Effect of different preparation procedures on the ultrastructure of gonococci

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SUMMARY Culture samples of *Neisseria gonorrhoeae* and *Escherichia coli* were studied by transmission and electron microscopy to evaluate the effect of different preparation procedures on the piliation of these organisms. Unfixed as well as fixed bacteria showed only few, long, filamentous appendages when investigated in ultrathin sections, negatively stained specimens, or critical-point dried preparations. Snap-frozen specimens of *E. coli* showed many short and thin pili after being shadowed with carbon and platinum whereas those of *N. gonorrhoeae* showed only some type-C-like pili. Thus, the number and morphological appearance of pili appear to be greatly influenced by the preparation techniques used for study by electron microscopy. Conclusions as to the type and the infectivity of a bacterial strain can, therefore, not be based on purely morphological criteria.

Introduction

The physical, chemical, and adhesive properties of pili in *Enterobacteriaceae* have been well documented in the past (Brinton, 1965; Duguid *et al.*, 1966, 1969). Consequently, it has been recognised that pili or morphologically similar structures occur in a wide range of Gram-negative organisms.

Many investigators have studied the surface of *Neisseria gonorrhoeae* in recent years. The presence of pili on Kellog type 1 and type 2 gonococci as well as their absence on other non-virulent types (Jephcott *et al.*, 1971, Swanson *et al.*, 1971) have stimulated discussion and theories. Recently, Haustein and Uehrlings (1976) suggested that non-piliated gonococci were important epidemiologically, since they had found non-piliated organisms in 20% of asymptomatic cases of gonorrhoea. The negative-staining technique had been used in these investigations. Using the same technique, however, we have seen differences in the number and in the appearance of pili—even in samples from the same culture—depending on the dye applied for the negative-staining.

We have therefore carried out a controlled study on gonococcal pili using different preparation procedures but following the same two-day culture techniques as Haustein and Uehrlings (1976) had used in their study.

Material and methods

Two-day-old cultures of gonococci cultured on Thayer-Martin agar and one-day-old cultures of *Escherichia coli* cultured on Bouillon agar were used for this study. For preparations of the bacteria the following techniques were used simultaneously:

- (1) Small pieces of agar with bacterial colonies were fixed with buffered glutaraldehyde and osmium tetroxide (OsO_4), dehydrated with alcohol, and embedded in Epon 812.
- (2) Gonococci and coliform bacilli grown on culture plates were placed in glutaraldehyde with a platinum loop, gently centrifuged, postfixated with OsO_4 , and embedded in Epon 812. Ultrathin sections of cultures by both methods were stained with uranyl acetate and lead citrate and examined in a Philips EM 200 electronmicroscope.
- (3) Glutaraldehyde-fixed and unfixed bacteria were placed on Formvar-coated electronmicroscope (EM) grids and stained with uranyl acetate or phosphotungstic acid (PTA). To place the bacteria on the EM grids two

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different techniques were used: (a) bacteria were suspended in water, Ringer solution, or glutaraldehyde and a drop of these suspensions was allowed to dry on the grids; and (b) the coated EM grids were directly put on the bacteria growing on the culture plate and after being dried, stained, and carefully washed in distilled water the preparations were examined in a Zeiss EM 9 electron-microscope.

- (4) For scanning electronmicroscopy the critical-point drying method described by Fromme *et al.* (1972) was used. Bacteria were fixed by suspension in glutaraldehyde. After filtration of this solution through a millipore filter the bacteria remaining on the filter were dehydrated with alcohol and transferred in Freon 11 and then in Freon 13 (pressure 39.36 mm Hg, temperature 28.8°C). After drying and decompression the bacteria were sprayed with carbon and gold and examined in a Cambridge scanning electronmicroscope.
- (5) EM grids with unfixed bacteria on the Formvar film were frozen in liquid nitrogen. These preparations were dried in a modified Leybold EPA 100 freeze-etching machine at -90°C and a pressure of about 2×10^{-7} millibars. After complete drying the grids were shadowed with a thin layer of carbon and platinum at -110°C and 2×10^{-7}

millibars. An angle of 15°C was chosen for the coating procedure in order to achieve sufficient contrast. Investigation of these specimens was performed in a Philips EM 200 electronmicroscope.

Results

TRANSMISSION ELECTRONMICROSCOPY OF ULTRATHIN SECTIONS

Between vacuolised and autolytical cells and cell membrane fragments well-preserved gonococci or coliforms could be seen. It was very difficult or nearly impossible in our preparations, however, to trace a long and thin, more or less unorientated filament like a pilus in an ultrathin section. Some osmiophilic filamentous structures or fragments of filaments could be seen extending between the cells or originating from their outer surface (Fig. 1a, b).

TRANSMISSION ELECTRONMICROSCOPY OF NEGATIVELY STAINED PREPARATIONS

With negatively stained preparations nearly identical results could be seen after the sections had been stained with uranyl acetate or with phosphotungstic acid. In the preparations of gonococci usually 20%–30% of the cells had long filamentous appendages corresponding to type A pili (Fig. 2a). These pili mainly stood in bundles and had a

Fig. 1 Transmission electronmicroscopy of ultrathin sections of (a) gonococci and (b) coliform bacilli fixed with glutaraldehyde and osmium tetroxide: (a) some thin filaments are visible extending from the outer surface of one organism to neighbouring bacteria ($\times 65\,000$ magnification); (b) several pearl-string-like fibres originate from the outer cell wall ($\times 52\,000$ magnification)

Fig. 2 Unfixed gonococci (a and b) and glutaraldehyde-fixed coliforms (c and d) negatively stained with PTA: (a) numerous long bundles of thin filaments are present around the organisms ($\times 24\,000$ magnification); (b) a number of parallel membranes arranged in bundles is visible ($\times 100\,000$ magnification); (c) well-defined, long, curved pili are present ($\times 25\,000$ magnification); (d) cross-overs and ramifications of pili are shown ($\times 140\,000$ magnification).

diameter of about 10 nm and a length of several μm . The same preparations frequently also exhibited bundles of parallel membranes along these aggregates of pili, which, because of their thinner diameter, corresponded to structures described as type B pili (Fig. 2b).

Simultaneous preparations from pilated *E. coli* strains also showed very long pili. They were curved and irregularly arranged; at a higher magnification they showed some crossovers (Fig. 2c, d).

SCANNING ELECTRONMICROSCOPY OF FREEZE-DRIED PREPARATIONS

With scanning electronmicroscopy the critical-point dried preparations of gonococci showed no recognisable pili. Only rarely were some thin fibres seen extending from one organism to a neighbouring one (Fig. 3) similar to the structures present in the ultrathin sections.

TRANSMISSION ELECTRONMICROSCOPY OF SHADOWED PREPARATIONS

Finally, in the platinum-carbon shadowed preparations of the samples mentioned above no type A pili could be detected at all (Fig. 4a). Only a few cells showed single long pili with a knob at the end (Fig. 4b). These structures have been described as type C pili (Novotny and Turner, 1975). Most of the gonococci had no recognisable pili, however,

Fig. 3 Scanning electronmicrograph of critical-point dried gonococci on millipore filter; only a few filamentous structures are present between the cells ($\times 19\,000$ magnification)

but only a pronounced outer membrane (Fig. 4a). In preparations of *E. coli* more very short and thin pilar structures could be seen all over the surface of the cells (Fig. 5a, b). These were also seen wrapped around the cells and, occasionally, at some distance from the membrane (possibly after having lost their contact with the cell itself).

Discussion

Our investigations into the comparative effects of different preparation procedures on the ultrastructural appearance of gonococci and coliform bacilli gave varied results. Transmission electronmicroscopy of ultrathin sections gave the impression that only occasionally was a pilum tangentially cut, but it was never possible to follow these structures over a longer distance. Thus, this technique itself is not suitable for the evaluation of pilar structures at all. Scanning electronmicroscopy of critical-point dried material was also not satisfactory in our preparations.

Negative-staining mainly produced long bundles of type A pili, after staining of gonococci and coliforms with phosphotungstic acid and uranyl acetate. The same cultures, however, exhibited only type B and C pili or even no pili when examined after freeze-drying and shadowing with platinum.

These discrepancies in the number and morphological appearance of pili indicate strongly that the pilation of both *N. gonorrhoeae* and *E. coli* is greatly influenced by the mode of preparation. Similar variations were seen by Duguid *et al.* (1966) when different culture conditions were applied. These authors discussed their findings in relation to the changing external factors and to ageing processes and the infectious potentials of the bacteria. Our results show that preparation conditions also play an important part in the ultrastructural appearance of the outer bacterial zone.

Conclusion

From these experiments and a consideration of the factors influencing the pilation we were not able to endorse the opinion that simple electronmicroscopical examination of negatively stained gonococci permits definite determination of the strain investigated. The fact that (according to Haustein and Uehrlings) 20% of asymptomatic cases of gonorrhoea are caused by non-piliated organisms does not necessarily indicate infection with type 3 or 4 gonococci. Too many factors influence the morphological appearance of pili, and many more compartments of the glycocalyx, besides the pili, are necessary to

build up a suitable environment for the multiplication of the bacteria and to form the aggregates called 'infection units' (Novotny *et al.*, 1975).

Electronmicroscopical examination of micro-organisms has made an important contribution to our present knowledge of bacterial structure, and

further progress is to be expected. Further standardisation of the preparation techniques, however, are necessary before typing and quantification of gonococcal pili can be used to determine the infectivity of the strain on a purely morphological basis.

Fig. 4 Carbon-platinum shadowed preparations of snap-frozen gonococci: (a) no pili can be recognised in this specimen ($\times 20\,500$ magnification); (b) a thick filament with a knob at its end seems to extend from the cell ($\times 51\,500$ magnification)

Fig. 5 Carbon-platinum shadowed preparations of snap-frozen coliform bacilli: (a) numerous, short and thin pili are visible ($\times 27\,000$ magnification); (b) numerous pili covering the surface are shown ($\times 61\,500$ magnification)

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