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Attachment of *Neisseria gonorrhoeae* to human sperm

Microscopical study of effect of trypsin and iron

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SUMMARY Pilated *Neisseria gonorrhoeae* of colony type 1 (T1) and non-pilated bacteria of colony type 4 (T4) were observed by transmission (TEM) and scanning electron microscopy (SEM). No pili were observed on T4 gonococci, but two types of pili—straight, type a, and bent, type b—were seen on T1 by TEM. When incubated with human sperm and examined by either TEM or SEM, T1 gonococci were seen to attach by individual pili, by several pili wound together as a rope, or by direct contact. Gonococci from T4 colonies attached only by direct contact. Treatment with typsin (1 mg/ml) damaged or removed pili from gonococci. After incubation with trypsin, attachment of pilated gonococci to sperm was decreased significantly, but such treatment did not affect attachment of non-pilated gonococci. Incubation of gonococci from either colony type in 0·1 mmol/l ferric nitrate, followed by incubation with sperm, significantly increased attachment of only T4 bacteria. No pili were seen on T4 gonococci treated with ferric ntrate; thus, it appears that factors other than pili alone are concerned in attachment of these gonococci to sperm.

Introduction

Occurrence of pili in the genus *Neisseria* was first reported by Wistreich and Baker (1971). *Neisseria* with pili caused haemagglutination, and scanning electron microscopy (SEM) showed that bacterial pili were adhered to the erythrocytes. Novotny *et al.* (1975) distinguished three types of pili on negatively stained *Neisseria gonorrhoeae*. He named these types a, b, and c, according to the occurrence, structure, and serological properties. The most common, type a, showed no tendency to form constant-angle, layered crystal aggregates and could be classified as type II or III according to the nomenclature of Brinton (1965, 1967). Another type composed of shorter units with structures usually bent and segmented was called b, and pili which showed small knobs at the end were designated type c.

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Kellogg *et al.* (1962, 1968) described four morphological types of *N. gonorrhoeae*. Colony type 1 (T1) and type 2 (T2) were isolated primarily from exudates of patients and type 3 (T3) and type 4 (T4) developed in laboratory strains. Virulence was associated with T1 and T2, since only these types caused gonorrhoea when inoculated into human volunteers (Kellogg and Thayer, 1969). Swanson *et al.* (1971) later demonstrated the presence of pili on *N. gonorrhoeae* from colony types 1 and 2 that were associated with virulence; pili were not present on the avirulent colony types 3 and 4. Gonococcal pili were purified (Buchanan *et al.*, 1973; Buchanan, 1975) and shown to exist in antigenically (Buchanan, 1975; Robertson *et al.*, 1977) and immunologically (Novotny and Turner, 1975) distinct groups.

The pathophysiology of gonorrhoea has been a puzzle to investigators. Many studies have shown that pili are important in the interaction of gonococci with cells of the potential host. Pili mediate interaction *in vitro* between gonococci and tissue cells (Swanson, 1973), urethral mucosal cells (Ward and Watt, 1972), red blood cells (Punsalang and Sawyer, 1973), leucocytes (Punsalang and Sawyer, 1973;

Swanson *et al.*, 1974), human epithelial cells (Punsalang and Sawyer, 1973), and human sperm (James-Holmquest *et al.*, 1974). Attachment of gonococci to human sperm *in vitro* (James-Holmquest *et al.*, 1974) was studied as a model for the host-parasite interaction. Such attachment *in vivo* could partly explain transmission of the bacteria and spread of the disease in the host.

Attachment of gonococci to human sperm is influenced by a variety of physical and chemical factors (James *et al.*, 1976). The enzyme, trypsin, reduced attachment of T1 bacteria to the level of T4, apparently by action on the proteins of pili. Iron salts, in contrast, enhanced attachment. In the present investigation, we examined by electron microscopy the interaction of gonococci with human sperm after treatment of the bacteria with either trypsin or iron salts.

Materials and methods

GROWTH AND STRAINS OF BACTERIA

Colony types 1 and 4 of *N. gonorrhoeae* strain WP were obtained from a clinical isolate stabilised in this laboratory after 30 transfers (James *et al.*, 1973). Cultures were incubated in candle jars for 18-20 hours at 35°C and were maintained by transfer on GC Agar Base Medium (Baltimore Biological Laboratories, Cockeysville, Maryland) plus 1% IsoVitaleX (BBL), a medium we term GCB. Colonies were typed by the method of Kellogg *et al.* (1968) using oblique illumination from beneath the Petri dish. Stocks of *N. gonorrhoeae* were frozen in a solution of four parts of Trypticase Soy Broth (BBL) to one part of glycerol (Mallinckrodt Inc., Paris, Kentucky) and stored at −60°C.

Isolated T1 and T4 colonies of gonococci were picked from the agar surface and suspended in either of two buffered solutions. Alsever's solution (AS) was composed of dextrose, 2·05 g; sodium citrate ($Na_3C_6H_5O_7 \cdot 2\ H_2O$), 0·80 g; NaCl, 0·42 g; and citric acid ($H_3C_6H_5O_7 \cdot 2\ H_2O$), 0·55 g in 100 ml of deionised water (Alsever and Ainslie, 1941). Ringer's solution (RS) contained NaCl, 8·6 g; KCl, 0·3 g; and $CaCl_2 \cdot 2\ H_2O$, 0·33 g per litre of deionised water (Diem and Lentner, 1970). No significant difference in attachment of gonococci to sperm was found when either of the two buffers was used. AS was used in most of the work because better results were obtained in electron microscopy studies, especially when uranyl acetate (UA) was used as a stain.

For experiments, bacterial suspensions were mixed on a Vortex mixer (Vortex Genie Mixer, Scientific Products, Evanston, Illinois) to obtain an even distribution of cells. The concentration of bacteria per ml was determined in a Petroff-Hausser chamber (Hausser Scientific, Blue Bell, Pennsylvania); then suspensions were diluted to achieve a final concentration of 2×10^8 organisms per ml.

PREPARATION OF SPERM

Samples of human semen with normal sperm counts were provided by the immunology laboratory of the obstetric and gynaecology department of Baylor College of Medicine, Houston Texas. A pool of seminal fluid was diluted in RS or AS and centrifuged at 1500 × *g* (Model CL, International Equipment Co., Needham Heights, Maryland) for five minutes. The supernatant fluid was decanted, and more RS or AS was added to the pellet of sperm. This suspension was centrifuged again for five minutes and was finally resuspended in AS or RS. The number of washed sperm per ml was determined with a haemocytometer (Spencer Bright-Line American Optical, Instrument Division, Buffalo, New York) and dilutions were adjusted with the buffer solution to obtain a final concentration of 1×10^8.

MIXTURE OF BACTERIA AND SPERM

Equal volumes (0·5 ml) of suspensions of *N. gonorrhoeae* T1 or T4 (2×10^8/ml) and sperm (1×10^8/ml) were mixed and incubated for 15 minutes at 35°C in a waterbath shaker (Model 676, New Brunswick Scientific Co. Inc., New Brunswick, New Jersey). Samples were then processed for observation by either transmission electron microscopy (TEM), scanning electron microscopy (SEM), or light microscopy (LM).

USE OF PHYSIOLOGICAL AGENTS

The effects of adding trypsin and ferric nitrate to T1 and T4 organisms alone and in combination with sperm were determined. Trypsin from bovine pancreas type III (Sigma Chemical Co., St Louis, Missouri) was prepared as a stock solution of 10 mg/ml by dilution in water and stored at 0°C. A final concentration of 1 mg/ml was used with bacteria.

Ferric nitrate ($Fe(NO_3)_3 \cdot 9\ H_2O$; Fisher Scientific Co., Fairlawn, New Jersey) was prepared by dilution in water to 1 mmol/l, sterilised by filtration (Nalgene filter 0·20 μm, Nalge-Sybron Corp., Rochester, New York) and stored at 4°C in a light-proof container. A final concentration of 1 mmol/l was used in each test.

Both T1 and T4 gonococci were incubated separately for 15 minutes at 35°C with the agent to be tested. After this treatment, the bacteria were washed twice by centrifugation at 19 000 r/min (RC2-B, Sorvall Inc., Norwalk, Connecticut) for 15 minutes each and then suspended to a volume of 1 ml in RS or AS.

PREPARATIONS FOR ELECTRON MICROSCOPY

Scanning electron microscopy

Samples of bacteria, either alone or with sperm suspended in AS or RS, were placed on glass slides and exposed to 1% osmium tetroxide (OsO_4) vapours for 30 minutes. Specimens were dehydrated through 25, 50, 75, 95, and 100% ethanol (v/v) for five minutes each and amyl acetate (100%) for five minutes. The slides were transferred to a critical-point drying apparatus (CPD-1, Denton Vacuum Inc., Cherry Hill, New Jersey), where the amyl acetate was replaced by CO_2. The critical temperature and pressure changed the liquid to gas in about 30 minutes. This procedure avoided surface tension distortion (Anderson, 1951). The specimens were then coated with gold-palladium and examined with an ETEC scanning electron microscope (ETEC Corp., Hayward, California) with a 20-30° angle of tilt, at an accelerating voltage of 20 kV.

Transmission electron microscopy

Negative staining. Aqueous phosphotungstic acid (PTA) was used in one procedure. A grid coated with carbon-collodion was floated on top of a drop of sample in RS for one minute. The excess fluid was absorbed with blotting paper, and the grid was inverted on a drop of 1% PTA (pH 7·2) for one minute. The excess fluid was blotted up and the grids were allowed to dry in air.

Positive staining. A pseudoreplicative technique (Smith *et al.*, 1961) was used, in which one drop of sample in AS was placed on top of a 2·5-mm thick square of 2% (w/v) Noble agar (Difco Laboratories, Detroit, Michigan) on the end of a glass slide. The preparation was allowed to stand until dry. Samples were exposed to OsO_4 vapours for 15 minutes. The specimen was then covered with 0·75% (w/v) collodion, the excess collodion was drained off, and the preparation was allowed to dry in a vertical position. The preparation was gently immersed in UA (1-2%), whereby the collodion film with embedded organisms floated from the agar block and remained on the stain for two minutes. A carbon-collodion-coated grid was centred over the specimen. The grid was then removed by means of a small peg, and, by one continuous motion, the grid was brought out of the stain with the organisms now on the top. Excess fluid was drained off, and the preparation was allowed to dry in air before being examined in a Siemens 1A electron microscope at an accelerating voltage of 80 kV.

EVALUATION OF ATTACHMENT OF BACTERIA TO SPERM

Percentage of attachment of bacteria to sperm was evaluated by light and transmission electron microscopy. Statistical analyses showed no significant difference between the two methods.

For LM, clean slides were prepared. Three drops of the mixture of sperm and bacteria after incubation were pipetted on to the top of the slide and allowed to dry in air. The slides were then fixed in 95% (v/v) aqueous ethanol for seven minutes and rinsed with water. Slides were stained with crystal violet, 2 g in 20 ml of 95% ethyl alcohol (Piekarski and Ruska, 1939) for two minutes, rinsed with water, dried in air, and then examined with the oil immersion lens by LM. Several fields were examined until 100 sperm had been counted. The sperm with bacteria attached were enumerated and the percentage of attachment was calculated (James-Holmquest *et al.*, 1974).

For evaluation of attachment by TEM, several grids were stained by the UA method and were observed at a magnification of × 8000. One hundred sperm were examined. Sperm with or without bacteria attached were counted to calculate the percentage of attachment.

DETERMINATION OF PILI DIAMETER

The transmission electron microscope was first calibrated by means of a grating replica with a spacing of 462·9 nm. Micrographs of both the grating replica and pilated bacteria were taken at × 20 000 magnification. The thickness of pili was obtained by measuring the centre-to-centre width of pili lying side by side. Dimensions were calculated from the grating replica standard.

PERCENTAGE OF GONOCOCCI WITH PILI AND MEAN NUMBER OF PILI/BACTERIUM

T1 bacteria were obtained from pure cultures after incubation at 35°C for 20 hours and were suspended in AS to a concentration of 2×10^8/ml. Grids were prepared, stained with UA, and examined by TEM. A total of 84 bacteria was examined for the presence or absence of pili. The percentage of bacteria with or without pili was calculated. The number of gonococci with pili divided by the total number examined and multiplied by 100 gave the percentage with pili. The combined total number of pili divided by the number of bacteria from three experiments gave the mean number of pili/bacterium.

Results

Approximately 71·43% of T1 gonococci had pili (Table). The number of pili per bacterium varied considerably from as few as one to more than 40 with an average of 10 per gonococcus. The thickness of pili was 4·5-5·5 nm. Designation of pili was made according to different types observed in TEM. Straight, type a (Fig. 1) pili and bent, type b (Fig. 2)

Table *Mean number of gonococci with pili from T1 colonies and mean number of pili/gonococcus as determined by TEM*

Experiment no.	Total no. of gonococci	With pili		Without pili		Total no. of pili	Mean no. of pili/gonococcus (range)
		No.	%	No.	%		
1	37	25	67·57	12	32·43	239	9·56 (1-40)
2	14	10	71·43	4	28·57	92	9·20 (1-25)
3	33	25	75·76	8	24·24	272	10·88 (1-24)
Total	84	60	71·43	24	28·57	603	10·05

pili, as described by Novotny (1975), were observed. Spherical structures were seen but not at the end of pili (Fig. 2). Many of these structures appeared as isolated blebs that might be extensions of the bacterial membranes, as suggested by McGee *et al.* (1976).

ATTACHMENT TO SPERM

Pilated *N. gonorrhoeae* mixed with sperm and incubated for 15 minutes at 37°C showed various forms of attachment to sperm. The gonococci attached to sperm tails by several pili twisting together as a rope (Fig. 3a and 3b), by pili in a spiderweb arrangement around the bacteria (Fig. 4a and 4b), by direct contact alone or along with pili, and by a combination of pilus, ropelike bundles, and direct contact (Fig. 5). In some preparations, the tails of sperm appeared to be wound up by the pilated gonococci (Fig. 6). When non-pilated gonococci and sperm were observed, direct contact was the only type of attachment seen, whereas pilated organisms attached to sperm heads directly or by ropes of pili.

Statistically significant differences in attachment to sperm between gonococci from colony types 1 and 4 were observed (Fig. 7). The mean percentage of sperm with pilated organisms attached was

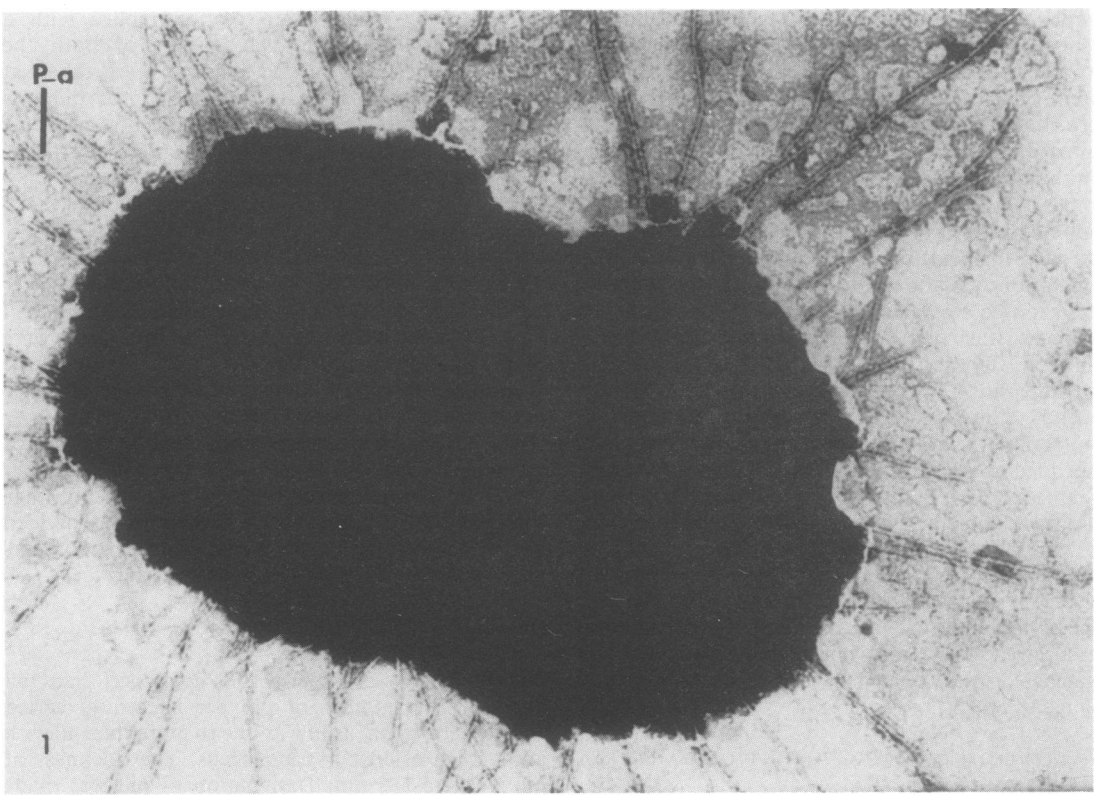


Fig. 1 *TEM micrograph of pilated* Neisseria gonorrhoeae *negatively stained by PTA showing pili of type a (P-a). (× 80 000 magnification).*

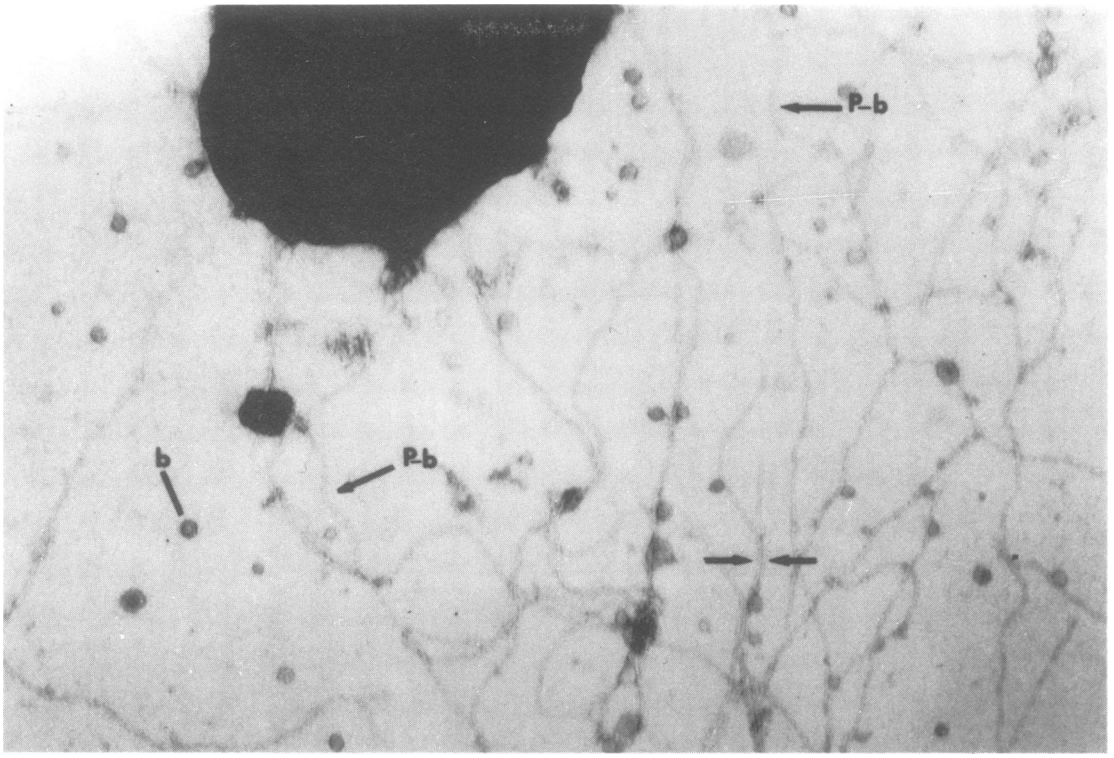
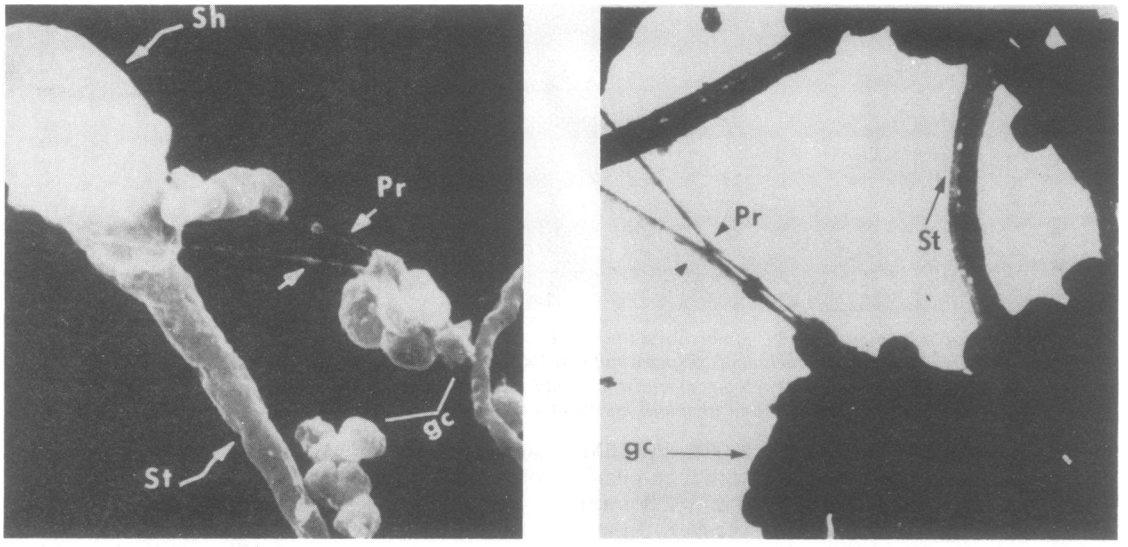


Fig. 2 *TEM micrograph of pilated* N. gonorrhoeae *negatively stained with UA. (× 31 000 magnification). Diameter of pili was obtained by measuring the distance from centre to centre of pili lying side by side (arrows). Pili type b (P-b) and blebs (b) are visible in this micrograph.*



(a) *(b)*

Fig. 3 *Micrographs of pilated* N. gonorrhoeae *attached to sperm by direct contact and by ropes of pili: (a) SEM micrograph (× 9000 magnification); (b) TEM micrograph (× 13 500 magnification). Arrows indicate sperm head (Sh), sperm tail (St), gonococci (gc), and ropes of pili (Pr).*

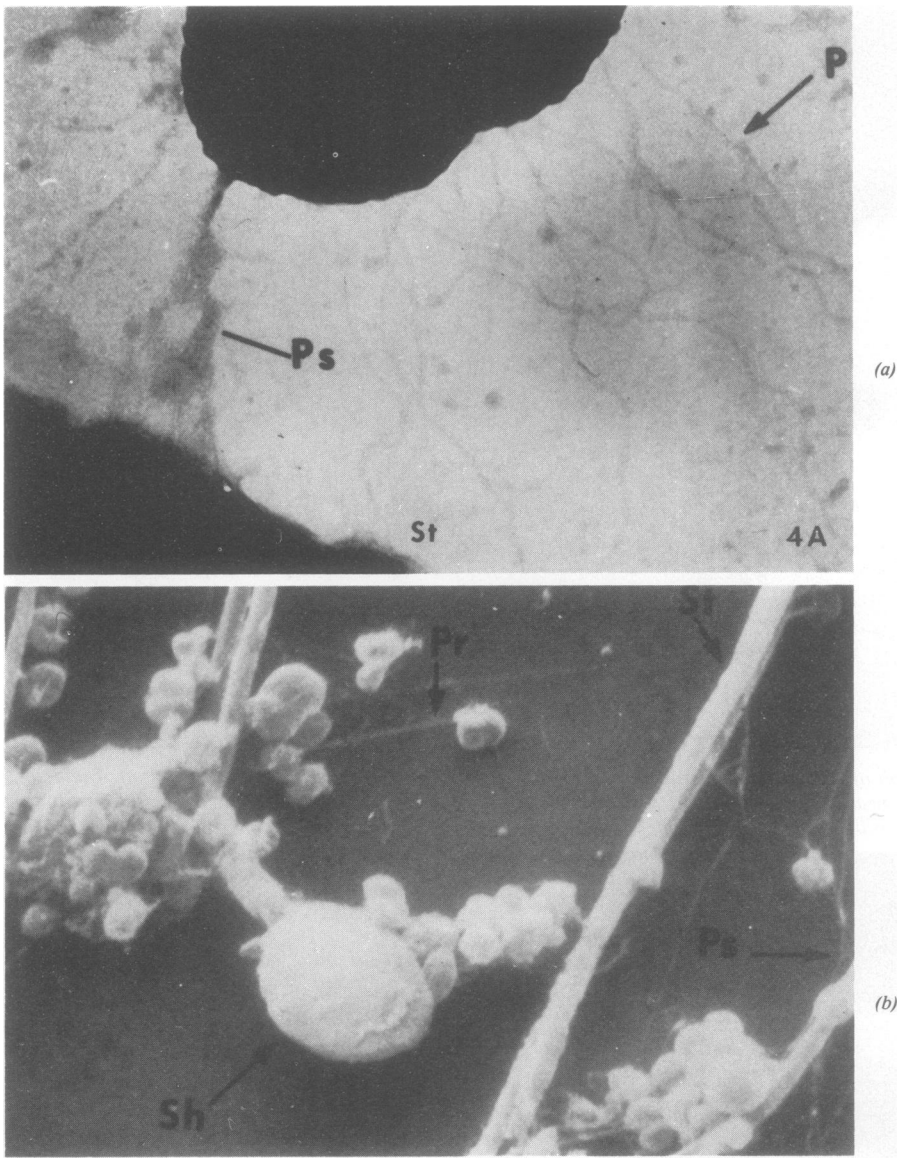


Fig. 4 *Micrographs of pilated (P)* N. gonorrhoeae: *(a) TEM micrograph showing spiderweb (Ps) arrangement of attachment to sperm tail (St) (× 52 000 magnification); (b) SEM micrograph showing spiderweb arrangement (Ps) and ropes of pili (Pr) and direct attachment to sperm head (Sh) and to sperm tail (St) (× 7000 magnification).*

61·8 ± 2·4% by LM and 62·6 ± 2·06% by TEM. The mean percentage of sperm with non-pilated gonococci attached was 41·3 ± 3·2% and 41·5 ± 2·1% by LM and TEM respectively. Results analysed by Student's *t* test showed a significant difference ($P = <0·0005$ for both LM and TEM) between the percentage of attachment. The two methods of microscopy were comparable because no significant variations were detectable in the results obtained by either LM or TEM.

EFFECT OF TRYPSIN

Pilated gonococci treated with trypsin showed both degradation and loss of pili (Fig. 8). Adherence to sperm was mostly due to direct contact, and mediation by pili was rarely observed. After

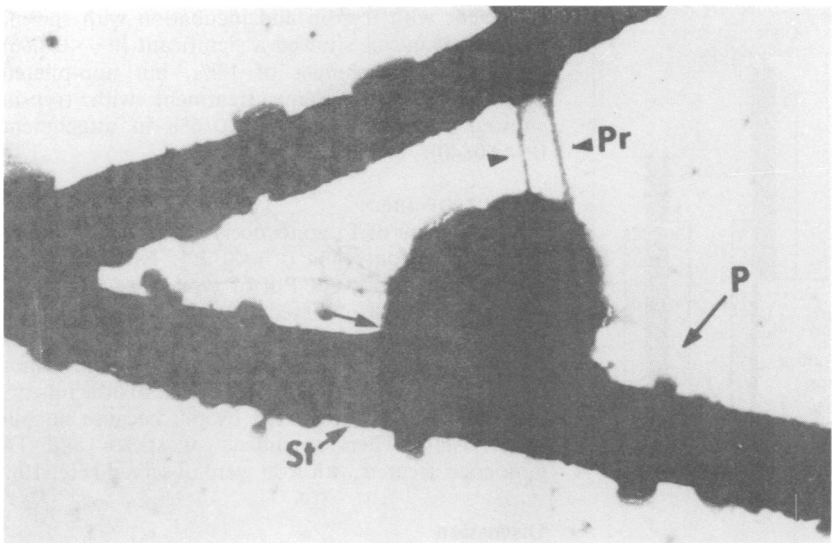


Fig. 5 *TEM micrograph of pilated* N. gonorrhoeae *attached to sperm tail (St) by direct contact (arrow), individual pilus (P), and ropes of pili (Pr). (× 31 500 magnification).*

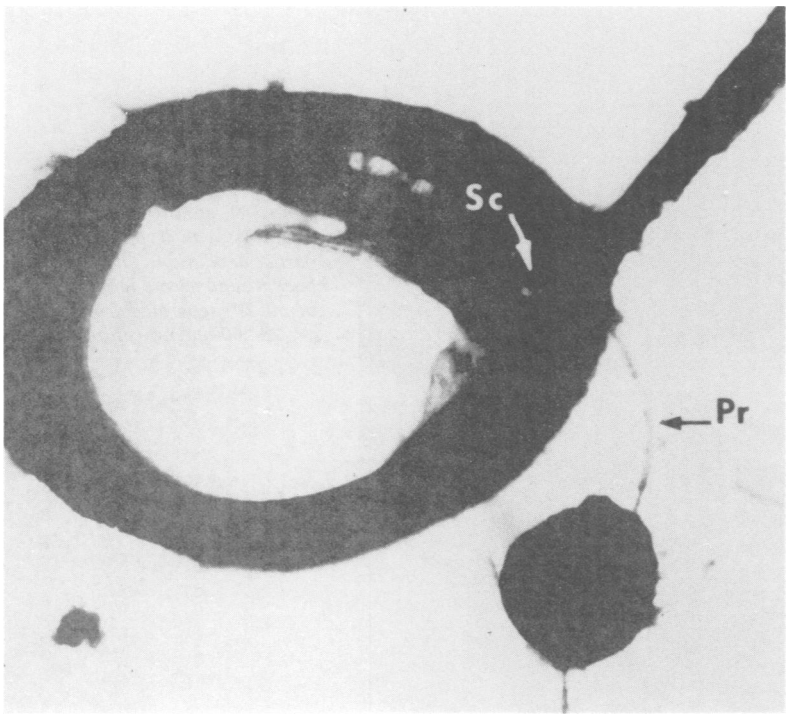


Fig. 6 *TEM micrograph of pilated* N. gonorrhoeae *attached to a coiled sperm tail (Sc) by ropes of pili (Pr). (× 26 400 magnification).*

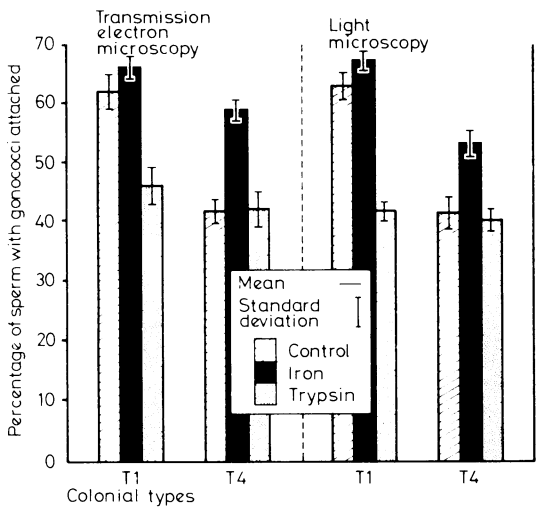


Fig. 7 *Comparison by TEM and LM of the percentage of sperm with attached gonococci from T1 or T4 colony types. Attachment by bacteria treated with iron, or trypsin, before incubation with sperm is contrasted with attachment by untreated gonococci (control).*

treatment with trypsin and incubation with sperm, pilated organisms showed a significant ($P = <0{\cdot}005$) decrease in attachment of 18%, but non-pilated bacteria after the same treatment with trypsin showed a decrease of only 0·6% in attachment ($P = <0{\cdot}40$).

EFFECT OF IRON

Iron treatment of T1 gonococci enhanced attachment to sperm by only 4% ($P = <0{\cdot}20$). No increase in pilation was observed. Pili of type b, as well as of type a, were still observed (Fig. 9). In contrast, T4 gonococci pretreated with iron salts attached to 15% more sperm than did the control, a significant difference ($P = <0{\cdot}0005$). The increase in attachment, however, was not mediated by pili because no pili were visible when specimens of sperm and T4 gonococci treated with iron were observed (Fig. 10).

Discussion

The interaction of gonococci with sperm was examined by three types of microscopy. Each type gave a different dimension to studies of attachment. Specimens dried on glass-slide fragments by the

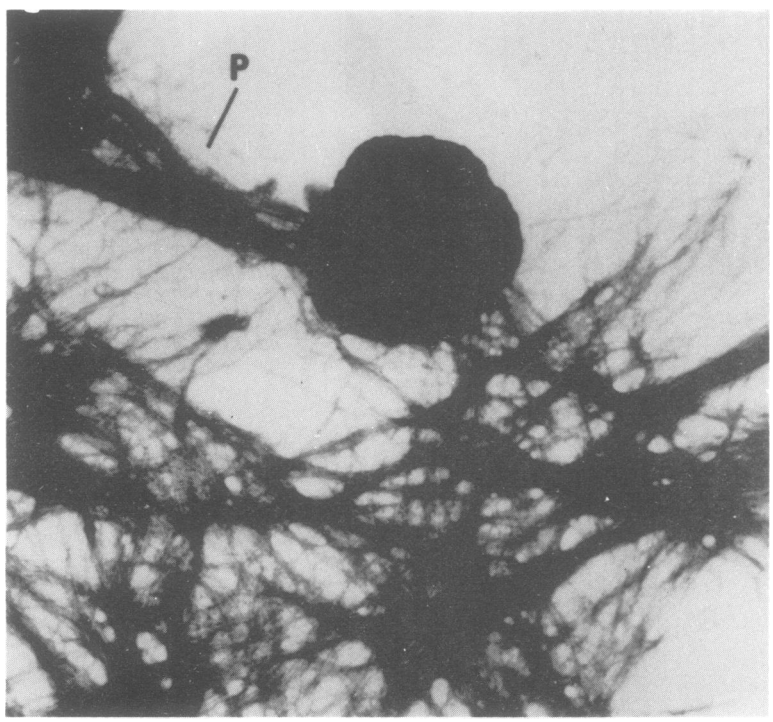


Fig. 8 *TEM micrograph of pilated* N. gonorrhoeae *after treatment with trypsin showing partial depilation. The background shows large numbers of pili (P) separated from cells. (× 28 500 magnification).*

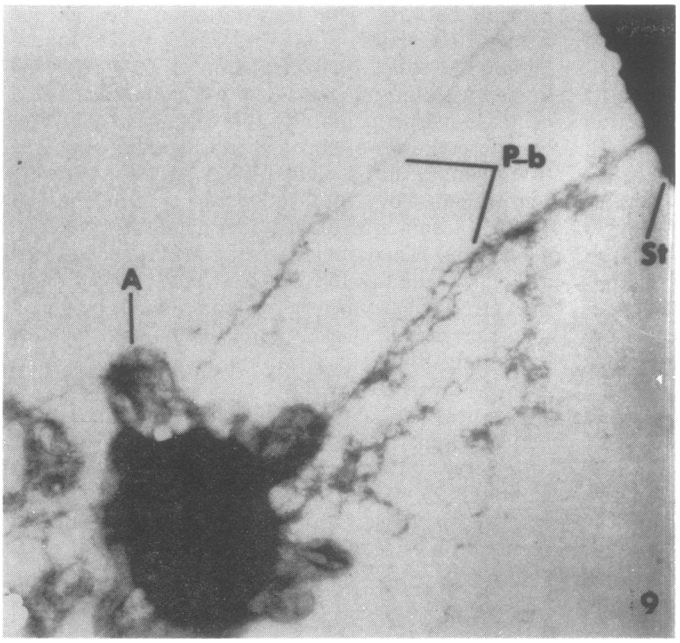


Fig. 9 *TEM micrograph of pilated (type b, P-b)* N. gonorrhoeae *treated with iron, incubated with sperm (St), and positively stained with UA. (× 36 600 magnification).*

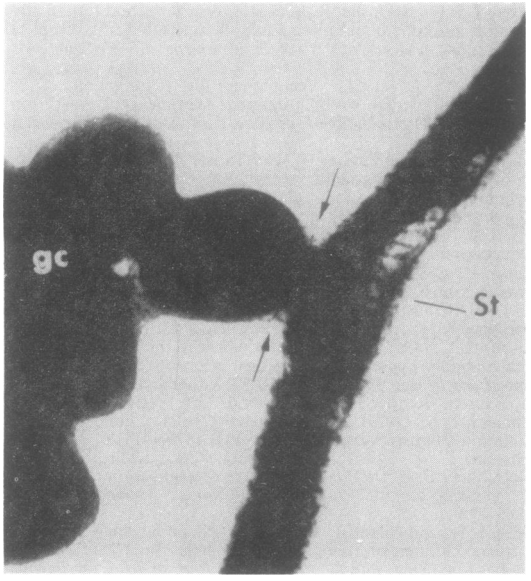


Fig. 10 *TEM micrograph of non-pilated* N. gonorrhoeae *treated with iron, incubated with sperm (St), and then negatively stained with UA. Only direct contact (arrows) was observed. (× 34 300 magnification).*

critical-point drying procedure gave the best results for SEM. This type of microscopy is important for observations of the tridimensional structure of both prokaryotic and eukaryotic cells. TEM was useful in documenting adherence of pilated and non-pilated gonococci to sperm. Light microscopy confirmed the results of James-Holmquest *et al.* (1974) and James *et al.* (1976).

TEM was used in most of the work because the resolving power is greater (0·4-0·8 nm) than SEM (10·0-12·0 nm). The difference in resolution allowed us to observe the ultrastructure of pili and the interaction of gonococci, with or without pili, with sperm. For TEM, several techniques were investigated. The best results were obtained with specimens in Alsever's solution, fixed with osmium vapours, and either negatively or positively stained with uranyl acetate. The application of the pseudoreplicative technique provided a more consistent preparation and minimised interference from the background. Breakage of the coated grid around the bacterial periphery was avoided in most grids by this method. In addition, positively stained pili were well defined. The measurements of pilar diameter in both positively and negatively stained preparations closely agreed.

The diameter of pili varied little from 4·5-5·5 nm. The results agreed with the observations of Jephcott *et al.* (1971), Novotny *et al.* (1975), and McGee *et al.* (1976). Results obtained by Swanson *et al.* (1971) (8·0—10·0 μm) were not comparable, possibly owing to different methods of calibration.

Both straight pili of type a and bent pili of type b were seen (Novotny *et al.*, 1975). These types were observed in different experiments and by the

different techniques (Figs 1, 2, and 6). In contrast to the findings of Novotny *et al.* (1975), type a pili did not appear to be part of the cell wall.

Pili of type c that have a knob at the end (Novotny *et al.*, 1975) were not included in our classification because, although some of our preparations showed similar spherical structures, these rarely occurred at the end of a pilus. Isolated blebs and possible membranous extensions of different sizes and shapes were seen in many micrographs (Figs 2, 4a, and 10). Other investigators have described similar structures Grimble and Armitage, 1974; DeVoe and Gilchrist, 1975; McGee *et al.*, 1976), and lipopolysaccharides were isolated from comparable blebs (Novotny *et al.*, 1975). These blebs and membrane extensions were present on the external surface of the cell wall and occasionally wrapped around the cell (Fig. 10).

Each gonococcus from colony type 1 that possessed pili had an average of 10. We do not know why some lacked pili, although treatment of the specimen for electron microscopy could have removed appendages. The number of pilated gonococci (71%) was greater than the number without.

Removal of pili from gonococci by trypsin reduced attachment to sperm to the level expected for non-pilated bacteria (Punsalang and Sawyer, 1973; James *et al.*, 1976). Ninety per cent of T1 bacteria treated with trypsin attached directly to sperm rather than through the usual mediation by pili. These findings supported the hypothesis that pili play a part in the adherence of gonococci to eukaryotic cells (Swanson, 1973). The important role of pili in attachment is emphasised by the work of James-Holmquest *et al.* (1974) in contrast to the conclusion of Novotny *et al.* (1977).

Attachment of gonococci to sperm appears to involve other factors besides pili. Treatment of avirulent, T4 gonococci with iron salts significantly increased attachment to sperm. In contrast, similar treatment of T1 bacteria had little effect on attachment. We speculate that, if iron is mostly concentrated in the cell membrane (Bartsch, 1968; Lankford, 1973), the polarity of the membrane surface may be changed thereby enhancing adherence (Heckels *et al.*, 1976). Also, changes in the configuration of the cell membrane might make receptor sites more available for adherence by non-pilated gonococci. Of interest for these speculations is the recent report that iron salts increased the killing power of avirulent T3 and T4 gonococci after intravenous inoculation into chicken embryos but had little or no effect on the virulence of T1 and T2 gonococci (Payne and Finkelstein, 1975).

The attachment of pilated gonococci to sperm *in vivo* could affect the fertility of the host as well as provide bacteria with transportation through the female urogenital tract (Howard, 1971; James-Holmquest *et al.*, 1974). Isolation of T-mycoplasma in men with infertile marriages was correlated with a decrease in sperm motility (Fowlkes *et al.*, 1975b). Our observations of coiled sperm tails that appeared to be wound up by pilated gonococci (Fig. 6) were reminiscent of the reported association of T-mycoplasma with coiled sperm tails (Fowlkes *et al.*, 1975a). A high incidence of gonorrhoea has been associated with low fertility (Arya *et al.*, 1973). Thus, one effect of gonococcal disease could be direct interference with sperm motility by entanglement of sperm tails by the bacteria.

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