Nonpilar Surface Appendages of Colony Type 1 and Colony Type 4 Gonococci

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The nature and distribution of surface appendages morphologically distinct from pili were examined in three strains of gonococci. As reported previously by others, large appendages were seen emanating from colony type 1 gonococci when negatively stained preparations were examined by electron microscopy. Whereas the diameter of pili was 4 to 6 nm, the appendages varied from 8 to 40 nm in diameter with bulbous enlargements of up to 130 nm. However, in contrast to previous findings, the appendages were not limited to virulence-associated colonial types but occurred with almost equal frequency on organisms from colonial types 1 and 4. Thus, the presence of these appendages on gonococci was unrelated to colonial morphology, and there is currently no reason to associate them with gonococcal virulence. The large appendages were seldom seen if the organisms were suspended in isotonic fluid rather than in distilled water, which was used in previous studies. This suggests that the large appendages may occur infrequently in the isotonic environment of tissues or in slightly hypertonic purulent exudates. Nevertheless, we did observe small appendages or cell wall blebs on the surface of gonococci suspended in isotonic medium and noted similar structures as spheres free in the surrounding milieu. These findings suggest that the material of which the large appendages consist may form small appendages or cell wall blebs and be shed into the milieu under isotonic conditions. Such cell wall blebs have been demonstrated on meningococci and been shown to consist of endotoxin.

Despite increased research on gonococcal infections, the mechanisms by which gonococci are able to avoid host defenses and produce tissue damage are unknown. In 1963 Kellogg and colleagues (11) described four distinct types of colonies of *Neisseria gonorrhoeae* and later found that gonococci of colony types 1 and 2 were virulent when inoculated into the urethra of male volunteers, whereas those of colony types 3 and 4 were not (10). Subsequently, attention was given to surface structures which might be related to the virulence of gonococci. Swanson et al. (15) and Jephcott et al. (8) showed that gonococci of colony types 1 and 2 had pili on their surfaces whereas those of colony types 3 and 4 lacked pili. Therefore pili, like colony type 1 and 2 morphology, were associated with virulence for humans. More recently, Grimble and Armitage (7) reported that large surface appendages, distinct from pili, occurred on the surface of colony type 1 but not colony type 4 gonococci. Therefore these surface appendages, like pili and type 1 or 2 colonial

morphology, were associated with virulence. Interest in nonpilar surface structures of gonococci was stimulated further by Gibbs and Roberts (5), who reported nonpilar surface factors that appeared during the log-phase growth of gonococci in the allantoic cavity of chicken embryos and inhibited phagocytosis by human polymorphonuclear leukocytes.

We used the technique of Grimble and Armitage (7), as well as a method developed by us, to examine by electron microscopy the nonpilar surface appendages of gonococci and their relationship to colonial morphology.

MATERIALS AND METHODS

Microorganisms. Colony types 1 and 4 of strains 192A, 2686, and F62 of *N. gonorrhoeae* were used.

Cultivation of microorganisms. Gonococci were grown on ordinary chocolate agar with X and V enrichment added (PLS Manufacturing Co.) and on a clear medium consisting of GC agar base (Difco) plus 2% (vol/vol) IsoVitaleX (BBL). Colonies of specific morphology were picked under direct observation through a dissecting microscope with incident

light as described by Kellogg et al. (10). Inocula consisting of three or more colonies were streaked over the surface of agar plates, which were incubated at 37 C in 2% CO₂ in air. After 18 to 22 h of incubation, the organisms were scraped from the tops of colonies of typical morphology with a platinum loop under direct observation through a dissecting microscope.

Techniques of negative staining. Specimens for examination by electron microscopy were negatively stained by two different methods. The first method was that of Grimble and Armitage (7). Gonococci scraped from plates with a platinum loop were gently agitated for 20 s in 5 drops of distilled water. One drop of 3% (wt/vol) potassium phosphotungstate, pH 6.0, was added, and the mixture was transferred immediately to a Formvar-carbon-coated grid, which was kept still for 30 s before the fluid was blotted from the edge with filter paper, leaving the grid with a dry appearance. The entire procedure took approximately 80 s.

The second procedure for negative staining was performed as follows. Organisms scraped from the tops of colonies growing on agar plates were dis-

persed in a drop of Eagle minimal essential medium containing Earle salts and 0.05 M *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (pH 7.45) (HEPES-MEM). A Formvar-carbon-coated grid was inverted and floated on the surface of a drop of the organism suspension for 1 min. It was blotted from the edge with filter paper and floated for 2 min on the surface of a drop of 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. The grid was again blotted from the edge with filter paper and floated for a few seconds in each of two drops of distilled water. After the grid had been blotted, it was next floated on a drop of 1% sodium phosphotungstate, pH 6.0, for 20 s. It was then blotted thoroughly around the edge and allowed to dry. The grids were examined with a Phillips 300 electron microscope at 60 kV.

Technique of examination. The preparations were examined at approximately $\times 25,000$ magnification for the presence or absence of pili and other surface appendages. Surface structures were considered to be "large appendages" if they emanated from the organism and extended from the surface for a distance equal to or greater than at least half the diameter of the organism. These projections were

FIG. 1. Morphology of gonococcal colonies designated colony type 1 (a) and colony type 4 (b). *N. gonorrhoeae* 192A viewed with incident light, $\times 50$ magnification.

studied not only for their morphology but also for their frequency of occurrence. As the grid was moved in one direction, the first 50 organisms encountered were examined and the number that had one or more appendages was noted.

RESULTS

Colony types of gonococci. Colonies designated type 1 were refractile with typical high-domed configuration, whereas those designated type 4 were larger in diameter and low-convex and lacked refractivity (Fig. 1).

Characteristics of large appendages. Exam-

ination of gonococci negatively stained by the method of Grimble and Armitage (7) revealed large appendages that were distinct from pili (Fig. 2a and c). These appendages were occasionally broken off, unattached to microorganisms. Whereas the diameter of pili was uniformly 4 to 6 nm, the diameter of the appendages varied from 8 to 40 nm but most often was about 20 nm. Their length varied from approximately 0.5 to 5 μm . Swellings or beads, which occasionally occurred along the length of the appendages or as terminal bulbs, varied from 60 to 130 nm in diameter (Fig. 2a and c). The

FIG. 2. Electron micrographs of negatively stained preparations of *N. gonorrhoeae* 2686. (a) Preparation made from type 1 colonies by the method of Grimble and Armitage, showing pili (P) and large appendages (LA) with beading and terminal bulbs. Note also small structures resembling pieces of appendages unattached to organisms. (b) Organisms of type 1 colonies from the same culture plate as those in (a) but prepared by the method of McGee et al. Note lack of large appendages but, as in (a), the presence of pili and small spherical structures. (c) Colony type 4 organisms lacking pili but having large appendages with beading and terminal bulbs. Preparation by the method of Grimble and Armitage. (d) Colony type 4 organisms from the same culture plate as those in (c) but prepared by the method of McGee et al. Note the lack of both pili and large appendages. Negatively stained, $\times 32,000$.

appendages usually occurred singly or in pairs (Fig. 2a), but occasionally an organism was seen which had as many as 5 to 10 appendages emanating from its surface (Fig. 2c).

Relationship of appendages to gonococcal colony type. Because Grimble and Armitage (7) reported that "large appendages" were seen on organisms from type 1 colonies but were not seen on organisms from type 4 colonies, we quantitated the proportion of organisms from type 1 and type 4 colonies that had large appendages. With all three gonococcal strains tested, the proportion of organisms that had large appendages was about the same whether they were from colonies of type 1 or type 4 (Table 1). This was true whether the organisms were stained by the method of Grimble and Armitage, when approximately 25 to 50% of them had large appendages, or by the method of McGee et al., when only a few organisms had large appendages. Furthermore, the use of organisms grown on chocolate agar or the use of 3% sodium phosphotungstate at pH 6.0 or 7.4 with the technique of Grimble and Armitage did not affect the appearance of the large appendages on gonococci of colony type 1 and colony type 4.

Effect of suspending fluid on the occurrence of appendages. Although the large appendages occurred in negatively stained preparations made by the method of Grimble and Armitage (7), they seldom occurred in preparations made by the method of McGee et al. (Table 1). It is of note that with the former method the gonococci are suspended in distilled water, whereas with the latter method they are suspended in an isotonic fluid, HEPES-MEM. Although we used organisms from chocolate agar

rather than clear agar, interchanged sodium and potassium phosphotungstate at pH 6.0 and 7.4 in the two staining techniques, and omitted the glutaraldehyde fixation in the technique of McGee et al., the large appendages were seen frequently only when the organisms were suspended in distilled water.

In preparations made by either technique, small appendages or cell wall blebs were seen on the surface of the gonococci. Similar structures, which were usually spherical, were also seen unattached to microorganisms in preparations made from type 1 or type 4 gonococci. These spheres, which varied from approximately 10 to 30 nm in diameter, had the appearance of pieces of large appendages.

DISCUSSION

In the current studies, approximately 25 to 50% of the organisms seen in negatively stained preparations of three gonococcal strains had large appendages emanating from the cell surface. The appendages varied in width, had occasional areas of beading along their length, and sometimes ended in a terminal bulb. These features clearly distinguish the appendages from flagellae or pili (4, 8, 14, 15).

Grimble and Armitage (7) reported that they found large appendages in negatively stained preparations of colony type 1, "virulent" gonococci, but not in preparations of colony type 4, "laboratory" strains. Their findings, if confirmed, would indicate that the large appendages, like pili, were associated with gonococcal virulence. However, we repeatedly found that gonococci from type 4 colonies had appendages as frequently as organisms from type 1 colonies. Furthermore, the morphological appearance of appendages and their number on individual organisms was the same for gonococci from type 1 and type 4 colonies. This was true with two well-known laboratory strains, F62 and 2686 (2, 11), and a more recent clinical isolate, 192A (12). Neither variation in the medium used to grow the organisms nor in the negative stain affected the appearance of the appendages on organisms from type 4 colonies. Therefore it is difficult to understand why the results of this study differ from those of Grimble and Armitage. In this regard it is of note that the "pathogenic" gonococci in some of their electron micrographs lacked pili. This suggests that these gonococci, which had appendages, may have come from colonies with type 4 morphology. Although the finding of appendages on nonpilated, colony type 4 gonococci does not exclude a role for these structures in the pathogenesis of gonococcal infections, there is currently no particular reason to relate them to the ability of

TABLE 1. Frequency of large appendages^a among gonococci of colony types 1 and 4 negatively stained by two methods

<i>N. gonorrhoeae</i>		Organisms (%) with large appendages after staining by the method of:	
Strain	Colony type	Grimble and Armitage	McGee et al.
192A	1	34 ^b	0
192A	4	42	2
2686	1	28	4
2686	4	38	2
F62	1	54	6
F62	4	48	8

^a An organism was considered to have large appendages if one or more projections emanated from its surface and extended for at least half the diameter of the organism.

^b Percentage of 50 organisms examined at $\times 24,700$ magnification.

gonococci to cause disease.

A more pertinent question raised by these studies is whether the development of large appendages is strictly a laboratory phenomenon. The appendages were frequently seen if the gonococci were suspended in distilled water prior to negative staining, as in the method of Grimble and Armitage, and bring to mind the bizarre forms produced by osmotic hemolysis of erythrocytes (1). The large appendages were seldom seen if the organisms were suspended in isotonic fluid, which suggests that their formation depends on the suspending medium having a low osmolality. Therefore, the large appendages may occur infrequently, if at all, in tissues where the milieu is isotonic or in purulent exudates, which are slightly hypertonic.

The physicochemical nature of the large appendages on gonococci is unknown. They seem to be related to the outer surface of the organisms where most of the endotoxin lipopolysaccharide is thought to reside (6, 9, 13). Small appendages or "cell wall blebs" have been described on the outer surface of *N. meningitidis*. These blebs, which were also seen free in the medium, were shown by chemical analysis to contain endotoxin (3). Similar cell wall blebs were seen on the surface of gonococci and free in the isotonic medium in negatively stained preparations in the present studies. They have also been observed upon thin-section electron microscopy of gonococci infecting human fallopian tubes in organ culture (unpublished data). These findings suggest that, although large appendages may form infrequently in vivo, the material of which they consist, perhaps endotoxin, may form cell wall blebs which, in vivo, are shed from the surface of gonococci as small spheres.

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