STUDIES ON GONOCOCCUS INFECTION

I. Pili and Zones of Adhesion: Their Relation to Gonococcal Growth Patterns*

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Neisseria gonorrhoeae is certainly one of the most frequent causative agents of human disease. Although many data are available on the epidemiology of gonococcal infections, relatively little is known about the physiology or the pathogenicity of the causative organisms. Unanswered are numerous questions concerning the mechanisms by which the gonococcus establishes itself in the human urogenital tract and what role is played by humoral or cellular immune mechanisms that occur during infection and recovery from this disease.

Virulence of gonococci has been shown to correlate with the colonial form displayed by the organism when grown on agar medium. Kellogg et al. (1) have described criteria by which gonococci can be divided into four distinct colonial types numbered 1, 2, 3, and 4. Gonococci freshly isolated from clinical material most often have type 1 or type 2 colonial morphology. Upon repeated nonselective transfers these gonococci will give rise to a population of which the majority belong to colonial types 3 and 4; however, colony type 1 can be maintained in vitro if selectively transferred on solid media, and even after 700 transfers the organisms are virulent if inoculated into human volunteers. In contrast, gonococci of type 4 are unable to cause infection.

In the present study it was found that, when gonococci were grown in liquid medium, these organisms could grow either as an even suspension or with various degrees of clumping. This difference of growth pattern in liquid medium was found to be correlated with the colonial morphology on solid medium. The

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reasons for these different patterns of growth, both on agar and in liquid medium, and the association with virulence have not been previously difined.

The four colonial forms of the gonococcus were studied by electron microscopic techniques to define ultrastructural characteristics related to the colonial variability in the hope that these might be relevant to the pathogenetic potential of the organism.

Materials and Methods

Strains of Neisseria gonorrhoeae.—All strains freshly isolated from patient material were initially cultured on Thayer-Martin selective media (2). They were further identified as Gram-negative diplococci that produced a positive oxidase reaction with *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride and fermented glucose but not maltose or sucrose. Fermentations were performed using cystine tryptic agar (Baltimore Biological Laboratories, Baltimore, Md.) containing 1% of the test sugar. After the minimal number of passages to ensure purity, certain strains (strains 15, 16, 18R, and 18T) were preserved either by lyophilization or freezing in a medium consisting of 5% w/v bovine serum albumin and 5% w/v monosodium glutamate (3). Strains 15 and 16 were isolated from the exudate of men with gonococcal urethritis. Strain 18 was isolated from the pharynx (18T) and rectum (18R) of a patient with gonococcal arthritis. By selective transfers on solid medium the four colonial variants were isolated from each of these strains. Colony types 1, 2, 3, and 4 of strain 2868 were generously supplied by Dr. Douglas S. Kellogg of the Venereal Disease Research Laboratory.

Growth of the Organisms.—

Solid media: Organisms were plated on GC agar base supplemented with Iso-Vitalex (R) (Baltimore Biological Laboratories) and grown for 16–18 hr at 37°C in a candle-extinction jar.

Liquid media: Liquid cultures were performed in the defined meningococcal medium described by Frantz (4) supplemented with 0.4% dialyzed yeast extract (Difco Laboratories, Detroit, Mich.). Organisms from solid media were removed with sterile cotton swabs, suspended in 50 ml of liquid media contained in a 250 ml Erlenmeyer flask, and incubated at 37°C for 4 hr on a rotary platform shaker revolving at about 150 rpm.

Colony Morphology.—The organisms were grown on solid media as described above. The colony-typing system was that of Kellogg et al. (5), with the exception that type 3 produced a rough suspension in saline. The other qualities of type 3 and the features of the other types were those outlined by Kellogg.

Electron Microscopy.—

Thin sectioning: Gonococci grown in fluid medium were fixed by adding 1/10 volume of fixative (2% glutaraldehyde in 0.1 M sodium cacodylate, pH 6.9) to the culture which was then centrifuged. The pellet was resuspended gently in fresh fixative. Subsequent steps included rinsing with cacodylate buffer, osmication, en bloc uranyl acetate staining, and embedding the dehydrated specimens in Epon. Individual intact colonies were prepared for electron microscopic examination by flooding the plate with fixative in order to preserve the colonies while still *in situ*. The details of this method have been published previously (6). Thin sections were stained with uranyl acetate and lead citrate. Lanthanum nitrate staining was done by fixing colonies in 2% glutaraldehyde–0.1 M *s*-collidine–4% lanthanum nitrate (final pH 7.7) and rinsing and postosmication in lanthanum containing collidine-buffered solutions (final pH 7.7) (7).

Negative Staining.—Gonococci of a particular colony type were evaluated by negative staining of both fluid cultures and buffer suspensions of agar cultures. Fluid cultures were inoculated with organisms that grew as a single colony type on solid medium and were incu-

bated for 4–6 hr, and aliquots were used for negative staining. Liquid cultures of each colony type from stains 15, 16, 18T, 18R, and 2686 were examined in this way. Also, specific colonies of the desired type were selected from a 16–20 hr culture on agar medium, suspended in a small volume of water, and aplied to grids along with negative stain. By this method at least two colonies of each type derived from different strains were studied. Furthermore, the primary cultures on selective media of two female and one male genitourinary exudate were also examined. Both 1% potassium phosphotungstate (pH 6.0) and 2% aqueous uranyl acetate (pH 3.5) were utilized as negative strains. Use of uranyl acetate necessitated preliminary washing of the specimen with water after application to the grid and removal of excess suspending fluid to prevent the coprecipitation of uranyl and phosphate ions. Carbon-coated, collodion-covered grids were used for the negative staining studies. All electron microscopic observations were made with an AEI EM801 microscope (AEI Scientific Apparatus Inc., White Plains, N.Y.) operating at 80 kv.

RESULTS

Colonial Morphology.—Differences in colonial morphology are readily appreciated when 16–18-hr agar cultures of gonococci are studied with a dissecting microscope and oblique lighting. Two forms are most easily recognized: small and large colonies. Differentiation of type 1 from type 2, both of which are small forms, and type 3 and type 4, both of which grow as large colonies, depends on additional criteria, such as opacity, sharpness of perimeter, color, and consistency. The characteristics that permit identification of each colony type are summarized in the photomicrographs and legends of Figs. 1 and 2. Repeated selective passages of each colonial type result in cultures whose colonies are nearly all of the same type.

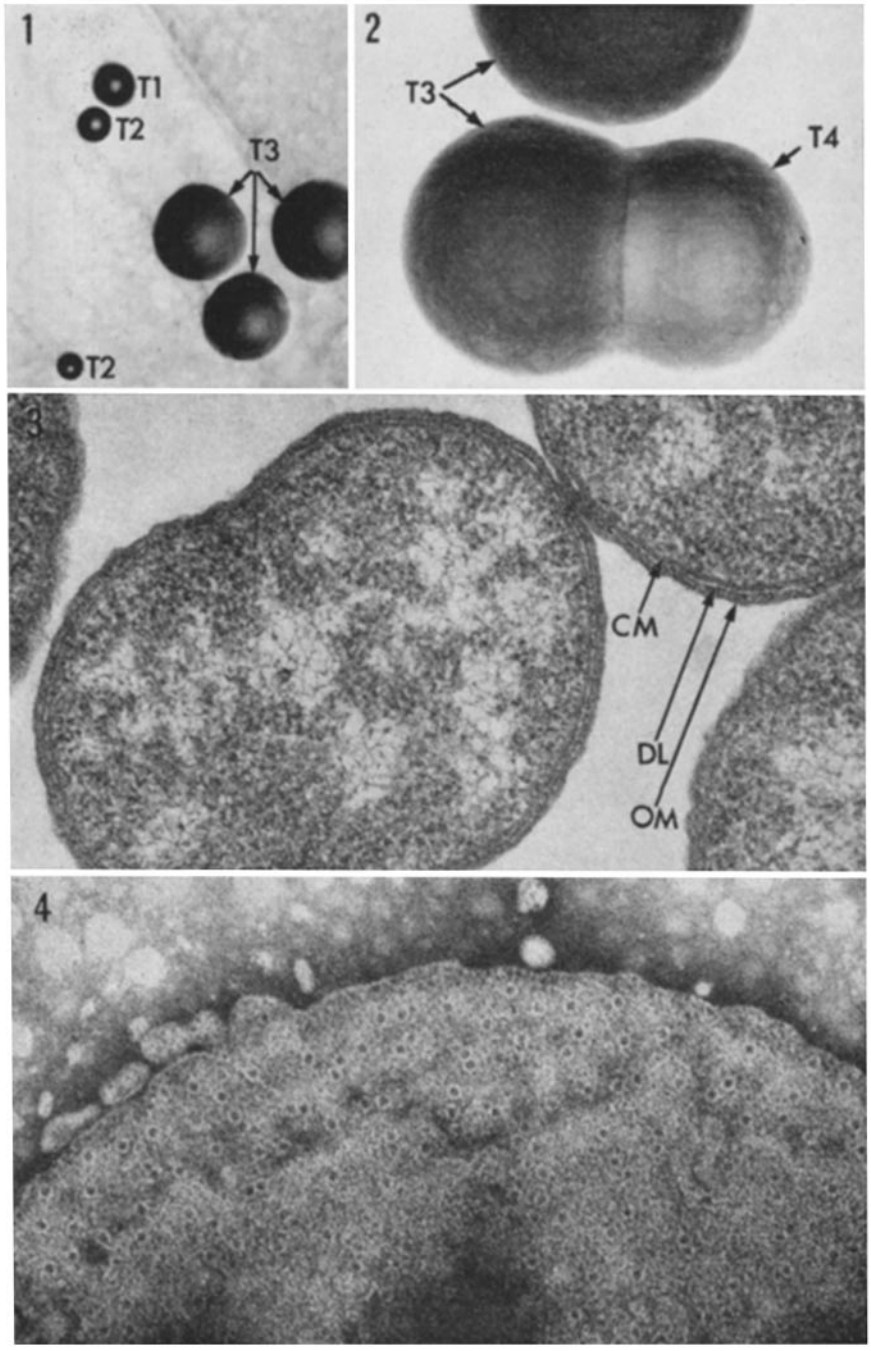
Ultrastructural Differences among Gonococcal Colony Types.—The general electron microscopic appearance of a gonococcus (8, 9) is similar to that of a meningococcus (10). In thin sections the nucleoid region and the cytoplasm of these species are very similar. The cell wall consists of the structural elements

FIG. 1. Gonococcal colonies on agar medium display differences in morphology as described by Kellogg (1). The small colonies (T1 and T2) appear very dark in the oblique lighting used with the dissecting microscope. These small colonies are from 0.2–0.5 mm in diameter. Type 2 (T2) colonies are differentiated in that their edges are sharper in outline than those of type 1 (T1). Several large type 3 (T3) colonies are shown for comparison to the small colony types. × 10.

FIG. 2. Type 3 and 4 colonies, both of the larger colony variety, are differentiated on the basis of the granularity of the surface and their color as seen with oblique lighting. The large colony types are quite pale as compared with the small colony types. Type 3 (T3) colonies are slightly more pigmented (light brown) than the type 4 (T4) colonies. The greater granularity of the surface of type 3 colonies as compared with the type 4 colony is also seen. × 40.

FIG. 3. Gonococci in thin section exhibit internal and surface layer architecture typical from Gram-negative bacteria. The cell wall consists of two parts, the outer membrane (OM) and a dense lamina (DL), and surrounds the cytoplasmic membrane (CM). × 62,500.

FIG. 4. A gonococcus negatively stained with phosphotungstate exhibits numerous focal accumulations of stain on its surface. The electron-opaque foci are 80–100 A in greatest dimension and are surrounded by an electron-lucent halo. × 100,000.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T1
T2
T3
T2
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T4
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CM
DL
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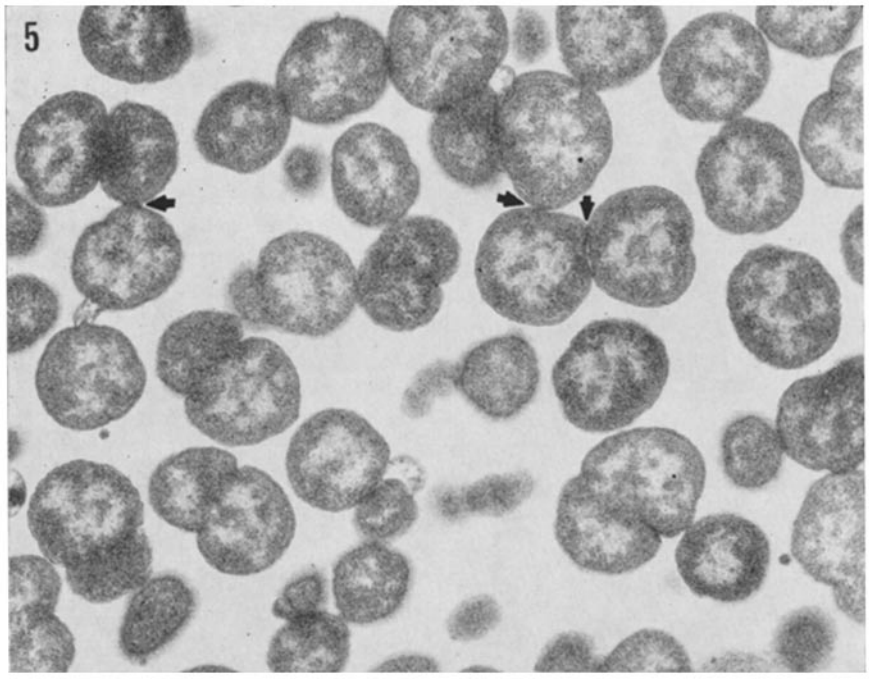
typical for Gram-negative bacteria (Fig. 3). The outer membrane and the middle dense layer are morphologically identical to those of meningococci (10). Negatively stained gonococci are also similar in morphology to meningococci except that the former have more abundant foci of negative stain accumulation on their surfaces (Fig. 4) (10). These foci, which have the appearance of "holes" or "pits," are 80–100 A in greatest dimension, are slightly elliptical in outline, and do not seem to be distributed in any particular geometric pattern on the surface of gonococci.

Thin sections of type 4 gonococi fixed in colonies *in situ* on the agar surface are similar to those of cells growing in liquid culture. The majority of the bacteria appear as single or diplococcal forms (Fig. 5). The latter appearance derives from two daughter cells being incompletely separated or divided from one another. Infrequently, one observes a tetrad arrangement or a streptococcal-like arrangement of gonococci in these colonies. Very few zones of adhesion (see description below) are observed. If the cultures have been inoculated 20 or more hours before examination, the gonococci in the center of the colonies may display degenerative changes with virtually complete loss of their internal cellular architecture. In these "old" cultures the cells at the periphery of the colony retain their typical architectural features and appear to be in states of active growth and division.

Types 1, 2, and 3 exhibit differences from type 4 colonies in the manner by which individual gonococci are arranged with respect to one another. The cells appear to associate with their neighboring gonococci by rather curious zones of focal adhesion of their cell walls' outer membranes (Figs. 6–9). These zones of adhesion are composed of flattened apposing segments of adjacent cells' cell walls and are of variable length. The outermost electron-opaque laminae of the apposed cells' outer membranes are separated by a gap 20 A in width along the entire length of the focus of adhesion (Fig. 9). Individual gonococci may be adherent to two, three, or more neighboring bacteria through these foci (Figs. 7 and 8). These zones or foci of adhesion, described in detail later in this report, resemble the gap junctions that have been seen in several animal systems. These foci of adhesion, while occasionally seen in type 4 colonies (Fig. 5), are much more common in the other types; therefore, their paucity serves as a criterion to differentiate type 4 gonococci. The ultrastructural feature which distinguishes type 1 and type 2 colonies from types 3 and 4 can be seen in thin sections as wisps of extracellular material superficially resembling flagella in the type 1 or 2 specimens (Fig. 10).

Negative staining with uranyl acetate and phosphotungstate has revealed pili on the surfaces of every specimen of type 1 and type 2 gonococci but never on gonococci of types 3 or 4. Both liquid cultures and suspensions of organisms were examined and no difference in the degree of pilation is obvious when comparing the two kinds of preparations. The number of pili per gonococcus is



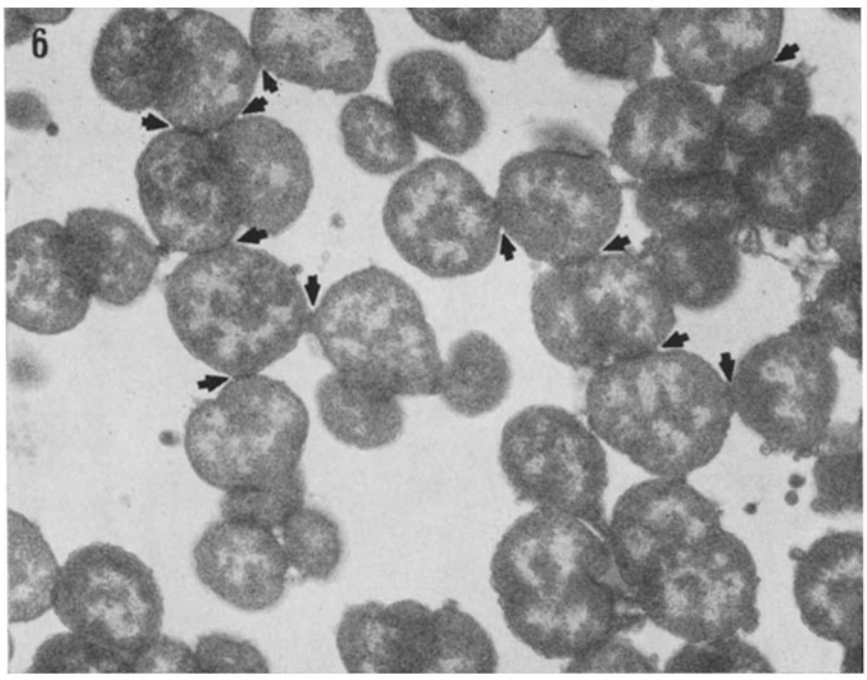
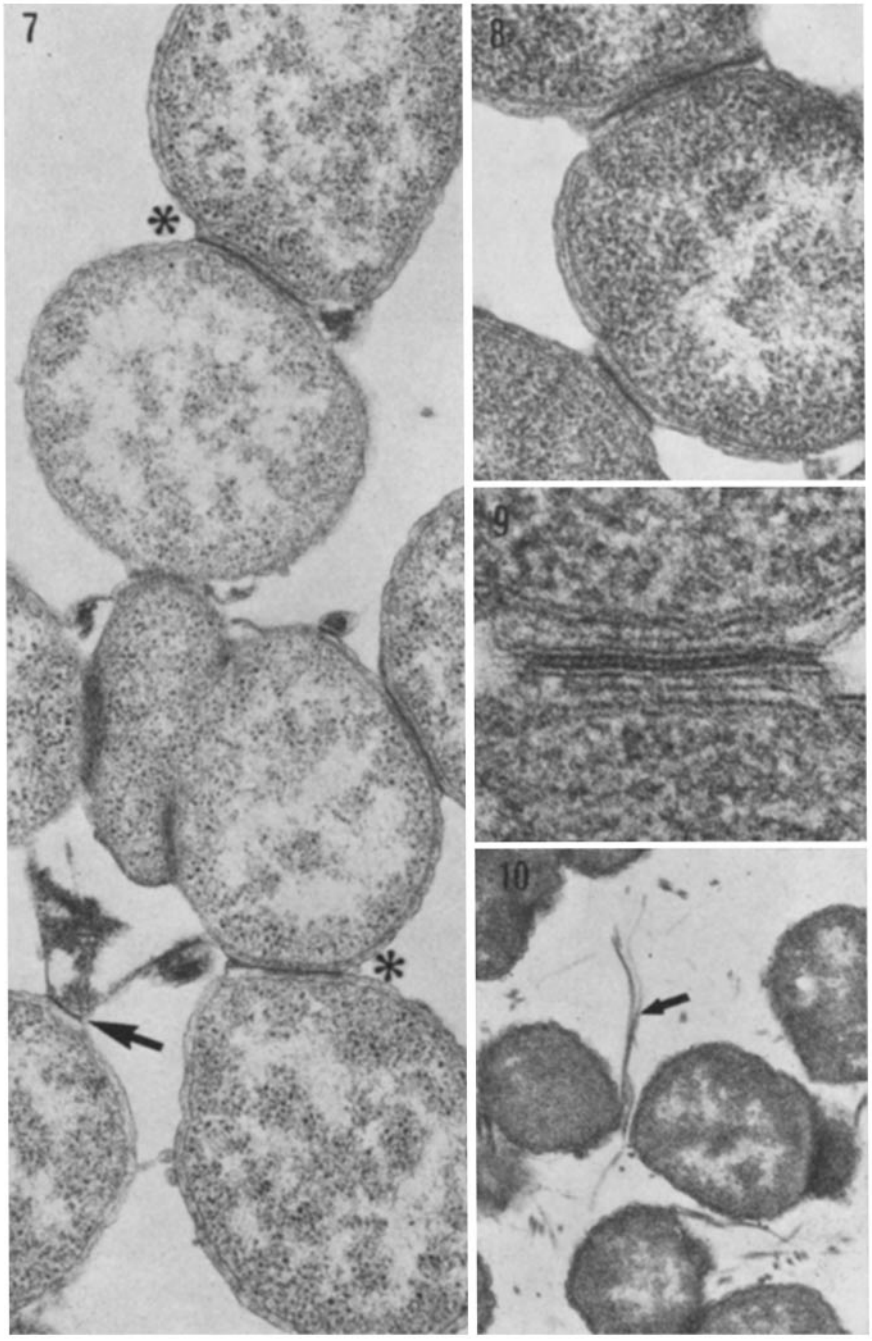


FIG. 5. Type 4 gonococci in colonies on agar medium are arranged mainly as single or diplococcal forms. In a few instances the cells are connected by zones of adhesion (arrows). × 16,000.

FIG. 6. Type 3 gonococci in colonies on agar medium are often adherent to another to form lattice-like arrangements. The cells appear bound to one another by zones of adhesion, some of which are indicated (arrows). × 16,000.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difficult to establish because the organisms are usually found in clumps. In some specimens only a few pili (approximately 2–6) appear to arise from each gonccoccus (Fig. 13). In other specimens many more (approximately 25–50) pili are found in tangled masses surrounding a small clump of cells (Figs. 11 and 12). The pili exhibit a marked tendency toward lateral aggregation and, when the bacteria are extensively pilated, crystalloid arrays of the pili may be found detached from the gonococci (Fig. 14). In the aggregates there is often visible a faint periodicity of approximately 50 A along the length of the pili (Fig. 15). This formation of laterally aggregated arrays is seen predominantly when acidic (pH 3.5) uranyl acetate is used for negative staining. Phosphotungstate negative staining at pH 6.8 yields a pattern of dispersed, nonaggregated pili (Fig. 17). Uranyl acetate staining also is associated with extensive kinking and bending of individual pili (Fig. 18) which tend to follow a straighter course in phosphotungstate-stained preparation (Fig. 17). In the curved portions of pili stained with uranyl acetate, 50 A periodicity is sometimes visible along the length of the pilus (Fig. 19) corresponding to the faint periods seen in laterally-aggregated pili (Fig. 15).

Gonococcal pili vary in length and may be as long as 4 μ (Fig. 13). The majority of pili appear 0.5–2 μ long. These variations in length do not suggest more than one "class" of pilus but rather that many of the surface appendages have fractured or are in different stages of protrusion or synthesis. The pili from all strains of gonococci have the same diameter of 80–85 A. This dimension is derived from center-to-center measurements of two or more pili lying side by side. Individual pili often appear 90–100 A in width and seem to narrow in areas of decreased stain concentration or, in some instances, when the pilus is curved or kinked (Fig. 18). The narrowed portion in these regions is 80–85 A in diameter. These data suggest that the pili may be slightly flattened in cross-section, but the findings could also derive from apparent differences in diameter due to variations in the depth of stain surrounding the pili.

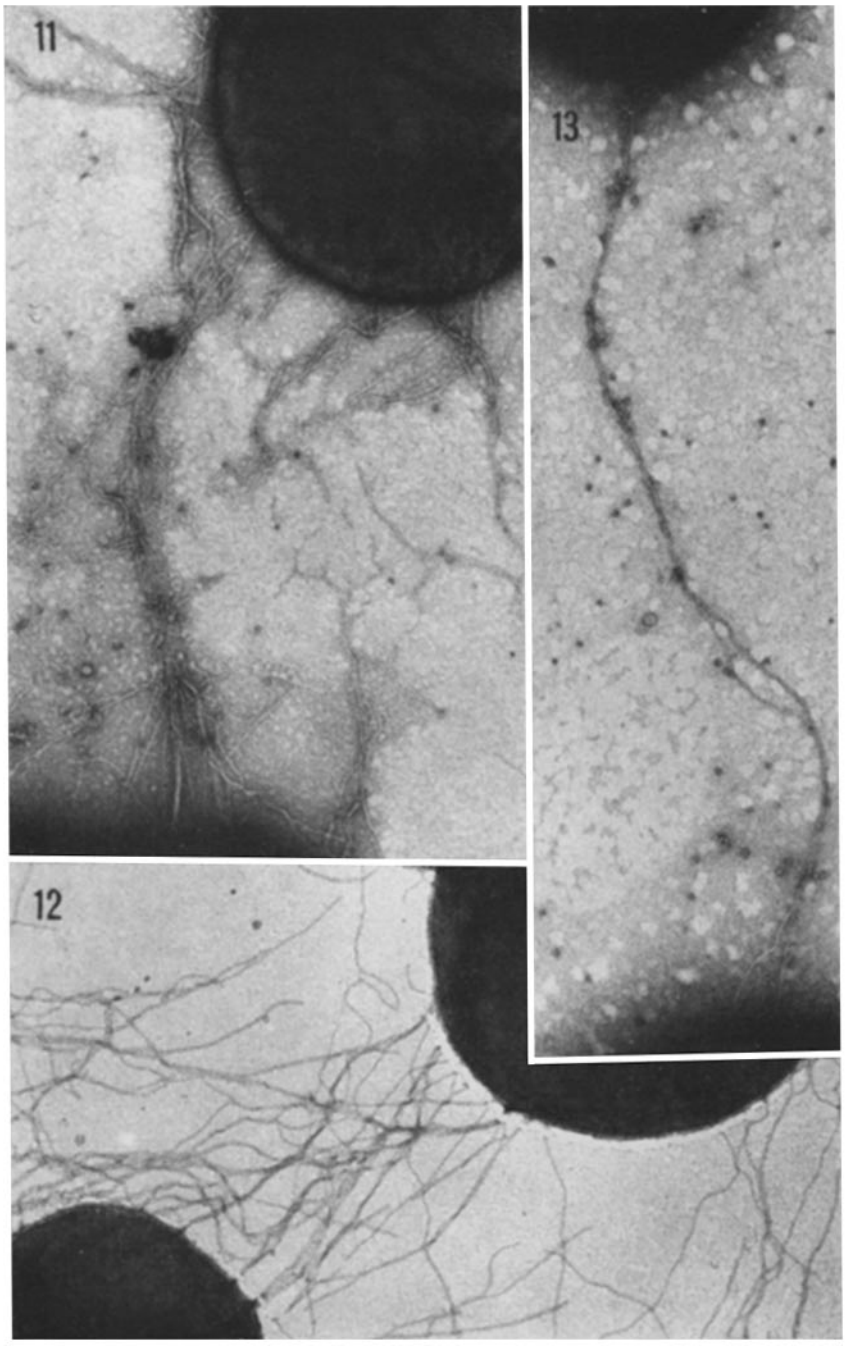
The lateral aggregation of pili noted above does not occur solely in negatively

FIG. 7. Gonococci from a type 2 colony fixed *in situ* are connected by numerous zones of adhesion (asterisks). Electron-opaque material appears to fill the interspace between outer membranes of adjacent cells. Note the apparent tenacity of the adhesion zone (arrow) comprised of a cell wall which appears pulled away from the underlying cytoplasmic membrane. × 40,000.

FIG. 8. Type 3 gonococci in fluid culture also exhibit many zones of adhesion. Several cells may be joined through these zones. × 62,500.

FIG. 9. Higher magnification of cells seen in Fig. 8 reveals a radiolucent area about 20 A wide separating the apposed surfaces of gonococci joined by zones of adhesion. The adhesion zone deforms the outer contour of participating cells and has a straightened profile. × 200,000.

FIG. 10. Type 2 gonococcal cultures from either fluid or solid medium can often contain numerous electron-opaque wisps of extracellular material (arrows). These structures are seen only in type 1 and type 2 cultures. × 25,000.



FIGS. 11–13. Gonococci from type 1 or 2 have pili visualized by negative staining with uranyl acetate. Some cells are surrounded by many tangled pili (Figs. 11 and 12) whereas other gonococci seem to give rise to only a few pili as in Fig. 13. × 40,000.

stained preparations. Thin sections also may contain such aggregates of pili which appear as longitudinally striated structures (Fig. 16) that superficially resemble bacterial flagella. True flagella have never been observed in negatively stained preparations of gonococci in which their terminal attachment apparatus should allow for their identification.

No pili have been found on or detached from gonococci in any of the type 3 or type 4 specimens studied by negative staining.

Ultrastructural Basis for Clumping in Fluid Cultures.—Strains of gonococci differ in their growth pattern in fluid medium. Some strains grow as a homogenous suspension whereas others display various degrees of clumping. This behavior was found to be relatively constant for a particular strain and was also observed in other fluid media supporting the growth of gonococci (11).[1] Systematic investigation of this phenomenon indicated that it was correlated with the colonial type of the strain. This relationship is illustrated in Figs. 20–24 and summarized and follows: type 1, slight or moderate clumping; type 2, marked clumping; type 3, moderate clumping; and type 4, no clumping. This held true in the instance where, because of selective transfers, the majority of the population of a strain belongs to a single colony type. In unselected strains containing more than a single type the growth pattern was unpredictable.

In an attempt to understand this behavior, several cultures in fluid medium which grossly displayed varying degrees of clumping were studied by negative staining and by thin-section techniques. The possible role of pili in promoting clumping was evaluated by studying several type 1 strains which displayed either slight or moderate clumping. All of the specimens were populated by gonococci that were pilated and no correlation between relative extent of piliation and degree of clumping was found. Moreover, type 3 gonococci which, as noted previously, do not have pili grow clumped. Gonococci grown in liquid medium were examined by thin-section techniques to evaluate the role of the zones of adhesion (see previous section) in promoting clumping. It was found that the specimens that showed gross clumping had extensive cell aggregation mediated through the adhesion zones on their cell walls. For any specific strain and colony type, the number of zones of adhesion is diminished in fluid medium as compared to agar cultures. For example, in fluid cultures of type 4 gonococci the zones of adhesion were rarely observed whereas colonies of the same strain fixed *in situ* exhibited more of these zones, though they were much less numerous than in the other types.

Lanthanum Nitrate Staining of Adhesion Zones.—When lanthanum nitrate solutions are adjusted with alkali to pH 7.7 a colloidal suspension results. This lanthanum colloid is electron opaque and its constituent particles appear to be 20 A or smaller in diameter (12). When alkaline lanthanum nitrate is added to fixative solution the colloidal material is trapped in spaces that approximate

[1] Kellogg, D. S. Personal communication.

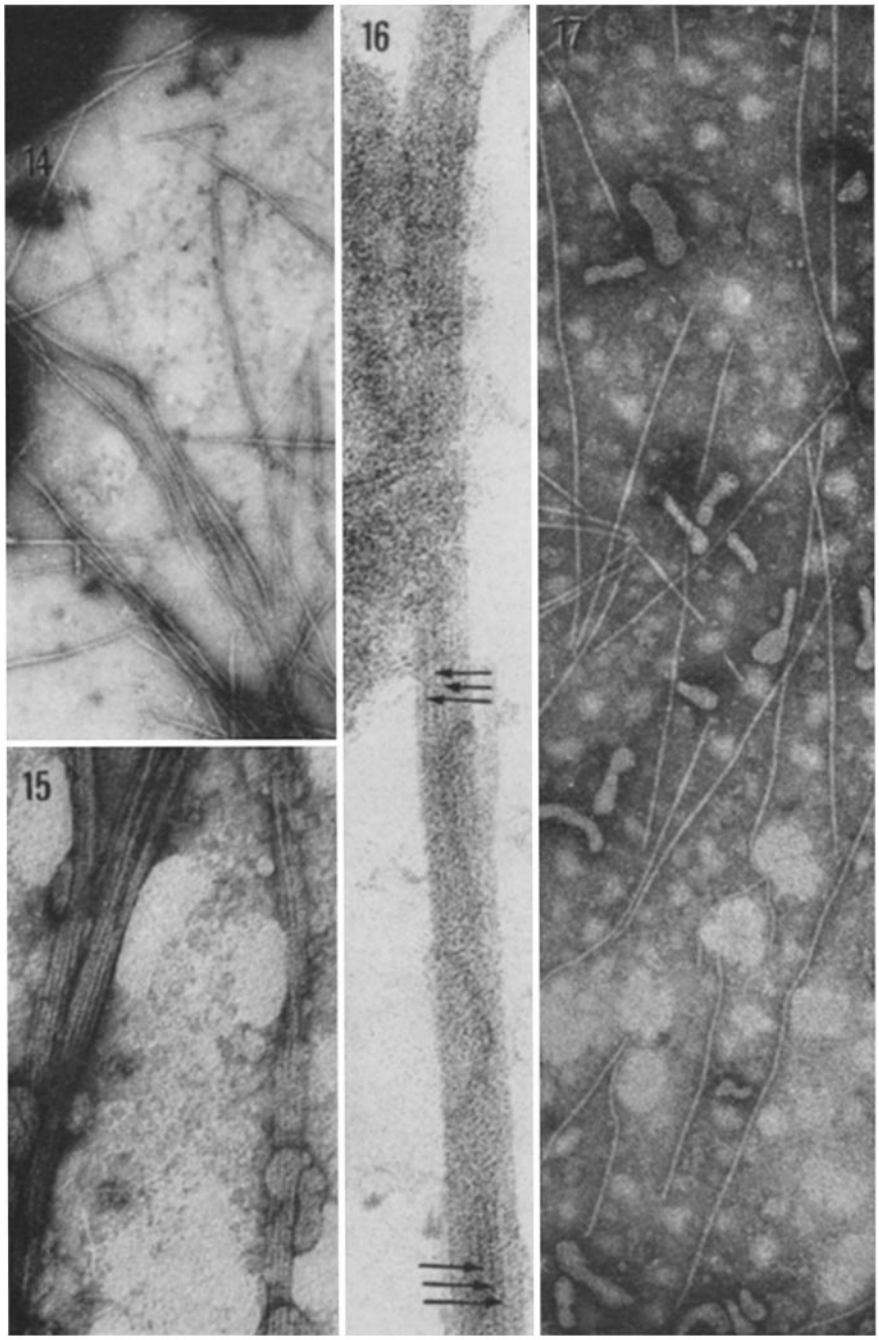


FIG. 14. Pili are exceedingly numerous in some type 2 gonococcal cultures and often form crystalloid aggregates when stained with uranyl acetate (pH 3.5). × 25,000.

FIG. 15. Crystalloid masses of pili exhibit faint periodicity along the length of individual pili as well as showing accumulations of negative stain (uranyl acetate) between the separate pili in the aggregates. × 125,000.

FIG. 16. In some thin sections, the electron-opaque wisps (see Fig. 10) can be seen to consist of laterally aggregated pili as indicated by the arrows. × 125,000.

FIG. 17. Pili negatively stained with phosphotungstate remain dispersed on the supporting grid. × 62,500.

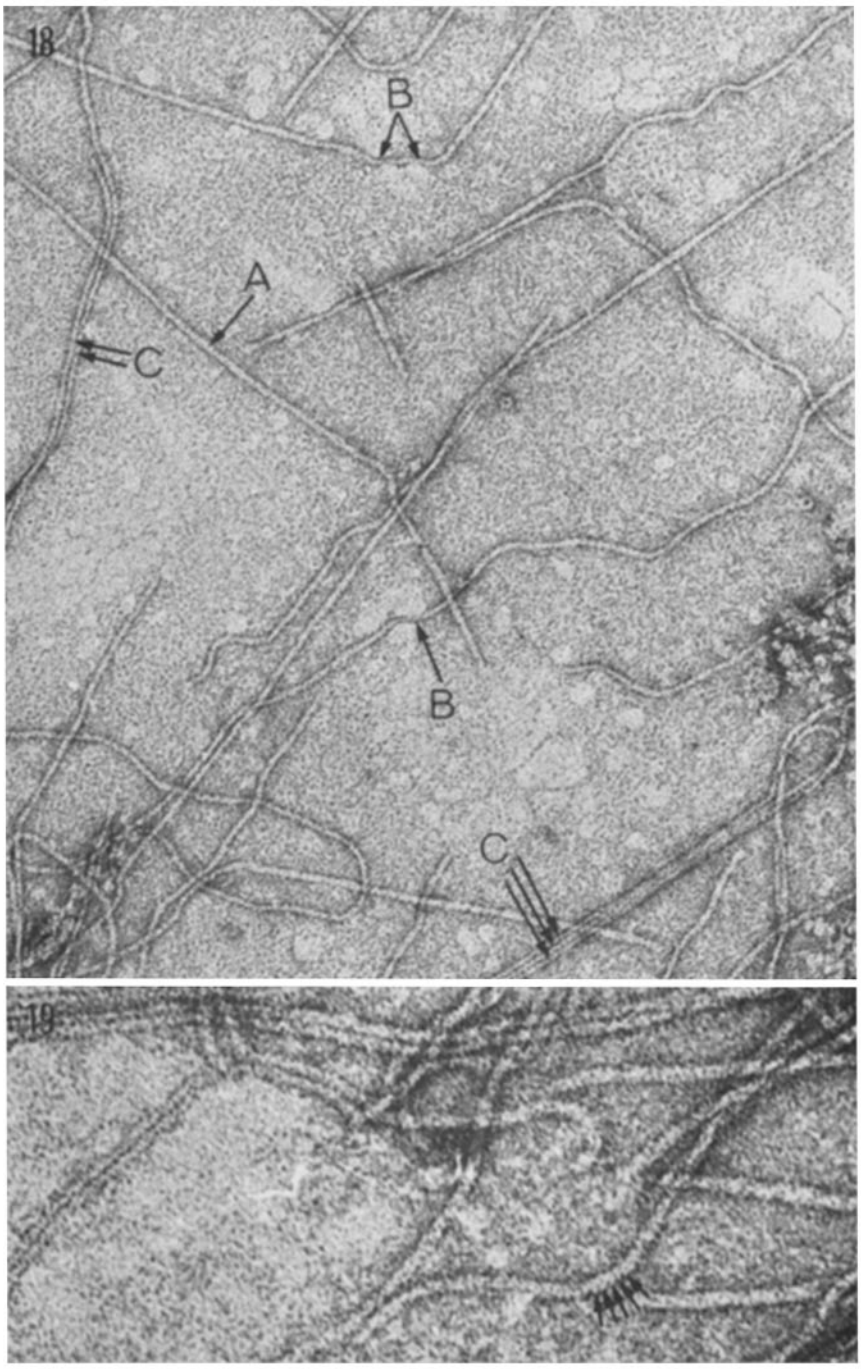


FIG. 18. Diameter of pili varies with their disposition on the supporting grid. Individual pili (A) may be 100 A in diameter. When the pili curve or kink, the diameter may be reduced to 80 A (B). A similar 80 A diameter is deduced from center-to-center measurements taken from laterally associated pili (C). × 125,000.

FIG. 19. At high magnification, pili may show (arrows) approximately 50 A periodicity along their length. × 250,000.

20 A in dimension. For this reason, it has been utilized to study the structure of gap junctions in animal systems (12). Because of the similarity of these structures to the zones of adhesion observed in gonococci, this technique was used to study in greater detail these intercellular zones by which gonococci adhere to one another.

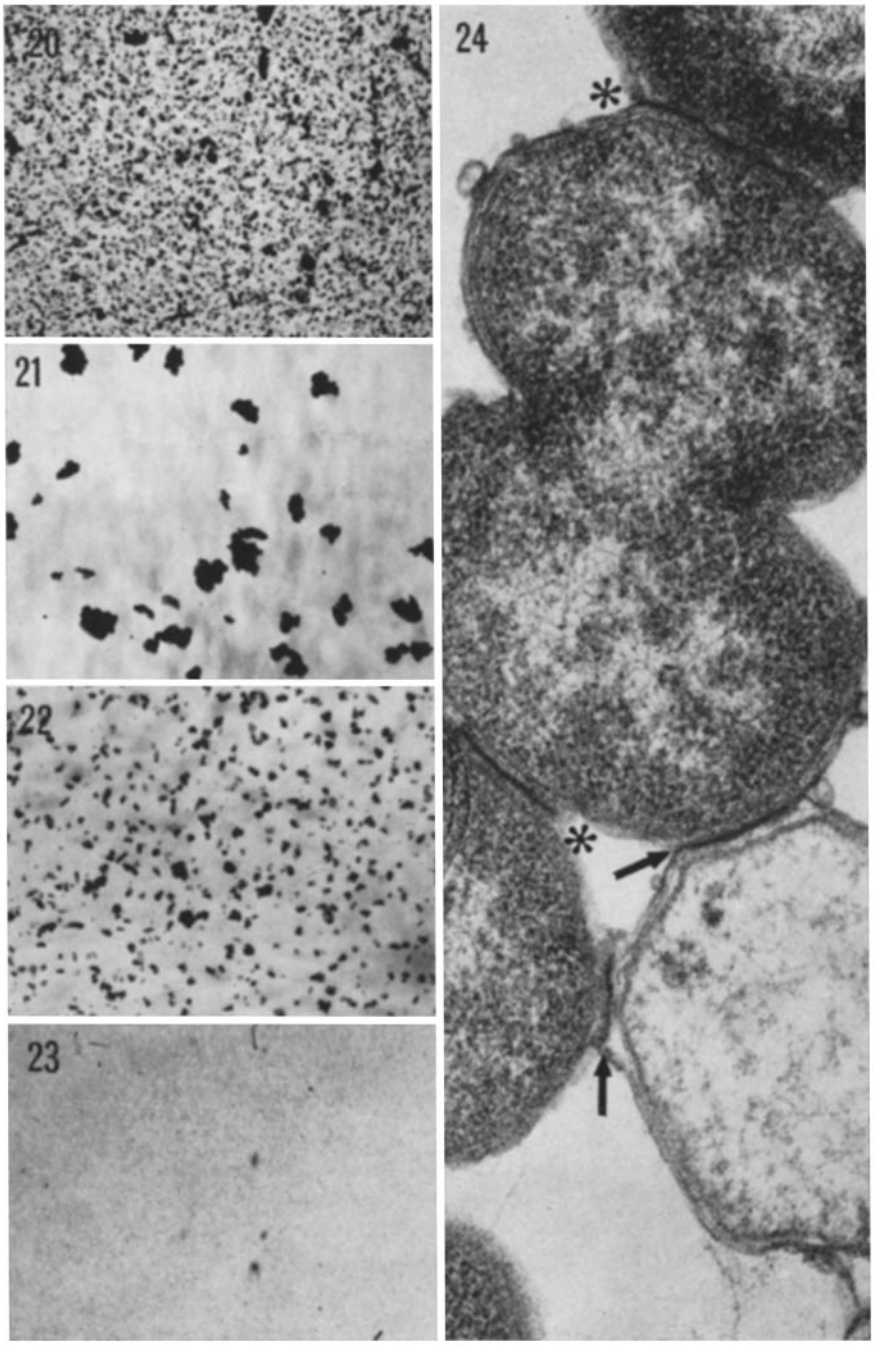
Unstained thin sections of gonococci fixed *in situ* in lanthanum-containing fixative (Figs. 25–28) show concentration of the electron-opaque colloid in patterns analogous to the intercellular space of the adhesion zones (Figs. 26–28). The width of the lanthanum deposit appears to be about 20 A although this measurement is not exact because of contribution of the outer leaflets of the cell walls' outer membranes to the density of the space. The amount of lanthanum present between cells varies with the portion of the intact colony that is examined. Near the colony's periphery and in its center, little lanthanum staining is observed. The former is probably because of loss of lanthanum during dehydration, whereas the latter can be ascribed to insufficient penetration of the colony by the lanthanum colloid. Lanthanum deposits are also seen in developing division septa (Figs. 27, 28). The spatial requirements for lanthanum retention are such that the marker is only retained where the outer membranes of adherent daughter cells, in the depths of the developing septum, are sufficiently closely opposed. In spite of extensive study of grazing sections, we have been unable to define arrays of subunits on the surfaces of membranes adjacent to lanthanum deposits. Thus, there does not seem to be the same type of membrane surface modifications present as in the gap junctions between animal cells (13).

DISCUSSION

Gonococci freshly isolated from the genitourinary exudate of patients with gonorrhea almost always grow as small colonies on agar (5, 14). These correspond to the colonial types 1 and 2 as defined by Kellogg (5) and are readily dis-

FIGS. 20–23. The four colony types of gonococci were examined by light microscopy of a thin layer (1–2 mm) of fluid cultures of the organisms. Type 1 gonococci (Fig. 20) exhibit moderate clumping consisting of cell aggregates of varying sizes. This correlates with slight to moderate autoagglutination by macroscopic examination. Marked clumping was present in the type 2 culture and is shown microscopically in Fig. 21. The type 3 specimen (Fig. 22) displays moderate clumping by microscopy and the culture was moderately clumped macroscopically. Type 4 organisms (Fig. 23) are evenly dispersed by microscopic examination and no aggregates are visible. This culture of type 4 organisms was homogenously suspended and no aggregates were visible macroscopically. × 180.

FIG. 24. Type 1 gonococci are adherent to one another through several zones of adhesion. Not only are cells whose morphology is entirely unremarkable joined by these zones (asterisks), but also gonococci that show different states of architectural intactness (arrows). The gonococcus in the lower right corner of the figure has lost much of its internal detail and is lysed. This disrupted morphology is in contrast to the intact, viable-appearing cells to which the lysed form is adherent. × 62,500.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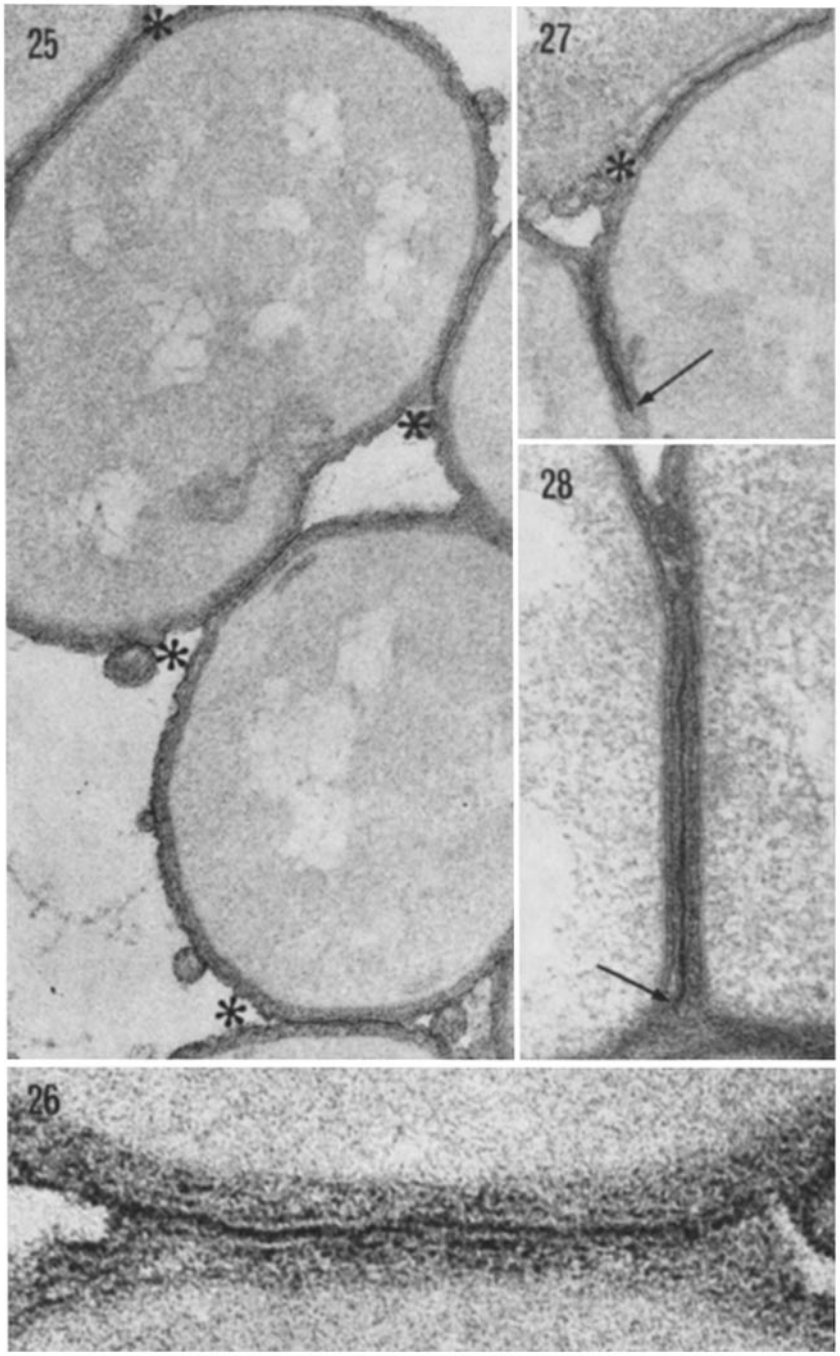
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tinguished from his type 3 and 4 gonococcal colonies which are considerably larger. In the course of this study all the primary isolates except 18R contained predominantly type 1 and type 2 gonococci. The single exception was strain 18R isolated from the rectum of a homosexual male and the predominant colonial type was type 4; however, it should be noted that it was possible to isolate from this strain colonies of the other 3 types by selective transfers on agar.

Kellogg (1) in a very important study has provided clear evidence that if the small colonial types are maintained in vitro through as many as 700 selective transfers the strain maintains its virulence for human volunteers. His results also indicate that the large colony types 3 and 4 have lost their virulence as demonstrated by their inability to produce infection in inoculated volunteers.

In the present study evidence has been provided that the main difference between the small colony types 1 and 2 and the large colony types 3 and 4 is that the former bear pili on their surfaces. This relationship between pilation and colony morphology has been seen in *Escherichia coli* (15) and in *Moraxella nonliquefaciens* (16), an organism capable of causing eye infections. In both instances, just as with the gonococcus, colony variants lacking pili appear upon subculture. Many Gram-negative bacterial species such as *Shigella*, *Salmonella*, *Klebsiella*, *Proteus*, and others have been shown to possess pili (15). These pili have been divided into several classes by morphological criteria such as diameter, length, and number of pili per bacterial cell (15). Brinton (17) has purified type 1 pili of *E. coli* and defined in great detail the structure and the chemistry of these. He found that they consisted of protein and that they could be dissociated into subunits of about 17,000 mol wt which he named pilin. Brinton has also in some instances defined the function of pili. They may serve as receptor sites for certain bacteriophages and, in the case of the F pilus, represent the organelle which transports the DNA from one cell to the other during bacterial mating (17).

To date little attention has been paid to the possible pathogenetic role of bacterial pili. Brinton (15) notes that approximately 90% of Gram-negative bacteria belonging to several species isolated from patients with urinary tract infections were pilated. Bovre et al. (16) have shown that strains of *Moraxella nonliquefaciens* recently isolated from clinical material tend to be piliated. To

FIGS. 25–28. Type 1 gonococci fixed in the presence of lanthanum nitrate display accumulation of the electron-opaque material in zones of adhesion (asterisks and in Fig. 26) and in developing division septa (arrows). The width of electron-opaque regions in adhesion zones between cells is 45–50 A and appears to be both the lanthanum in the space and portions of the outermost electron-opaque leaflets in the adjacent cell wall membranes which are poorly defined in these unstained sections. Lanthanum fills the division septa from the open, peripheral portion of the septum to the point (arrows) where the septal cell wall joins dividing cells. The widths of these spaces in the division septa are similar to those of lanthanum-filled regions of adhesion zones. × 62,500; × 250,000; × 62,500; × 100,000.

these examples one may now add that only those gonococcal colony types which are piliated are able to cause infection in human volunteers (1). At present there are inadequate data to define the mechanisms whereby pili may alter the host-parasite relationship. One possibility is that they hinder phagocytosis of the bacteria by the host's leukocytes. The role of the pili would then be similar to bacterial capsules (18) or to streptococcal M protein (19). The recent study of Thongthai and Sawyer (20) suggesting that type 1 are more resistant than type 4 gonococci to phagocytosis by human leukocytes would be in line with this hypothesis. Another hypothetical manner in which pili could influence pathogenicity is by altering the adherence of the bacteria to the epithelial cells of the host. Most pili display "stickiness" for other pili, for polystyrene particles, and for red cell membranes (15). Although to date it has not been possible to demonstrate hemagglutination by gonococcal pili, these structures do have a strong tendency to laterally aggregate and to bind membrane-limited vesicles probably consisting of the endotoxin of this organism. An enhanced ability of an organism to attach to epithelial cells might be necessary to start colonization of a site such as the eye or the urinary tract which is subject to continuous or periodic flushing and cleansing.

The pili of other bacterial species have been shown to be antigenic. Gillies and Duguid (21) have shown that the pili of several *Shigella* strains are antigenically indistinguishable and that they cross-react to some extent with pili of *E. coli* but not with pili of several other species. No information is available as yet on the antigenic structure of gonococcal pili, and it is impossible to determine whether any of the previous antigenic analyses of the gonococcus have dealt with pili. Studies are in progress to determine whether a single or many antigenic types of gonococcal pili exist and to what extent they cross-react with pili of other bacterial species.

Another phenomenon which was encountered in the course of this study was that different strains of gonococci autoagglutinated to varying degrees when grown in liquid media. Investigation of this autoagglutination phenomenon during growth indicated that it was correlated with the colonial morphology with type 2 having the most clumped growth followed by type 3, then type 1, and, finally, type 4 which showed little, if any, evidence of macroscopic clumping. This pattern held true in two other liquid media which were tested and for buffer suspensions of organisms grown on solid medium.

Type 4 gonococci, probably because of the smooth growth, do grow better than the other types in liquid media. This may help explain some of the results previously obtained with liquid media. The success with these media was variable; good growth was reported with complex (22) or defined (11) media, and granular or filamentous growth occurred with two other complex media (23). The qualitative differences in growth may have been because of the use of different colony types for the inoculum. The previous observation of

granular or filamentous growth occurred when the inocula were only several in vitro passages from the patient (23), a time when most of the colonies would by types 1 and 2 (5, 14). Conversely, the previously reported good growth of gonococci in liquid media occurred with laboratory strains that had been passed for periods of 2 wk to 32 yr (1, 23). Unselected serial passage of this duration would eventuate in a predominance or pure culture of type 4.

Because of the "sticky" nature of pili previously mentioned, their role in promoting clumping needs to be considered. The fact that type 3 gonococci grow clumped but are not piliated precludes ascribing all gonococcal autoagglutination to the presence of pili. Rather, the clumping of gonococci in liquid medium appears to be mediated by zones of adhesion on the gonococcal cell walls. It is possible that the presence of pili may accentuate the clumping as suggested by the finding that type 2 organisms consistently bear the greatest number of pili and show the most marked clumping. On the other hand, clumping may favor the development of pili.

Zones of adhesion exhibit the same intercellular space seen in gap junctions of animal cell systems. This has been demonstrated by conventional methods of electron microscopic study as well as by the experiments utilizing lanthanum nitrate. We have been unable to demonstrate any morphological counterpart to the geometric arrays of subunits seen in classical gap junctions. Additional freeze-etch and freeze-cleavage studies may reveal surface alterations in the zones of adhesion.

Lanthanum nitrate also fills the early division septa formed by the invagination of cell wall and cytoplasmic membrane components. The lanthanum colloid fills the extracellular portion of the septum to the point at which cell wall is shared by the developing daughter cells. These findings suggest a relationship between the zones of adhesion and the division septa. One might argue that zones of adhesion are merely views of division septa in thin sections which do not include the still incomplete central portions of the septa. Several lines of evidence suggest that this is not the most probable explanation. The zones of adherence seen in all, except type 4 gonococci, seem more numerous than could be explained on the basis of division planes alone. Furthermore, to explain the scarcity of zones of adhesion in type 4 gonococci, it would be necessary to postulate that division planes are rapidly completed, whereas in the other types the septa are frequently incomplete leaving the cytoplasm of the daughter cells in communication. This postulate is rendered untenable by the fact that often a morphologically normal cell is connected by means of a typical zone of adhesion to a lysed gonococcal cell. If the zones of adhesion are related to former division planes these septa are quite mature and between cells that are physiologically separated.

We suspect that the zones of adhesion represent specialized regions of stickiness of the gonococcal cell walls. Recent evidence has shown that the cell-to-cell

adherence can be abolished by ultrasonic perturbation without compromising the structural integrity or viability of the gonococci.[2] It will be of interest to investigate whether the regions of stickiness can be demonstrated after ultrasonic disaggregation. If these regions of the gonococcal cell wall retain their sticky character and can become reassociated with one another, they may also become adherent to human epithelial cells by the same mechanism. Further, it will be of interest to determine whether the zones of adhesion are found in other bacterial species or whether their presence in gonococci in unique.

SUMMARY

The four colony types of several different strains of gonococci were isolated by selective transfers on agar. These colony variants differed in the degree of autoagglutination which occurred when they were grown in fluid medium. It was found that this clumping behavior was related to the colonial type, with type 2 isolates exhibiting the greatest autoagglutination followed by types 3, 1, and 4.

Electron microscopic examination of thin sections indicated that the clumping in fluid medium was mediated by peculiar zones of adherence of the outer membranes of gonococci. These resembled the gap junctions seen in animal cell systems but differed in that the gonococcal membranes involved in the zone of adherence did not bear typical surface modifications.

Electron microscopic study of negatively stained specimens of gonococci revealed that pili with a diameter of approximately 85 A and a length up to 4 μ were present on the surfaces of all type 1 and type 2 gonococci examined, and were not seen on any type 3 or 4 gonococci. The consistent presence of pili on type 1 and type 2 gonococci which are virulent colony forms and the lack of pili on avirulent colony types 3 and 4 suggests a relationship between the gonococcal pili and pathogenetic potential of the organisms.

Note Added in Proof.—After preparation of this manuscript, two articles dealing with pili of *Neisseria spp.* have come to our attention. In one report, Jephcott et al. (Jephcott, A. E., A. Reyn, and A. Birch-Andersen. 1971. Brief Report: *Neisseria gonorrhoeae*. III. Demonstration of presumed appendages to cells from different colony types. *Acta Pathol. Microbiol. Scand. Sect. B.* **79**:437.) has noted extracellular "bundles of fibrils" in gonococcus type 1 and type 2 colonies. These fibrils were not seen in type 3 or type 4 colonies. Except for a slight difference in the reported diameters, the structures in the published micrographs are identical to laterally-aggregated pili demonstrated in the present paper. Wistreich and Baker (Wistreich, G. A., and R. F. Baker. 1971. The presence of fimbriae (pili) in three species of Neisseria. *J. Gen. Microbiol.* **65**:167.) have examined several *Neisseria spp.* by electron microscopy and have found pili in *N. catarrhalis*, *N. perflava*, and *N. subflava* all of which are generally considered to be nonpathogenic organisms.

[2] Swanson, J. Unpublished observations.

BIBLIOGRAPHY

1. Kellogg, D. S., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. 1968. *Neisseria gonorrhoeae*. II. Clonal variation and pathogenicity during 35 months *in vitro*. *J. Bacteriol.* **96**:596.
2. Thayer, J. D., and J. E. Martin, Jr. 1964. A selective medium for the cultivation of *N. gonorrhoeae* and *N. meningitidis*. *Pub. Health Rep.* **79**:49.
3. Greaves, R. I. N. 1960. Preserving living cells by freeze-drying. *Ann. N.Y. Acad. Sci.* **85**:723.
4. Frantz, I. D. 1942. Growth requirements of the meningococcus. *J. Bacteriol.* **43**: 757.
5. Kellogg, D. S., W. L. Peacock, Jr., W. E. Deacon, L. Brown, and C. I. Pirkle. 1963. *Neisseria gonorrhoeae*. I. Virulence genetically linked to clonal variation. *J. Bacteriol.* **85**:1274.
6. Swanson, J., and M. McCarty. 1969. Electron microscopic studies on opaque colony variants of group A streptococci. *J. Bacteriol.* **100**:505.
7. Doggenweiler, C. F., and S. Frenk. 1965. Staining properties of lanthanum on cell membranes. *Proc. Nat. Acad. Sci. U.S.A.* **53**:425.
8. Fritz-James, P. 1964. Thin sections of dividing *Neisseria gonorrhoeae*. *J. Bacteriol.* **87**:1477.
9. Kellogg, D. S., E. M. Turner, C. Callaway, L. Lee, and J. E. Martin, Jr. 1971. Particulate fractions of *N. gonorrhoeae*. *Infec. Immunity.* **3**:624.
10. Swanson, J., and I. Goldschneider. 1969. The serum bactericidal system: ultrastructural changes in *Neisseria meningitidis* exposed to normal rat serum. *J. Exp. Med.* **129**:51.
11. Kenny, C. P., F. E. Ashton, B. B. Diena, and L. Greenberg. 1967. Chemically defined protein-free medium for the cultivation of some species of *Neisseria*. *Bull. World Health Organ.* **37**:569.
12. Revel, J. P., and M. J. Karnovsky. 1967. Hexagonal array of subunits in intercellular junctions of the mouse liver and heart. *J. Cell Biol.* **33**:C7.
13. Goodenough, D. A., and J. P. Revel. 1970. A fine structure analysis of intercellular junctions in the mouse liver. *J. Cell Biol.* **45**:272.
14. Sparling, P. F., and A. R. Yobs. 1967. Colonial morphology of *Neisseria gonorrhoeae* isolated from males and females. *J. Bacteriol.* **93**:513.
15. Brinton, C. C. 1967. Contributions of pili to the specificity of the bacterial surface and a unitary hypothesis of conjugal infections heredity. *In* The Specificity of Cell Surfaces. B. D. Davis and L. Warren, editors. Prentice-Hall, Inc., Englewood Cliffs, N.J. 37.
16. Bovre, K., T. Bergan, and L. O. Froholm. 1970. Electron microscopical and serological characteristics associated with colony type in *Moraxella nonliquefaciens*. *Acta Pathol. Microbiol. Scand. Sect. B.* **78**:765.
17. Brinton, C. C., Jr. 1965. The structure, function, synthesis and genetic control of bacterial pili and a molecular model for DNA and RNA transport in Gram negative bacteria. *Trans. N.Y. Acad. Sci.* **27**:1003.
18. Wood, W. B., Jr., and M. R. Smith. 1949. The inhibition of surface phagocytosis by the "slime layer" of pneumococcus type III. *J. Exp. Med.* **90**:85.

19. Lancefield, R. C. 1957. Differentiation of group A streptococci with a common R antigen into three serological types with special reference to the bactericidal test. *J. Exp. Med.* **106**:525.
20. Thongthai, C. F., and W. D. Sawyer. 1971. Resistance to phagocytosis and colonial morphology of *Neisseria gonorrhoeae*. *Bacteriol. Proc.* **71**:110. (Abstr. M272).
21. Gillies, R. R., and J. D. Duguid. 1958. The fimbrial antigens of *Shigella flexneri*. *J. Hyg.* **56**:303.
22. Stokinger, H. E., H. Ackerman, and C. M. Carpenter. 1944. Studies on the gonococcus. I. Constituents of the cell. *J. Bacteriol* **47**:128.
23. Lazor, M., M. Gavrilescu, I. Porojan, and T. R. Circiumarescu. 1969. A new medium for culturing gonococci. *Dermato-Venerologia*. **14**:47.