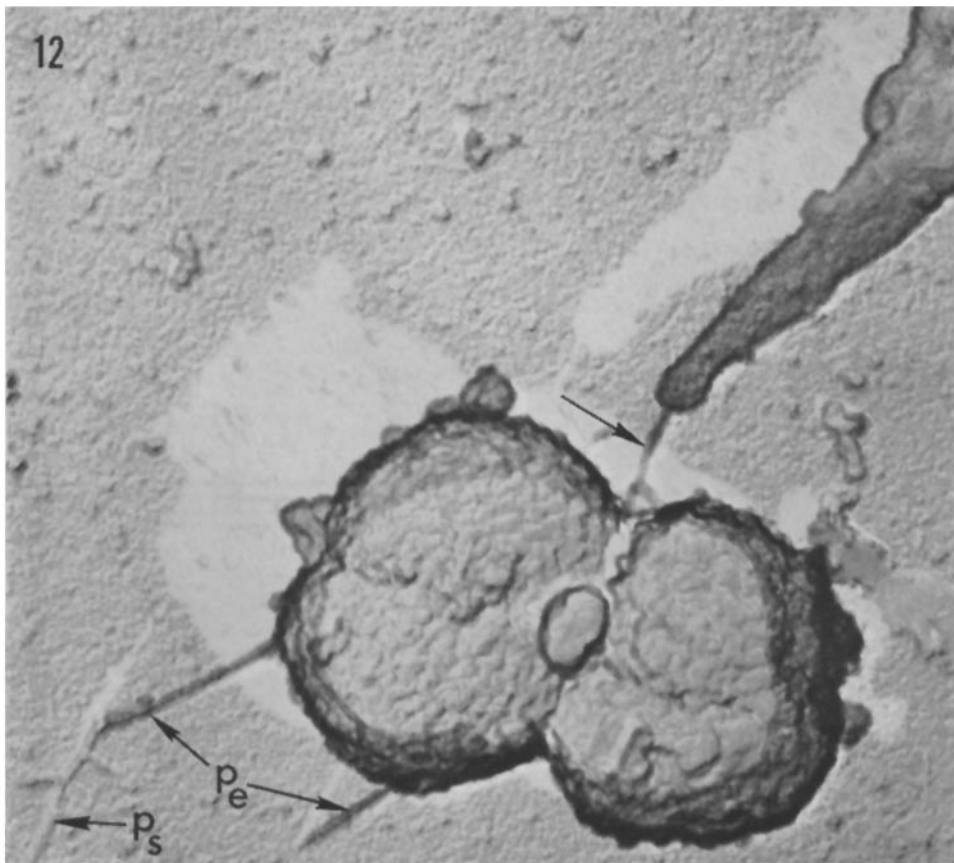
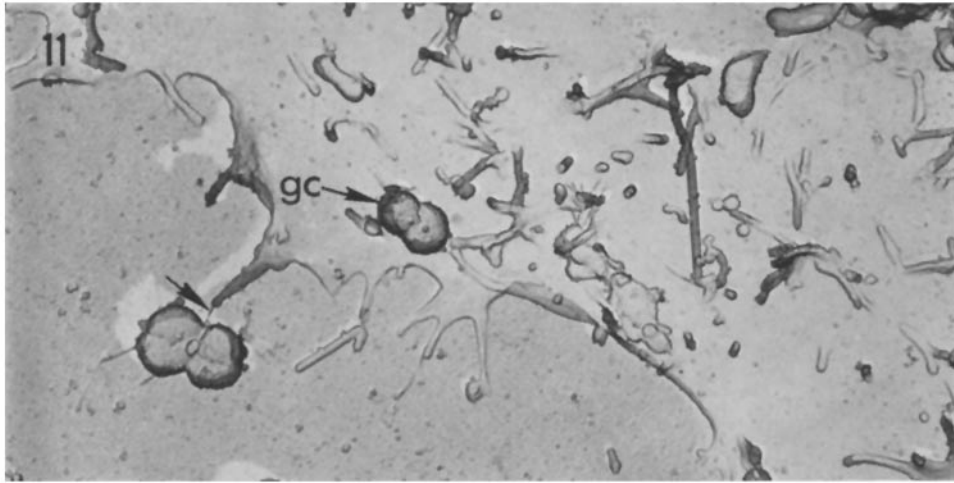


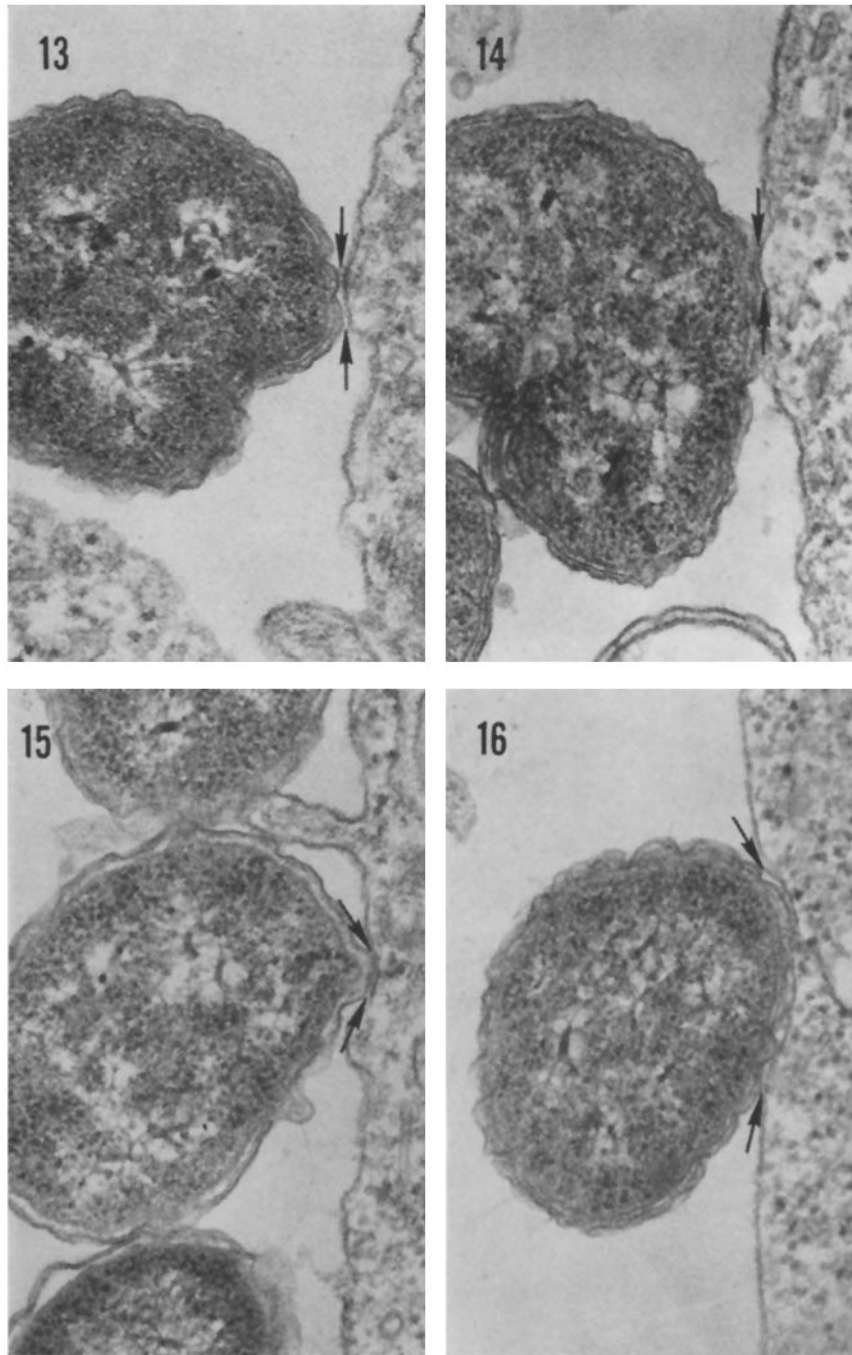
bacterium from the amnion cell. With the latter, T-4, nonpilated organisms the cell wall appears to abut directly on the amnion cell surface. The difference may be explicable by virtue of the adherent, surface-coating orientation of some gonococcal pili, demonstrated recently by freeze-fracture, freeze-etching (6). Thus, the space present between T-2 gonococci and amnion cell membranes could represent pili difficult to resolve by electron microscopy in this particular orientation and location. It is unclear whether the two morphological types of gonococcus-amnion cell association represent a sequence of events. It is possible that the initial attachment of gonococci to the eukaryotic cells is mediated through the organisms' elongated, radially extended, sticky pili. This may provide temporary attachment for bacterium to amnion cell; eventually the two cells may come together in the more intimate contact mediated by their respective external membranes. The differing proximity of cell walls of T-2 and T-4 to the amnion cell plasma membrane seems somewhat paradoxical. T-2 gonococci adhere more avidly to the tissue culture cells; yet their cell walls are separated from the plasmalemma of the amnion cells by spaces not present with T-4 gonococci. It is possible that this close or intimate association of bacteria with eukaryotic cells is related to phagocytic uptake of the bacteria although the small number of intracellular organisms seen in our experiments makes this unlikely. It is also possible that survival and multiplication of gonococci are, in some manner, inhibited when they are in such close contact with eukaryotic cells.

The role of pili in promoting attachment of gonococci to eukaryotic cells is not novel. Duguid and Gillies (10) demonstrated that *Shigella* that bear pili adhere to intestinal epithelial cells in vitro. These authors correlated the presence of pili and attachment of these organisms to epithelial cells to virulence of these *Shigella* sp. More recently Silverblatt has shown a correlation between virulence and pilation of *Proteus* sp. and his micrographs of experimental infections strongly suggest that attachment of the organisms to epithelium of the urinary tract is mediated by pili.² These pilus-attachment correlations may have general implications regarding bacterial pathogenicity. Brinton (9) demonstrated the presence of pili on bacterial isolates of various genera from clinical urinary tract infections. It is conceivable that pilation is a requisite for virulence

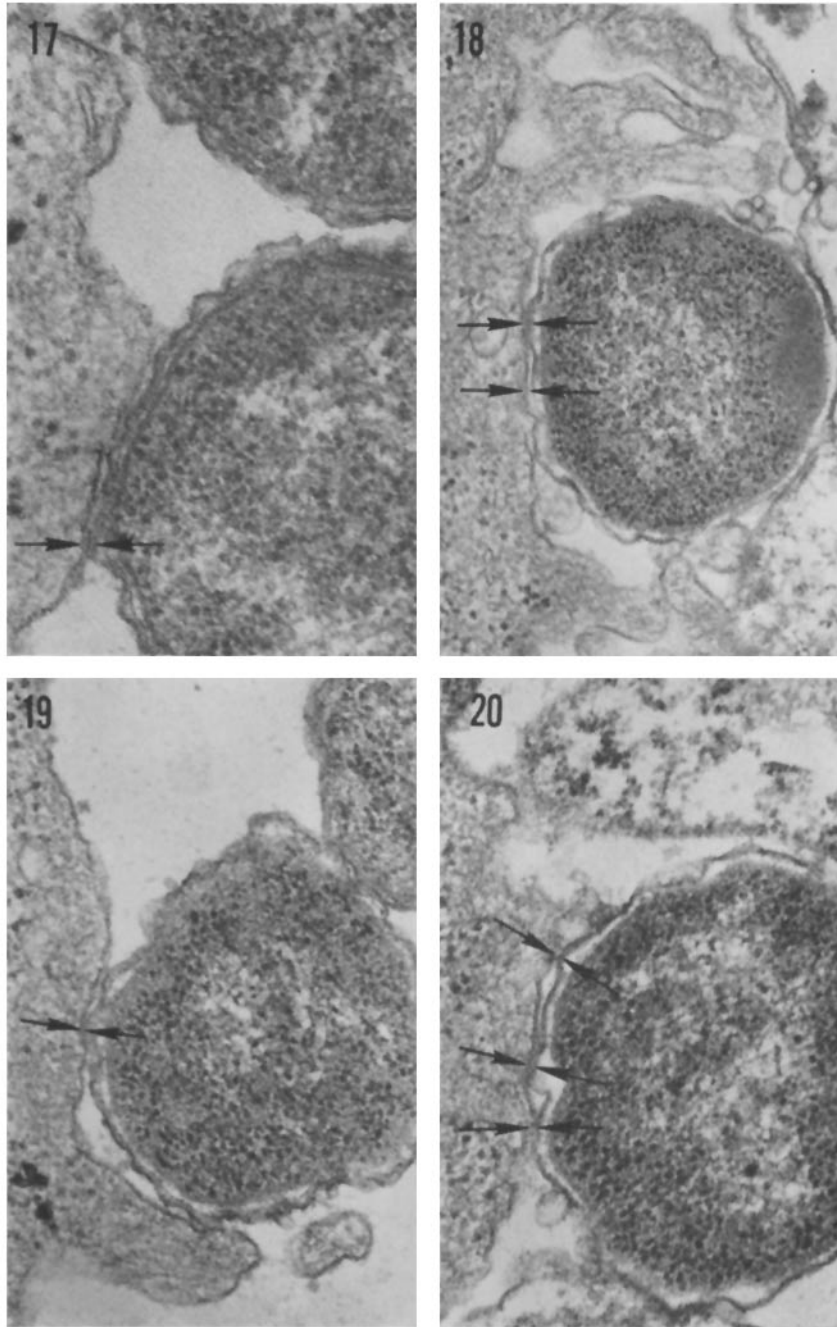
² Silverblatt, F. Personal communication.

FIGS. 11 and 12. Pilus-mediated attachment of gonococci can be visualized also on amnion cell projections that are above the level of the substrate (compare position of the shadow of projection to which organisms are attached with those of projections lying to the right of that projection in Fig. 11). Gonococci sometimes appear to be completely above the plane of the substrate, have pili extending to amnion cell projections (arrows), and radiate other pili that are partially adherent to the substrate (P_s and P_e). Note also the absence of discernible pili radiating from gonococci (gc) that lie on the surface of the amnion cell. (Fig. 12 is an enlarged portion of Fig. 11.) $\times 12,000$ (Fig. 11) and $\times 75,000$ (Fig. 12).

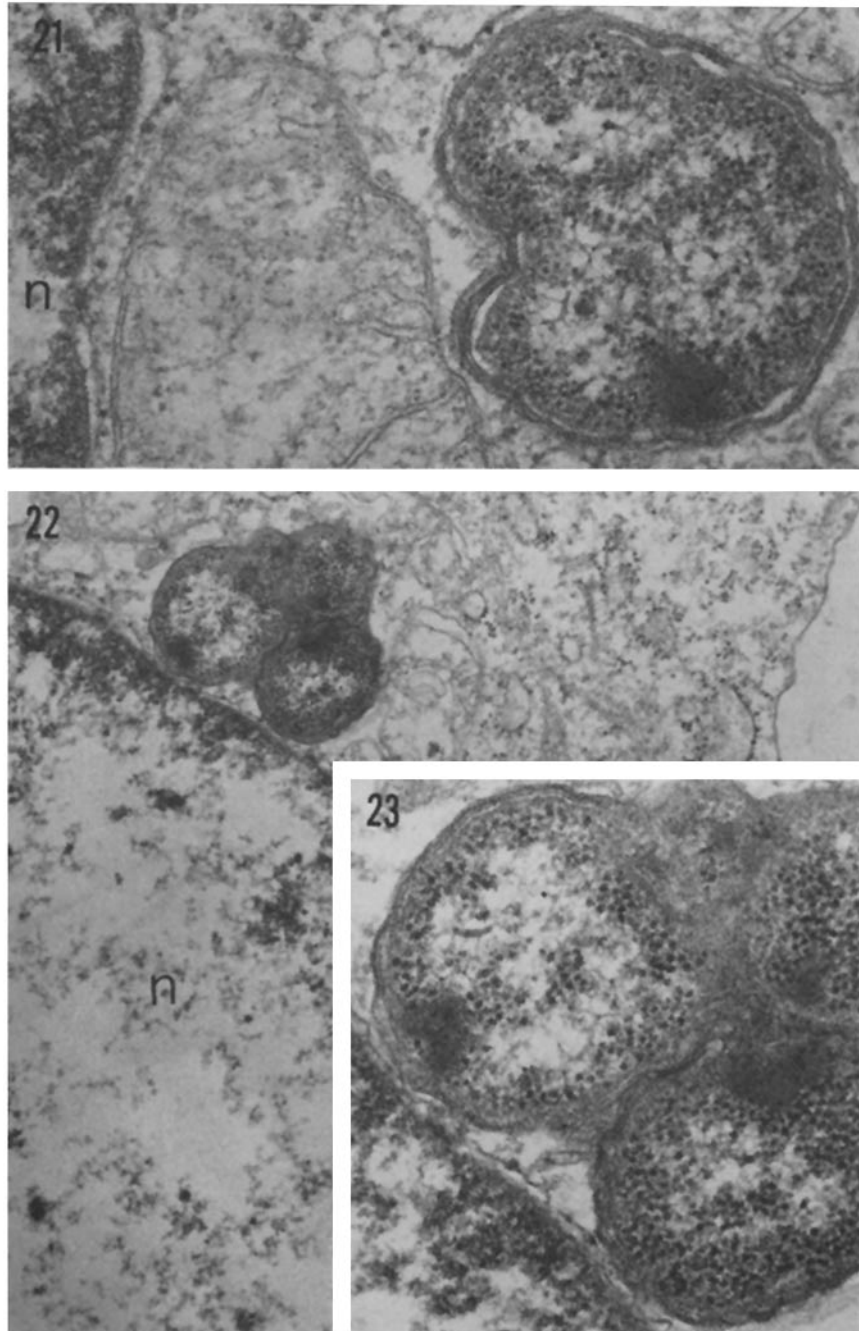




FIGS. 13-16. In ultrathin sections pili are not visualized on the surfaces of these pilated, T-2 gonococci. The cell walls of the gonococci are focally apposed to the plasma membranes of amnion cells, but an electron-lucent space (arrows) is seen between the limiting membranes of bacteria and tissue culture cells. $\times 60,000$.



Figs. 17–20. Occasional nonpilated, T-2 gonococci are seen adherent to amnion cells. The cell walls of these organisms appear to impinge directly on foci of the amnion cell membranes (arrows). $\times 75,000$ (Figs. 17 and 20) and $\times 60,000$ (Figs. 18 and 19).



Figs. 21-23. Gonococci are sometimes observed within the confines of amnion cells. The bacteria are contained in membrane-limited vesicles and may lie at a distance from the nucleus (*n*) as in Fig. 21 or may be located immediately adjacent to the perinuclear cistern (Figs. 22 and 23). (Fig. 23 is an enlarged portion of the cell in Fig. 22.) $\times 60,000$ (Figs. 21 and 23) and $\times 22,000$ (Fig. 22).

of several kinds of gram-negative bacteria. It is doubtful that pili always endow organisms with pathogenic activity. Numerous "nonpathogenic" *Neisseria sp.* have pili (11) but are not associated with clinical infections of human hosts in whom the bacteria reside. On the other hand, *Neisseria sp.* are commonly encountered as part of the "normal flora" and it is possible that their maintaining a relationship with host cells and remaining a part of this flora depend on their possessing pili.

Although bacterial pili are long-recognized components of many gram-negative bacterial species, primary attention has centered on these structures as attachment sites for bacteriophages and as appendages possibly related to sexual mating of bacteria (9). Pili have also been studied as antigens, but only recently has this characteristic been exploited for diagnostic purposes. The study of Buchanan et al. (12) has shown that antibodies formed in response to gonococcal pili can be detected by a radioimmunoassay which may prove useful for diagnosing gonorrheal disease especially in unsuspecting, asymptomatic individuals. It is not clear at present whether antipilus antibodies function in modifying attachment of gonococci to eukaryotic cells. Preliminary studies show that these antibodies modify damage of tissue culture cells produced by T-2 gonococci.³ Whether this represents activity of the antibodies in modifying attachment of the pilated T-2 organisms or reflects agglutination of the organisms is not known.

The presence of gonococci within amnion cells is merely an incidental finding at this point. A recent study reporting an experimental animal model for gonorrhea (13) has shown intracellular organisms in the granulation tissue used for growth of gonococci in vivo. Further, the most complete histopathological description known to me (14) notes, in passing, the presence of intracellular gonococci in some biopsy specimens from patients with gonorrhea. These observations do not relate to intraleukocytic gonococci but to bacteria within epithelial or connective tissue cells. The intracellular location of gonococci has important possible implications to chronicity and to therapeutic refractiveness of gonococcal infections. Experiments on several aspects of intracellular gonococcal infections are under investigation. However, the relation of intracellular gonococci to genesis or continuation of gonococcal infections is currently unclear. One group of investigators has recently suggested that gonococci survive only if they remain on the mucosal surface of epithelium-lined tissues (15). Either this location of gonococci or intracellular sites might depend, initially, on anchoring of organisms to the epithelium.

SUMMARY

Attachment of *Neisseria gonorrhoeae* to amnion cells in tissue culture is facilitated if the gonococci bear pili. This has been determined by studying the

³ Swanson, J., and M. A. Siam. Unpublished observation.

number of pilated, colony type 2 gonococci associated with amnion cells after incubation in vitro as compared with the number of nonpilated, colony type 4 gonococci present with amnion cells under the same conditions. These data are supported by light microscope findings. Electron microscope studies provide visualization of fine structure of gonococcal attachment. Gonococci are also found within amnion cells in this in vitro system.

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Note Added in Proof.—Since submission of this paper, an article dealing with adherence of gonococci to urethral mucosal cells from individuals with gonorrhea has been published (Ward, M. E., and P. J. Watt. 1972. Adherence of *Neisseria gonorrhoeae* to urethral mucosal cells. An electron-microscopic study of human gonorrhea. *J. Infect. Dis.* **126**:601). In that study gonococci are shown to adhere to epithelial cells via extensive zones of intimate contact. It is not clear whether those zones of contact correspond to the attachment regions described in the present paper. Further, pili are not identified either by the authors of that paper or in their accompanying electron micrographs. However, the extent of gonococcal attachment, as well as apparent partial penetration of the gonococci into the epithelial cells in those specimens from humans with gonorrhea, is of considerable interest in relationship to the findings herein reported for an in vitro system.

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