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# A COMPARISON OF THE ASSOCIATION OF *NEISSERIA GONORRHOEAE* WITH HUMAN AND GUINEA-PIG GENITAL MUCOSA MAINTAINED IN ORGAN CULTURE

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**Summary.—Organ cultures of human and guinea-pig genital mucosa were inoculated with *Neisseria gonorrhoeae*, and the association of the bacteria with the epithelial surface of each tissue was studied by light microscopy and by scanning electron microscopy. Gonococci attached to the mucosa of human fallopian tube, adhering specifically to the surface of non-ciliated epithelial cells. In contrast, gonococci rarely attached to the mucosal surface of guinea-pig uterine horn, vagina or bladder, although organisms were occasionally seen associated with the submucosal tissue in areas where the epithelium had sloughed, and in extracellular mucus secretions. There is no evidence from this study that gonococci adhere to guinea-pig genital tissue in a manner analogous to that seen with human genital tissue.**

A CRITICAL STEP in infection by pathogenic microorganisms involves attachment to mucosal epithelial cells. Organisms which are unable to attach to a mucosal surface are washed away by extracellular fluids. Such considerations apply particularly to *Neisseria gonorrhoeae*, which is capable of colonizing the urethra despite frequent acts of micturition by the host. Electron microscope examination of urethral scrapings from men who had symptoms of gonorrhoea for less than 24 h has shown that gonococci become attached to the surface of epithelial cells and enter them by a process of phagocytosis (Ward and Watt, 1972). These workers suggested that during the incubation period of gonorrhoea, the organisms adhere to the urethral mucosa, and that this attachment is an essential prerequisite for the invasion of the subepithelial tissues which occurs later in the infection (Harkness, 1948).

A similar attachment of gonococci to genital epithelial cells has been seen in organ cultures of human fallopian tube inoculated experimentally (Taylor-Robinson *et al.*, 1974; Ward, Watt and Robertson, 1974). In these studies, gonococci attached to, and were taken up by, non-ciliated epithelial cells, and caused disruption of the epithelium and loss of ciliary activity. In contrast, it was found that gonococci had a markedly diminished ability to attach to and damage the epithelium of oviduct organ cultures of lapine, porcine and bovine origin (Taylor-Robinson *et al.*, 1974; Johnson, Taylor-Robinson and McGee, 1977). It was suggested therefore that the apparent inability of gonococci to infect species other than man and the chimpanzee (Chandler and Kraus, 1976) might be explained, at least in part, by the inability of gonococci to attach to, and thus colonize mucosal surfaces of non-human species (Taylor-Robinson *et al.*, 1974; Johnson *et al.*, 1977). At variance with this concept, however, was the observation of Tebbutt *et al.* (1976), who reported that gonococci adhered not only to organ cultures of human genital mucosa, but also to those of guinea-pig genital tissue. In their study, gonococci were inoculated on to pieces of tissue and after incubation

adherence was assessed by determining the number of viable organisms that remained associated with the tissues after they had been washed to remove non-attached bacteria. The aim of the present study was to investigate and compare the nature of the interactions occurring between gonococci and human and guinea-pig genital mucosal surfaces, respectively, using the techniques of light microscopy and scanning electron microscopy (SEM).

## MATERIALS AND METHODS

*Bacteria.*—*Neisseria gonorrhoeae* Strains 192A (a laboratory passaged strain) and E56831 (a recent clinical isolate) were used. Organisms were grown on GC agar base (Difco) supplemented with 2% IsoVitalex (Baltimore Biological Laboratories) in an atmosphere of 5% $CO_2$ in air at 37°. Only cultures which produced predominantly Type I colonies (Kellogg *et al.*, 1963) were used.

*Sources of organs for culture.*—Fallopian tubes were obtained from women undergoing hysterosalpingectomy for surgical indications. Only tubes which had vigorous ciliary activity when dissected (McGee, Johnson and Taylor-Robinson, 1976) were included in the study.

Adult female guinea-pigs were killed by i.p. injection of sodium pentabarbitone (Sagatal–May and Baker Ltd) followed by exsanguination by cardiac puncture. The fur covering the abdomen was shaved, and the urogenital tract of each animal was removed by dissection with sterile instruments, care being taken not to puncture the intestine.

*Preparation of organ cultures.*—The organs were placed in Petri dishes containing Eagle's minimal essential medium (MEM) (GIBCO) buffered to pH 7·3 with HEPES and supplemented with antibiotics. Vancomycin (5 μg/ml, Eli Lilly & Co.) and colistin (3 μg/ml, Pharmax Ltd) were used in experiments with human organs and either these antibiotics, or gentamicin (10 μg/ml, Roussel) and cephaloridine (20 μg/ml, Glaxo) were used in experiments with guinea-pig organs. The outer layers of serosal tissue were dissected away from each organ, a small pair of scissors was inserted into the lumen, and the tissue was cut longitudinally to expose the mucosal surface. Then pieces about 5 mm in width and 5–10 mm in length were cut and placed with their mucosal surface uppermost in Petri dishes. The tissue pieces were then rinsed several times either in medium free of antibiotics or in medium containing vancomycin and colistin.

*Inoculation of organ cultures.*—Gonococcal colonies were grown for 18–24 h on the solid medium described above and were then harvested into 3 ml of MEM free of antibiotics. The gonococcal suspension was agitated for 10–20 s on a Rotamix Deluxe at maximal amplitude to disperse clumps, after which it was used to inoculate the organ cultures. In some experiments, 20–60 μl of suspension was placed directly on to the mucosal surface of each tissue piece in Petri dishes not containing medium. In other experiments, the tissue pieces were immersed in MEM (either free of antibiotics or supplemented with vancomycin and colistin) and the gonococcal suspension was inoculated into the medium. In both types of experiment, organ cultures serving as controls did not receive gonococci but were inoculated instead with an equal volume of MEM.

The number of viable gonococci in the organ cultures at the beginning of each experiment was determined by plating out serial 10-fold dilutions of the inoculum on to solid medium and counting the number of colonies produced after incubation for 24 h.

*Assessment of adherence of gonococci to tissues.*—After inoculation, tissues pieces were incubated for various periods of time at 37°. Tissue pieces incubated for up to 2 h were kept in Petri dishes not containing medium. In contrast, in experiments in which the incubation period was longer, the tissue pieces were immersed in medium. At the end of the incubation period, MEM if present was removed, and the tissue pieces were washed to remove non-adherent bacteria by rinsing the mucosal surface 5–10 times with 1 ml of phosphate-buffered saline (PBS) delivered from a Pasteur pipette. This procedure was repeated 4 times for each piece of tissue using fresh PBS each time. After washing, the tissue pieces were fixed and the association of gonococci with the mucosa was examined by light microscopy and SEM.

*Light microscopy.*—Tissue pieces were fixed in formol sublimate, processed by routine histological methods, and sections stained by the Gram–methyl green–pyronin-light green stain, which was developed specifically for the purpose of detecting bacteria in tissue sections (Sowter and McGee, 1976). In some experiments, additional sections were stained with periodic acid–Schiff (PAS) reagent and Alcian blue, to detect mucins (Cook, 1974).

*Scanning electron microscopy.*—Tissue pieces were fixed with paraformaldehyde–glutaraldehyde fixative (Karnovsky, 1965). After washing with 0·1M cacodylate buffer (pH 7·3) they were post-fixed for 1 h on ice with 1% osmium tetroxide buffered with 0·1M cacodylate buffer (pH 7·3). The fixed tissues were dehydrated by passage through alcohol solutions of increasing concentration (25 to 100%) and then dried at the critical point of liquid $CO_2$ using a Polaron

E3000 critical point drying apparatus (Polaron Equipment Ltd, Watford, U.K.). The dried tissues were then mounted on aluminium stubs using "Electrodag 915" (Acheson Colloids Company, Plymouth, U.K.). The specimens were coated with a thin layer of gold using a Polaron E5100 sputter-coating apparatus and examined in a Phillips SEM500 scanning electron microscope.

## RESULTS

### *Sterility of organ cultures of human and guinea-pig genital tissue*

The human fallopian tube has no endogenous microbial flora, and thus tubes removed aseptically from patients without salpingitis are sterile. By incorporating vancomycin and colistin in the medium during preparation of organ cultures (McGee *et al.*, 1976), possible contamination with airborne microorganisms was inhibited and sterile cultures were readily produced. These antibiotics were chosen as they do not generally inhibit the growth of gonococci (Thayer and Martin, 1966). In contrast, cultures of guinea-pig genital organs were frequently bacterially contaminated despite the incorporation of vancomycin and colistin into the medium. However, antibiotic sensitivity testing of the contaminants indicated that they were generally sensitive to gentamicin and/or cephaloridine and, by incorporating these antibiotics into the medium in place of vancomycin and colistin, bacterial contamination was eliminated in subsequent experiments. Since gonococci are susceptible to gentamicin and cephaloridine, these antibiotics were removed before inoculation by rinsing the organ cultures thoroughly several times either with antibiotic-free medium or with medium containing vancomycin and colistin.

### *Association of gonococci with fallopian-tube organ cultures*

In an initial experiment, the mucosal surface of tissue pieces was seeded with $3 \times 10^6$ colony-forming units (c.f.u.) of *N. gonorrhoeae* Strain 192A. Examination by light microscopy of tissues fixed after incubation for 1 or 2 h showed that there was only very scanty attachment of gonococci to the mucosa, large areas of epithelium appearing free of adherent bacteria. A similar observation was made by SEM. However, a characteristic pattern was noted in areas of the mucosa where gonococci were detected. The bacteria, often in clumps, were associated primarily with non-ciliated cells, and the surface of these cells was often covered with organisms (Fig. 1a).

In other experiments, organ cultures maintained in MEM free of antibiotics were inoculated with gonococci so that the initial concentration of organisms in the medium was about $2 \times 10^5$ c.f.u./ml. Examination by both light microscopy and SEM of tissues fixed 18–24 h after inoculation showed close association of gonococci with extensive areas of the mucosal surface (Fig. 2*a*). The pattern of attachment seen by SEM, however, was similar to that seen in cultures incubated for 1–2 h, gonococci being attached to the non-ciliated epithelial cells only.

### *Association of gonococci with guinea-pig genital organ cultures*

A series of experiments were performed in which cultures of guinea-pig vagina, uterine horn and bladder were inoculated with $1 \times 10^6$–$2 \times 10^7$ c.f.u. of *N. gonorrhoeae*, either of Strain 192A or Strain E56831, and incubated for either 1–2 or 22–24 h. The results were similar irrespective of the length of time the tissues had been incubated following inoculation, the strain of *N. gonorrhoeae* used, and the presence or absence of vancomycin and colistin in the medium. The mucosal surface of all 3 types of tissue was generally free of adherent gonococci as seen by light microscopy (Fig. 2b, c, d), although diplococci or small clumps of gonococci were seen very occasionally on the surface of epithelial cells. However, in areas where the epithelium had sloughed, gonococci, occasionally in large numbers, were seen attached to the exposed surface of the submucosal tissue. Examination of the tissues by SEM confirmed that the

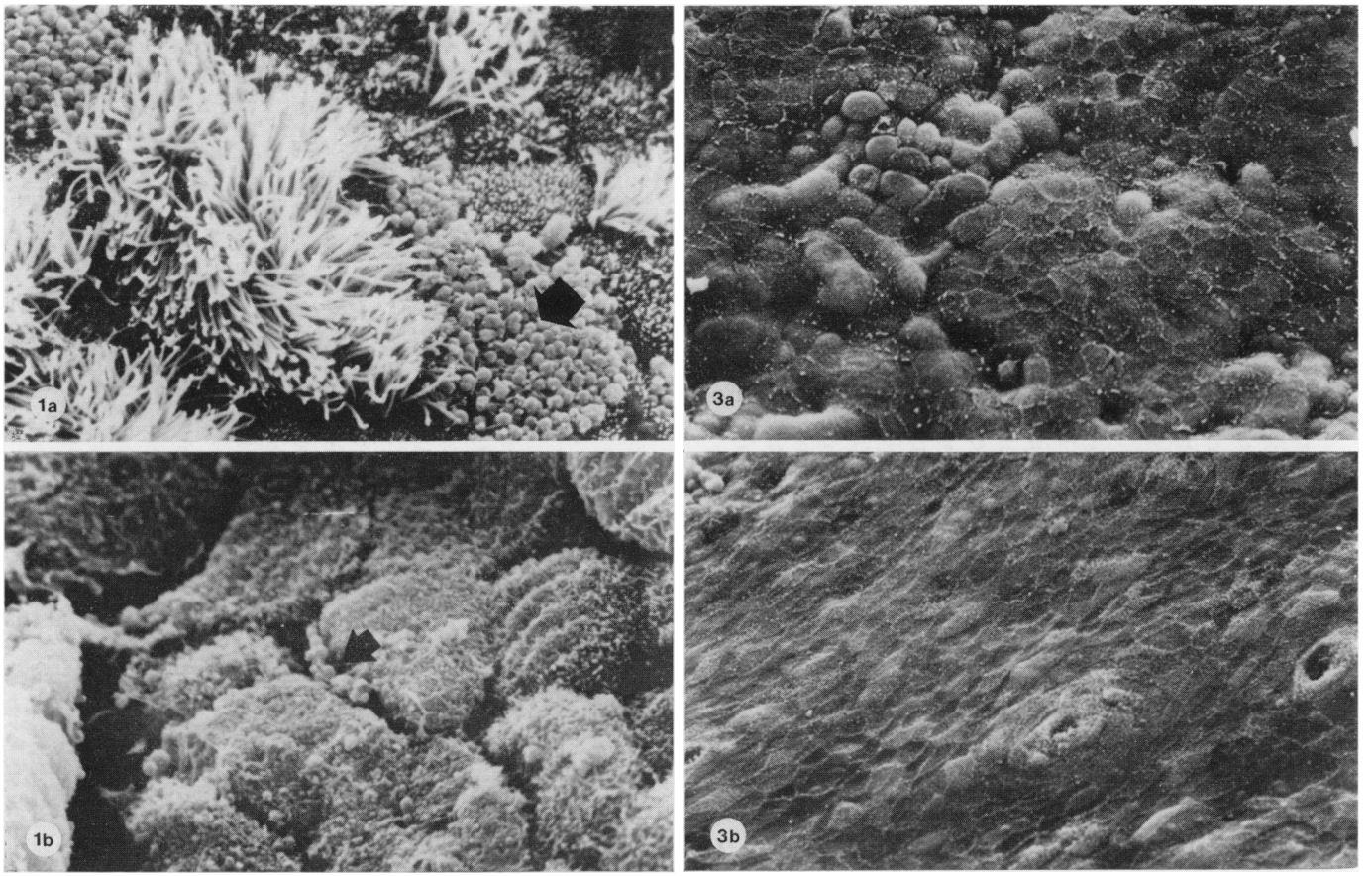


FIG. 1.—Scanning electron micrographs of organ cultures of genital tissue. (a) Human fallopian tube 1 h after inoculation with gonococci. Note gonococci (arrow) attached to surface of non-ciliated epithelial cells ( × 4250). (b) Guinea-pig bladder 1 h after inoculation with gonococci. Note gonococci (arrow) lying in groove between epithelial cells ( × 4250).

FIG. 3.—Scanning electron micrographs of guinea-pig genital tissues inoculated with gonococci. (a) Vagina 23 h after inoculation ( × 937). (b) Uterine horn 23 h after inoculation ( × 937). The surface of both tissues is free of adherent bacteria.

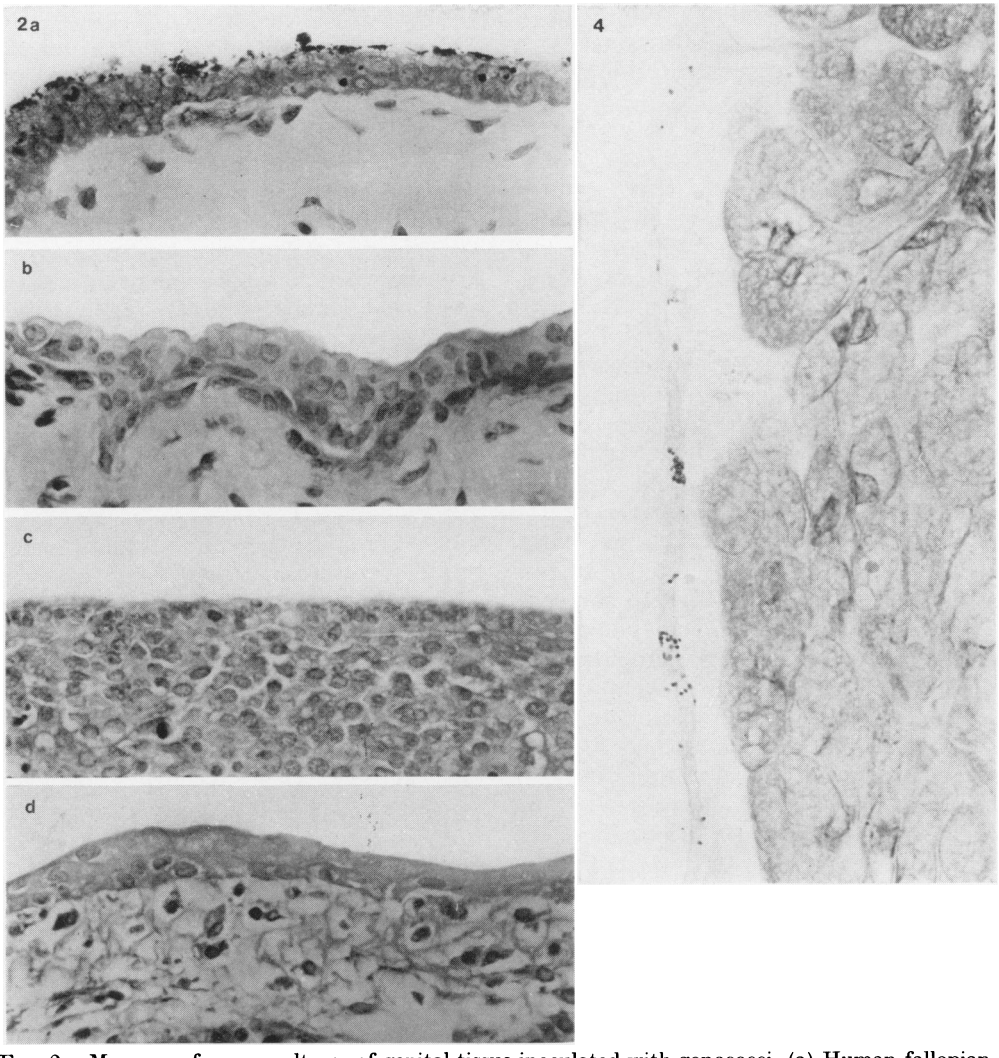


FIG. 2.—Mucosae of organ cultures of genital tissue inoculated with gonococci. (a) Human fallopian tube 18 h after inoculation ( × 490). (b) Guinea-pig vagina 23 h after inoculation ( × 490). (c) Guinea-pig uterine horn 23 h after inoculation ( × 490). (d) Guinea-pig bladder 23 h after inoculation ( × 490). Note that mucosa of fallopian tube is covered with gonococci, in contrast to mucosae of guinea-pig tissues, which are free of adherent bacteria. All sections stained by the Gram–methyl green–pyronin-light green stain.

FIG. 4.—Guinea-pig vagina 23 h after inoculation with gonococci. Numerous gonococci are located in the extracellular mucus, but the epithelial surface is free of adherent organisms. Gram–methyl green–pyronin-light green stain ( × 490).

epithelial surfaces were generally free of adherent gonococci (Fig. 3a,b). Moreover, the gonococci that were seen associated with the mucosal surface were usually found in small numbers in the grooves between adjacent epithelial cells (Fig. 1b) rather than on the surface of the cells.

Examination of vaginal tissue stained with PAS–Alcian blue showed that extracellular mucus secretions occasionally covered the mucosal surface. After Gram–methyl green–pyronin-light green staining, gonococci were seen within these mucus secretions (Fig. 4). However, de-

spite the localization of gonococci in mucus close to the mucosal surface, very few organisms were seen on the vaginal epithelium.

## DISCUSSION

The results presented here confirm earlier reports that gonococci attach to the mucosa of fallopian tube organ cultures, and that they adhere selectively to the non-ciliated cells (Taylor-Robinson *et al.*, 1974; Ward *et al.*, 1974; Johnson *et al.*, 1977). Only a few gonococci were seen attached to the mucosa of tissue pieces after incubation for 2 h, which may have been due to the mechanical barrier imposed by the actively beating cilia. By 18–24 h after inoculation, however, a large number of gonococci were seen associated with the mucosal surface, as reported previously (Johnson *et al.*, 1977). In contrast, Tebbutt *et al.* (1976) reported that a large number of gonococci attached to fallopian-tube tissue after incubation for only 1 h. The reason for the discrepancy between this result and those reported here is unclear. However, these workers did not report the presence of ciliary activity in the tissues they used and it is possible that, in the absence of ciliary activity, gonococci may have been able to attach to epithelial cells more readily than in our experiments.

Tebbutt *et al.* (1976) also reported that gonococci attached to the mucosa of guinea-pig genital tissues maintained in culture. The results presented here, however, show that there is a marked difference between the manner in which gonococci associate with human genital tissue and the manner in which they associate with guinea-pig genital tissue. The organisms appeared to make direct contact with the surface of non-ciliated human epithelial cells. In contrast, on the rare occasions when gonococci were seen on the mucosal surface of guinea-pig genital tissue, they were located in the grooves running between adjacent epithelial cells. Examination of guinea-pig vaginal tissue showed that gonococci could also be found in mucus secretions on the mucosal surface. Thus, gonococci that become associated with the mucosa of guinea-pig genital tissue may be trapped in folds in the epithelium, or in mucus, rather than attached to the surface of epithelial cells. Indeed, Tebbutt *et al.* (1976) reported that enhanced association of gonococci with guinea-pig genital mucosa was seen in tissues with complex mucosal folds and increased mucus secretion, which is compatible with this concept.

There is no evidence from the present study that gonococci attach to the mucosa of guinea-pig genital tissue in a manner comparable to that seen with human genital tissue. This finding is similar to the results of earlier studies using oviduct tissue from other non-human species (Taylor-Robinson *et al.*, 1974; Johnson *et al.*, 1977), results which have been confirmed recently by SEM (Johnson, Gunner and Clark, unpublished observations). Thus, the evidence available indicates that gonococci attach specifically to genital epithelium of human origin, and this factor may be responsible, at least in part, for man being the only known natural host for the gonococcus.

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