THE ADHERENCE OF PILATE AND NON-PILATE STRAINS OF \textit{NEISSERIA GONORRHOEAE} TO HUMAN AND GUINEA-PIG EPITHELIAL TISSUES

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The firm adherence of \textit{Neisseria gonorrhoeae} to mucosal surfaces, preventing dislodgement by tissue secretions or by the movement of material in the lumen, is a prerequisite for invasion of underlying tissue. Studies based on electron-microscopic examination of urethral scrapings have shown that adherence occurs (Ward and Watt, 1972). Presumably, specific surface components of both the gonococcus and the host are involved in this adherence as they are for other bacteria such as \textit{Streptococcus pyogenes} and \textit{Escherichia coli} (Ellen and Gibbons, 1974). However, the nature of these components is still unclear.

Kellogg et al. (1963, 1968) described four colonial types of gonococci; types 1 and 2 were virulent for man when large numbers of organisms were inoculated into the urethra, whereas types 3 and 4 were avirulent. Pili were found to be present on types 1 and 2, but not on types 3 and 4 (Jephcott, Reyn and Birch-Andersen, 1971; Swanson, Kraus and Gotschlich, 1971). These pili promoted the adhesion of gonococci grown \textit{in vitro} to tissue-culture cells; non-pilate strains adhered, but to a lesser extent (Swanson, 1973). Similarly, in studies with perfused preparations of isolated human fallopian tube, pilate type-1 gonococci grown \textit{in vitro} adhered (Ward, Watt and Robertson, 1974), but other studies demonstrated that non-pilate organisms also attached to fallopian tube held in organ culture (Taylor-Robinson et al., 1974). Anti-pili antibodies occur in natural infection (Buchanan et al., 1973) but pili comparable to those seen \textit{in vitro} are rarely seen on gonococci in infected urethral exudates (Novotny, Short and Walker, 1975), although larger appendages have been described (Grimble and Armitage, 1974). Thus, although some of the present evidence suggests that pili may be involved in adherence to the urogenital tract at an early stage in the infective process, this does not seem to be the only factor concerned.

In the present paper we have examined the adherence of pilate and non-pilate strains of gonococci to various human tissues: (i) adult human endocervix and fallopian-tube epithelium, which is susceptible to natural infection; (ii) epithelium of the ectocervix, which although exposed to gonococci is generally resistant to direct penetration (Harkness, 1948; Nolan and Osborne, 1973); and (iii) bronchial epithelium, which is not exposed to gonococci during natural infection. Specimens of human tissue from the posterior urethra were received 17 Oct. 1975; accepted 19 Nov. 1975.

also available but proved unsuitable because the epithelial surfaces were either badly damaged or completely lost. The possible relationship of gonococcal adherence to host specificity was studied by including some epithelial tissues from the uterus, cervix, male posterior urethra and bladder of the guinea-pig which is resistant to natural infection (Hill, 1944).

MATERIALS AND METHODS

Strains of Neisseria gonorrhoeae. Strains labelled AS and BS were isolated from different samples of urethral pus. Both formed small colonies, were pilate and readily infected guinea-pig skin chambers (Veale et al., 1975). Strain AL, obtained from a subculture of strain AS, formed large colonies, was non-pilate and did not infect guinea-pig chambers. Strain no. 005 was also isolated from urethral pus and resembled strain BS although its infectivity for guinea-pig chambers was not tested. A Kellogg strain (type 4) was obtained from Dr D. S. Kellogg jr, General Diseases Research Laboratory, National Communicable Disease Center, Atlanta, Georgia, USA.

Stock cultures were snap frozen and stored in liquid nitrogen (Ward and Watt, 1971). Working stock cultures were frozen in liquid nitrogen but were stored at -70°C.

Media. The inocula for adherence experiments were grown on a medium similar to that described by Amies and Garabedian (1967). GC Agar Base (Oxoid) received 10% v/v of supplements A [yeast extract (Oxoid) 10 g; glucose 8 g; water 95 ml] and B [foetal calf serum (Flow Laboratories) inactivated at 56°C for 30 min.]. Before use, the medium was incubated at 37°C for 24 h to reduce surface moisture and to check sterility. Viable counts were made on Diagnostic Sensitivity Test Agar (Oxoid) containing 5% lysed horse blood (Wellcome Laboratories).

Tissues. Pieces of human endocervix, ectocervix and fallopian tube were removed from specimens obtained after total abdominal hysterectomy for non-neoplastic conditions other than fibromyoma. All mucosae were intact, but mild cervicitis with some inflammatory changes around the squamocolumnar junction was present on some of the specimens. The patients were aged 29-48 years and none was postmenopausal. Human bronchus was obtained from lungs after thoracotomy and pieces of tissue furthest away from the neoplasm were chosen. From guinea-pigs the male posterior urethra, bladder, and the female uterine horn and cervix were removed within 15 min. of the animal’s death. All tissues were opened out and pieces about 1 cm² were placed in petri dishes (50x18 mm). The tissues were kept at 37°C in a moist atmosphere and used within 15 min. of preparation.

Dialysis tubing (Visking tubing, Scientific Instruments Centre Ltd, London) was used as a control. The tubing was opened out and cut into pieces about 1 cm², boiled in distilled water for 5 min. and then placed in petri dishes.

Preparation of inocula. The strains were grown on the modified Amies and Garabedian medium. The cultures were incubated at 37°C for 20-22 h in candle jars and the growth was harvested into 2% Proteose Peptone no. 3 (Difco) containing 10% of heat-inactivated foetal calf serum (PPCS). Clumps of bacteria were broken up by shaking with glass beads for 1 min. and mild sonication (Electrosonic bath, Headland Ltd, London) for 1 min. Electron microscopy, with potassium phosphotungstate (1%; pH 5.5) as the negative stain, showed that after this treatment some pili were shorter than in the untreated controls. Each suspension was agitated briefly for about 2 s on a Vortex mixer (Griffin and George Ltd, Wembley) before addition to the tissues in adherence experiments.

Measurement of adherence of gonococci to tissues. Adherence was measured by a modification of the method of Woodland, Griffiths and Pearce (1973). Each tissue was seeded with 4 μl of gonococcal suspension prepared in PPCS and was delivered from a chromatography micro-repette (Jencons Scientific Ltd, Hemel Hempstead). Generally the inoculum was c. 10⁶ viable gonococci, but in some experiments it varied from (5×10⁵)-(3×10⁶) organisms. A tissue was discarded if the inoculum did not remain on the mucosal surface. The total count (Helber chamber) and the numbers of viable gonococci in an equivalent

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inoculum were determined (Veale et al., 1975). The tissues were incubated at 37°C in a moist atmosphere containing 5% CO₂ for 1 h and then each tissue was gently washed with 3 ml of PPCS by tilting each petri dish four times. The supernatant fluids from the gentle washes were collected and held at 37°C for not more than 20 min. before viable counts were done. A further 3 ml of PPCS was added to each petri dish and each dish was tilted four times; the supernatant fluid from this wash, which contained less than 3% of the total numbers of viable organisms recovered, was discarded. The tissues were then vigorously washed in 3 ml of PPCS on a Vortex mixer for 1 min. and the supernatant fluids were kept for not more than 20 min. at 37°C before viable counts were done. To measure the numbers of gonococci remaining on the tissues after vigorous washing, the tissues were homogenised in 1 ml of PPCS in a Griffiths'-tube homogeniser. Parallel tests were done with dialysis membrane in place of tissue. The numbers of viable gonococci in the gentle wash (G), vigorous wash (V) and the homogenate (H) were determined and each count was expressed as a percentage of the total numbers (usually c. 10⁶ organisms) of gonococci recovered in the particular experiment. For example, the percentage of gonococci in the vigorous wash was expressed as:

\[
\frac{V}{G+V+H} \times 100.
\]

Counts were not expressed as percentages of the inocula because there was some variation (40-125%) in the percentages of the original inocula recovered after incubation on different tissues (see table I).

**Histology.** Sections were stained by haematoxylin–eosin and by the periodic acid-Schiff (PAS) method described by Drury and Wallington (1967). Some sections were treated with diastase prior to PAS staining. Sometimes human endocervix and ectocervix tissues were seeded with gonococci and after 1 h these were gently washed with PPCS as described previously and fixed in neutral buffered 10% formalin. The sections were treated with Alcian blue (pH 2.5; 5 min.) to stain any mucus and then with neutral red–fast green (Ollet, 1951) to identify gonococci on the surface.

**RESULTS**

The investigation of the adherence of gonococcal strains to human and guinea-pig epithelial tissues was divided into three parts. First, the anatomy and histology of the uninfected tissues were studied with particular reference to the type of epithelium lining the tissues and to the presence or absence of mucus. Second, the distribution of strains BS and AL on the epithelium of infected human endocervix and ectocervix was investigated histologically. Third, the adherence of the gonococcal strains to the various tissues was measured, and an attempt was made to relate the observed patterns of attachment to the surface structures of the gonococci and the epithelium involved.

**Anatomy and histology of uninfected tissues**

The human cervix is divided into two regions. The tissue adjacent to the uterus (endocervix) is covered by simple columnar epithelium which is thrown into folds to form clefts as it lines the endocervical canal. A prominent layer of mucus, which stained with PAS and Alcian blue and which varied in thickness with different specimens, covered the epithelial surface. The ectocervix is covered by stratified squamous epithelium. Glycogen is present in the upper layers but no mucous secretions were seen. The epithelium of the fallopian
tube is folded and each tube is lined with columnar cells, many of them ciliated, but no mucus-secreting cells were observed. Human bronchus is lined with columnar epithelium and a prominent surface layer of mucus (PAS and Alcian blue positive) was seen, with numerous mucus-secreting glands in the submucosa. Small areas of squamous metaplasia were observed in the epithelium.

In the guinea-pig the uterus is divided into paired uterine horns which join to form the body of the uterus. The latter consists of a cranial portion (upper cervix) containing two cavities separated by a median septum and a caudal undivided portion (lower cervix) projecting into the vagina. Each uterine horn is lined with columnar epithelium. The cervix bears columnar epithelium which is folded and the degree of mucosal folding varied with different specimens. More complex folds were associated with increased secretory activity when extracellular mucus was present. Sometimes squamous epithelium appeared to be predominant in the lower cervix. The male posterior urethra has transitional epithelium with an undulating surface. Numerous mucus-secreting cells and glands, lined with columnar epithelium, were stained by PAS and Alcian blue. Much of the epithelium was covered with extracellular material which contained spermatozoa, and mucus was also present around the openings of the urethral glands. The bladder is lined with transitional epithelium which was thicker than that present on the urethra and no mucus-secreting glands or cells were seen.

**Histology of infected human endocervix and ectocervix**

Organisms of strains BS and AL were embedded in mucus covering the endocervix. In a few specimens some attachment to red blood cells was also found. With strain BS, small compact clumps of organisms confined to a few sites on the tissue surface were seen, whereas strain AL organisms were more widely distributed as single organisms or in small groups of loosely clumped organisms.

On the ectocervix, organisms of strain BS were attached to the superficial layer of the stratified epithelium but with strain AL no organisms were seen.

**Measurement of adherence of gonococci**

Gentle washing presumably removed organisms that had settled on to the tissues but were not attached, although conceivably some gonococci had not come into contact with the tissue surface after 1 h. As the mucosae appeared intact after vigorous washing it was assumed that this treatment removed only organisms that had adhered to the tissue surface and did not remove epithelial cells which could have contained gonococci. To check that any differences in adherence between the pilate and non-pilate strains were not masked by too vigorous washing (1 min. on a Vortex mixer), the tissues in 3 ml of PPCS were either inverted by hand 20 times or were washed for only 5 s or 15 s on a Vortex mixer. The essential differences in the behaviour of the strains were unchanged.

Homogenisation disrupted guinea-pig urethra, uterus, cervix, bladder and human fallopian tube and bronchus but human cervix was more resistant;
microscopic examination showed that the columnar epithelium lining the human endocervix was disrupted but some deeper layers of the stratified epithelium of the ectocervix remained. Comparatively few gonococci—less than 5% of the total number recovered in our studies with most tissues—were present in the homogenates after 1 h and it was not possible to determine histologically whether these organisms were on the tissue surface or embedded in any remaining mucus or within the tissue. For this reason the number of gonococci in the vigorous wash, rather than in the homogenate—expressed as a percentage of the total number of organisms recovered—was used as a measure of the strength of attachment.

There was good recovery of organisms after incubation on the tissues (table I). Only in the case of strain BS in studies with fallopian-tube tissue was the number of organisms recovered appreciably lower than the number in the original inoculum. The recovery of a high percentage of the inoculum also
Survival of gonococci on human and guinea-pig tissues; viable organisms recovered after incubation for 1 h expressed as a percentage of the original viable inoculum

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percentage of viable gonococcal inoculum (± standard error) recovered with strain</th>
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<tbody>
<tr>
<td></td>
<td>BS</td>
</tr>
<tr>
<td>Human endocervix</td>
<td>101±4</td>
</tr>
<tr>
<td>Human ectocervix</td>
<td>97±7</td>
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<tr>
<td>Human fallopian tube</td>
<td>41±4</td>
</tr>
<tr>
<td>Human bronchus</td>
<td>77±7</td>
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<tr>
<td>Guinea-pig uterine horn</td>
<td>73±2</td>
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<tr>
<td>Guinea-pig cervix</td>
<td>80±9</td>
</tr>
<tr>
<td>Guinea-pig male urethra</td>
<td>125±10</td>
</tr>
<tr>
<td>Guinea-pig bladder</td>
<td>114±7</td>
</tr>
</tbody>
</table>

indicated that there was no gross killing of organisms by possible bactericidal tissue products released during vigorous washing or homogenisation.

In control studies, adherence to dialysis membranes was poor, with more than 90% of recovered organisms released by gentle washing. There was adherence—measured by the percentage of total organisms recovered that were released in the vigorous wash—of both pilate (strain BS) and non-pilate (strain AL) organisms to the endocervix (fig. 1); the difference was small, but strain BS adhered significantly better than strain AL (P<0.01). Strain BS adhered significantly better than strain AL to the ectocervix (P<0.001), the non-pilate strain adhering feebly, with more than 70% organisms recovered in the gentle wash (fig. 1). The pilate strain also adhered significantly better than the non-pilate strain to fallopian tube (P<0.001; fig. 1). Both strains adhered strongly and to similar extents to human bronchus (P>0.05; fig. 1). The homogenates of these tissues generally contained only a small proportion of the recovered gonococci. About 2% of the total of each of the two strains was recovered from the homogenates of human endocervix and bronchus and the difference in behaviour of the two strains was not significant for either tissue (fig. 1).

About 2% of recovered strain-BS organisms were present in homogenates of human ectocervix in comparison with less than 1% of recovered strain-AL organisms. The recovery of strain BS in homogenates of human fallopian tube (c. 10% of the total) was greater than the recovery of this strain in the homogenates of any of the other tissues, but less than 1% of strain AL was recovered in homogenates of fallopian tube (fig. 1). There was little difference between the percentages of the inocula recovered after incubation of either strain on the endocervix or ectocervix, but strain AL survived significantly better than strain BS (P<0.001) on the fallopian tube (table 1). Thus the better adherence of strain BS to these tissues was not merely a consequence of a better survival of this strain during the test.

In some experiments, promptly after sonication to break up the clumps of
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Human endocervix

Human ectocervix

Guinea-pig urethra

FIG. 2.—The influence of inoculum on the adherence of strains BS (○) and AL (△) to the human endocervix, ectocervix and guinea-pig urethra. Viable gonococci recovered after vigorous washing of the tissues (see Materials and Methods) are expressed as percentages of the total numbers of viable organisms recovered after incubation for 1 h.

bacteria (see Methods) the suspensions were diluted 1 in 100 in PPCS to minimise re-clumping and incubated for 1 h at 37°C; the length of pili increased but the strength of attachment of strain BS to human endocervix, ectocervix and guinea-pig urethra was similar to that observed in control preparations not incubated for 1 h.

When the number of organisms inoculated on to the endocervix or ectocervix was adjusted to vary from $10^5$ to $10^7$ there was no apparent relationship between the inoculum size and the strength of attachment (fig. 2), but there was some variation in results of individual experiments at a given inoculum size. This indicated that the larger inocula were insufficient to saturate all sites on these tissues available for attachment by gonococci (cf. guinea-pig urethra).

Strains BS and AL adhered to the posterior urethra of the male guinea-pig, but there was poor adherence to the bladder (fig. 3), and no significant difference between their strengths of attachment to these tissues was found. About 3% of the total of each strain recovered was present in homogenates of the urethra. Other small-colony forming types (strain AS resembling Kellogg-type 1 and strain no. 005 resembling Kellogg-type 2) gave similar results on guinea-pig urethra and bladder to those obtained with strain BS; a large-colony-forming type (Kellogg strain, type 4) gave similar results to those obtained with strain AL.

There was some variation between individual experiments at a given inoculum size in the strengths of attachment of strains BS and AL to uterus and cervix (fig. 3). Both strains adhered to cervix with complex mucosal folds and with increased mucous secretion, but there appeared to be rather less adherence to specimens with fewer folds and practically no mucus. These cyclic changes in the cervical epithelium have been described in detail by Jurow (1943). No significant differences between the strengths of attachment of the strains were apparent on these tissues.
In contrast to the results obtained in studies with human endocervix and ectocervix, the strength of gonococcal adherence to guinea-pig urethra was influenced by the size of inoculum (fig. 2). The best attachment was obtained with inocula of relatively small numbers of organisms, and the sites available on the tissue surface for the attachment of organisms seemed to be saturated by inocula of $10^7$ gonococci.

The results of the adherence studies are summarised in table II.

**DISCUSSION**

The test used here to measure relative strengths of attachment is probably a valid one in relation to natural infection of mucosae. The gonococci were inoculated directly on to the epithelial surfaces of freshly isolated tissues and the strength of attachment was tested after only 1 h. Whilst it is true that subtle changes in the epithelium could have occurred during this period in some tissues and may have affected the adherence, no significant changes were

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**FIG. 3.—Adherence of strains BS and AL to guinea-pig tissues.** Viable gonococci in the gentle wash (G), vigorous wash (V) and the homogenate (H) are expressed as percentages of the total number of viable gonococci recovered after incubation for 1 h. The viable inocula varied from $1-2-3-3 \times 10^6$. The number of experiments is indicated in brackets and each vertical bar represents one standard error.
observed in the histological appearance of the excised tissues during this time. Thus, although we cannot be certain that the test model truly reflects the situation in vivo, we believe that we have approached it as nearly as possible in these tests. Any organisms settling on to the tissues but not attached were presumably removed by gentle washing and therefore only gonococci that had adhered to the mucosal surfaces were counted in the vigorous washes. In most cases a high proportion of the original inoculum was recovered and therefore any differences detected between the strains were assumed to be due to differential attachment rather than differential survival.

With most tissues the numbers of organisms recovered in the homogenates were roughly proportional to those recovered in the vigorous washes, the one exception being the high percentage of strain BS recovered by homogenisation of human fallopian tube. The proportion of organisms recovered in the homogenate was not taken as an index of the strength of adherence because it was not clear whether these organisms had been strongly attached to the tissue surface or embedded in mucus, or were within the tissue.

Our results summarised in table II show that both pilate (strain BS) and non-pilate (strain AL) gonococci adhered to all human and guinea-pig tissues. Although strain BS adhered significantly better than strain AL to human endocervix, which is a primary site for gonococcal attack in the female, differences in the behaviour of the two strains in this context were small and probably of little biological significance. The slightly better adherence of strain BS could have been due to its pilation, but the adherence of non-pilate organisms indicates that pili were not the only factors involved in adherence. In this respect it should be noted that gonococci in urethral pus appear to possess few pili comparable with those found on gonococci grown in vitro (Novotny et al., 1975). The difference in adherence of the two strains to human ectocervix, which is not

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Viable gonococci in the vigorous wash expressed as a percentage of the total number of viable organisms recovered with strain BS</th>
<th>Viable gonococci in the vigorous wash expressed as a percentage of the total number of viable organisms recovered with strain AL</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human endocervix</td>
<td>75±3†</td>
<td>58±3</td>
<td>+(P&lt;0.01)</td>
</tr>
<tr>
<td>Human ectocervix</td>
<td>64±3</td>
<td>24±3</td>
<td>+(P&lt;0.001)</td>
</tr>
<tr>
<td>Human fallopian tube</td>
<td>60±3</td>
<td>27±6</td>
<td>+(P&lt;0.001)</td>
</tr>
<tr>
<td>Human bronchus</td>
<td>86±5</td>
<td>81±4</td>
<td>-</td>
</tr>
<tr>
<td>Guinea-pig uterine horn</td>
<td>31±7</td>
<td>29±6</td>
<td>-</td>
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<td>Guinea-pig cervix</td>
<td>42±6</td>
<td>38±3</td>
<td>-</td>
</tr>
<tr>
<td>Guinea-pig male urethra†</td>
<td>59±2</td>
<td>52±2</td>
<td>-</td>
</tr>
<tr>
<td>Guinea-pig bladder</td>
<td>33±4</td>
<td>38±3</td>
<td>-</td>
</tr>
</tbody>
</table>

* Student's t test.
† Values represent mean count ± standard error.
‡ With inocula ≥ 10⁷, gonococci adhered poorly; these results are not included (see fig. 2).
infected in gonorrhoea and to fallopian tube, which is infected only after spread of gonococci from the cervix, was considerably greater than for the endocervix. Thus pili might be involved in adherence to these tissues, but even with these tissues there was some adherence of the non-pilate strain. It is possible that other factors could have affected adherence. Some of these, such as temperature of incubation and inoculum medium were standardised for all tissues in the tests but others such as pH, the availability of divalent cations and possible inhibitory substances could have varied from tissue to tissue and might have been responsible for some of the differences observed.

The fact that gonococci adhered to all the human tissues whether they are infected in gonorrhoea or not suggests that adherence does not play a primary role in tissue specificity, at least in the female. With regard to the relevance of adherence to host specificity, both pilate and non-pilate gonococci adhered to guinea-pig tissues, especially to the posterior urethra and to cervix with complex mucosal folds and active mucous secretion. It seems, then, that the insusceptibility of guinea-pigs to gonorrhoea is not determined by lack of adherence at a primary site.

These results suggest that, although adherence is likely to be a prerequisite for gonococcal invasion, the final outcome of infection is determined by factors other than adherence. One factor that might promote infection on the human endocervix is the presence of mucus. The ectocervix is covered with stratified epithelium with no mucus-secreting glands. Pommerenke (1946) found that cervical mucus enhanced the growth of some strains of gonococci in vitro, and our histological observations showed gonococci to be embedded in mucus on the endocervix. This mucus might provide protection and nourishment to gonococci in the early stages of infection and the possible importance of this observation is being investigated further.

**SUMMARY**

Strains of *Neisseria gonorrhoeae* adhered to pieces of human endocervix and appeared to be embedded in the surface mucus. Although a pilate strain adhered better than a non-pilate strain, the difference was small and pilation did not appear to be exclusively responsible for adherence. The pilate strain showed better adherence to pieces of human ectocervix and fallopian tube, but both strains were similarly adsorbed to human bronchus and guinea-pig uterus, cervix, male urethra and bladder, although to different degrees for different tissues.

Since gonococci adhered to all tissues examined, their ability to infect human endocervix and fallopian tube and their failure to infect human ectocervix or guinea-pig urogenital tract mucosae are determined by factors other than a capacity for primary adherence to the tissue.

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