Studies of ciliated epithelia of the human genital tract

3: Mucociliary wave activity in organ cultures of human Fallopian tubes challenged with *Neisseria gonorrhoeae* and gonococcal endotoxin

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SUMMARY Quantitative determinations of the mucociliary activity of human Fallopian tube epithelium maintained as organ cultures were performed using a light beam reflex method. In non-infected organ cultures the mucociliary wave (MCW) frequency slowly decreased during the first 54 hours of culture maintenance. In organ cultures experimentally infected with fresh isolates of *Neisseria gonorrhoeae* producing T1/T2 colonies the MCW frequency either decreased to subnormal values or completely ceased whereas in organ cultures infected with a laboratory-adapted gonococcal strain the MCW frequencies remained within normal range. In organ cultures exposed to gonococcal endotoxin prepared from the laboratory-adapted strain, as well as in cultures in which cell-free filtrates of medium from organ cultures infected with *N. gonorrhoeae* (producing T1/T2 colonies) were added to the culture medium, the ciliary activity decreased and subsequently ceased. The same phenomenon occurred when organ cultures were challenged with *Escherichia coli* endotoxin. The ciliostatic effect appeared before any morphological changes in the surface epithelium, including the cilia, were demonstrable by scanning electron microscopy.

Introduction

Infection of the Fallopian tubes occurs in 10-15% of women with cervical gonorrhoea (Falk, 1965; Rees and Annels, 1969). The frequency with which *Neisseria gonorrhoeae* causes the salpingitis in these patients, however, may be considerably lower (Mårdh *et al.*, 1978a). It is not known what factors promote dissemination of gonococci from the cervix to the tubes, but it is generally accepted that the route of spread is canalicular—that is, *Neisseria gonorrhoeae* infects the tubes from the lumen. In the naturally occurring disease, there is little opportunity for obtaining tubal specimens for studies of the

effects of gonococcal infections on the epithelial lining of the tubes, particularly in mild cases and in the early acute stage of the disease.

Organ cultures of human Fallopian tubes have been used for studies of experimental gonococcal infections (Carney and Taylor-Robinson, 1973; Taylor-Robinson *et al.*, 1974; Ward *et al.*, 1974; Ward and Watt, 1975; Mårdh *et al.*, 1976; McGee *et al.*, 1976, 1978).

Apart from morphological studies, organ cultures of Fallopian tubes provide a means for studying tubal ciliary activity (McGee *et al.*, 1976; Weström *et al.*, 1977). In experimental gonococcal infection of such specimens, determination of this activity has been made with the aid of light microscopy (Taylor-Robinson *et al.*, 1974; McGee *et al.*, 1976). Great precision in measuring ciliary function may be achieved by registering the light reflections from mucociliary waves (MCW) caused by beating cilia (Toremalm *et al.*, 1974).

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The aims of the present study were: (a) to study the mucociliary activity, as shown by the MCW pattern, in organ cultures of human Fallopian tube epithelium with the aid of the light beam reflex method; (b) to study the effect of experimental infections with *N. gonorrhoeae* and of gonococcal and *Escherichia coli* endotoxins on the ciliary activity of these specimens; and (c) to study the tubular epithelium maintained as organ culture and the interrelationship between the epithelium and gonococci by means of scanning electron microscopy (SEM).

Material and methods

TUBAL SPECIMENS

Fallopian tubes were obtained from women who had undergone hysterectomy because of non-malignant disease or who were sterilised for non-medical reasons. All women were aged less than 45 years and were still menstruating. None of the women had received hormonal or radiological treatment. None had gonorrhoea. The operations were performed on different days of the menstrual cycles.

ORGAN CULTURE TECHNIQUE

The organ culture technique used was that already described in detail (Mårdh *et al.*, 1976). In the present series of experiments, four specimens were prepared from the ampullary portion of each tube. Two specimens served as controls and two were challenged. The medium used to transport the Fallopian tubes and to maintain them as organ cultures consisted of Earle's balanced salt solution supplemented with amino acids, glutamine, sodium bicarbonate, and vitamins (Flow Laboratories Ltd, Irvine, Scotland). Trimethoprim and polymyxin B were added to the organ culture medium in the same concentrations as used in the gonococcal culture medium. These antimicrobials were present throughout the experimental period.

GONOCOCCI

Some of the strains of *N. gonorrhoeae* used to infect the organ cultures were clinical isolates harvested from the primary isolation plate containing a gonococcal medium which included 25 IU polymyxin B and 3 mg trimethoprim per ml (Mårdh *et al.*, 1978b). The cultures were incubated overnight at 37°C in an atmosphere of 7% CO₂ and of constant humidity. After overnight incubation a few gonococcal colonies were selected and scraped off the agar surface and suspended in the organ culture medium to a density of approximately 5×10^4 organisms/ml. This suspension was used to infect the organ cultures.

In other experiments, two strains (5 and 7) obtained from the Neisseriae Department, Statens Seruminstitut, Copenhagen, Denmark, were used. These strains had been passaged on solid gonococcal culture medium daily for more than 10 years. During the last few years, the strains were cultured on the medium already mentioned (Mårdh *et al.*, 1978b).

The type of the gonococcal colonies (Kellogg *et al.*, 1963) used to infect the organ cultures was determined by examining the solid culture medium with the aid of a stereomicroscope using oblique illumination. Strains 5 and 7 produced T4 colonies. The fresh isolates of gonococci used produced T1 or T2 colonies.

Specimens of medium were collected daily from the organ cultures during the maintenance period. In these, both the number of colony-forming units (cfu) per ml and the colonial type of the gonococci were determined. The tissue culture medium was replaced by fresh (antibiotic-containing) medium after 48 hours of maintenance of the organ cultures.

All organ cultures were checked daily for secondary infection by aerobic and anaerobic culture of the medium on blood agar plates.

In some experiments, medium from organ cultures experimentally infected with freshly isolated gonococci and from non-infected control cultures were collected after 30 hours' incubation of the organ cultures. The specimens were centrifuged and filtered through a 0.45 µm millipore filter (Millipore, Molsheim, France) and kept at -20°C until used to challenge Fallopian tube organ cultures using a concentration of 1/10 (v/v) in fresh medium.

PREPARATION OF ENDOTOXIN

Endotoxin was prepared from strain 5 of *N. gonorrhoeae* by the method described by Maeland (1968). The gonococci were grown for 24 hours on a solid medium (Mårdh *et al.*, 1978b); they were then harvested by sweeping the agar surface with a glass rod which was subsequently shaken in saline. The suspension was centrifuged ($1000 \times g$) for 20 minutes. After 1.15 g (determined as wet weight) of the bacteria had been washed twice and suspended in 4 ml of saline and 8 ml of diethyl-ether the suspensions were kept in sealed tubes at 4°C for 48 hours and occasionally shaken. After centrifugation at $4000 \times g$ for 30 minutes the water phase was collected and 2 volumes of cold acetone were added. The deposit was collected by centrifugation as described and dialysed, using a dialysis bag (pore diameter 24×10^{-1} nm, for five days in distilled water which was changed every second day. The yield was 1 ml per 1.15 g of bacteria. The preparation was diluted to 1/5 in saline and added to the organ culture medium at a final dilution of 1/1000.

E. coli (0111:B4) endotoxin prepared according to Westphal and co-workers (1952) was purchased from Difco (Difco 3122). The endotoxin was dissolved in distilled water to concentrations of 10 and 100 µg/ml before it was added to fresh organ culture medium to final concentrations of 0.2 and 2.0 µg/ml.

DETERMINATION OF CILIARY ACTIVITY

The pattern and the frequency of the mucociliary waves caused by the beating cilia were registered by a light beam reflex method (Håkansson and Toremalm, 1965). The specimens were placed in a chamber at a steady temperature of 37°C and a relative humidity of 90%. A fibre optic system (Schott-Mainz, model KL 150, Zeiss, West Germany) was used as light source. The light reflections from suitable spots on the tubal epithelial surface were focused by means of an operating microscope (model OPMI 1, Zeiss), which had a diaphragm with a central aperture of 0.1 mm in diameter mounted on the ocular. A photomultiplier (EMI 9524 B, Middlesex, England) was fitted on the top of the ocular diaphragm. The photomultiplier was fed with 1000 V d.c. from a power supply (Oltronix A 2.5L-10 HR, Stockholm). The impulses were then filtered with a Krohn-Hite 3550 filter (Cambridge, Massachusetts, USA) in band pass mode 3-30 Hz. An Elema-Schönander Mingograph 34 (Stockholm) was used for recording the light reflection variations.

The MCW activity of both non-infected and experimentally challenged tubal specimens was registered after the organ cultures had been maintained for 6, 18, 30, 42, and, in some instances, 54 hours. For each specimen studied the numerical value given for the MCW frequency was the mean of six registrations from different spots on the tubal epithelium.

SPECIMEN PREPARATION FOR SCANNING ELECTRON MICROSCOPY

Organ culture specimens were fixed, dried, and subjected to critical-point drying (Mårdh *et al.*, 1976). After drying, the specimens were mounted on specimen stubs, covered with gold-palladium in a vacuum evaporator, and examined in a Cambridge Stereoscan Mark II A scanning electron microscope.

Results

MCW FREQUENCIES IN ORGAN CULTURES

Non-infected

The MCW frequencies of Fallopian tube epithelium in 54 non-infected organ cultures from 28 tubes of 14 patients were determined after 6, 18, 30, 42, and 54 hours of culture maintenance. The mean values and standard deviations obtained are shown in Figure 1.

Fig. 1 Mean value (mean) and standard deviation (SD) of MCW frequencies in 54 non-infected Fallopian tube organ cultures from 28 Fallopian tubes maintained for 54 hours. At each registration, the MCW frequency on six different areas of the specimen was registered.

These values were used for reference when studying the experimentally infected organ cultures. In the non-infected reference cultures, the MCW frequencies decreased from a mean of 1161 cycles per minute (c/min) to 894 c/min between six and 54 hours of culture maintenance. In no experiment was a frequency <600 c/min observed. When the frequency had decreased to about 600 c/min the ciliary activity in these non-infected specimens suddenly ceased.

In analyses of the MCW frequencies of the non-infected and experimentally infected organ cultures of epithelium from the same Fallopian tube, only experiments in which the control organ cultures had frequencies within the reference range are reported.

Experimentally infected with N. gonorrhoeae

In four out of eight organ cultures experimentally infected with freshly isolated gonococci (producing T1/T2 colonies), all ciliary activity ceased within six hours, in a further two within 30 hours, and in the remaining two before 42 hours (Table). MCW frequencies within the normal range were not seen after 18 hours.

In all six organ cultures experimentally infected with the gonococcal strain 5, ciliary activity was detectable after 54 hours of culture maintenance (Table). In 26 out of 30 registrations on infected tubal specimens, the MCW frequencies were within the reference range although they were lower than in the control cultures (Figure 2). The differences between the mean values of control and infected organ cultures increased with time (Figure 2).

Challenged with cell-free filtrates

When Fallopian tube organ cultures were maintained

Table Mucociliary wave activity in Fallopian tube organ cultures

Organ culture challenged with	MCW frequency*	Hours of organ culture maintenance				
		6	18	30	42	54
Freshly isolated gonococci (n = 8)	Within NR†	4	1	0	0	
	Below NR	0	3	2	0	
	None	4	4	6	8	
Laboratory-adapted gonococci (n = 6)	Within NR†	6	6	6	4	4
	Below NR	0	0	0	2	2
	None	0	0	0	0	0
Cell-free filtrates of other cultures infected with <i>N. gonorrhoeae</i> (n = 10)	Within NR	1	0	0	0	
	Below NR	5	6	4	1	
	None	4	4	6	9	
Gonococcal endotoxin (n = 14)	Within NR	6	4	0	0	0
	Below NR	5	5	8	7	4
	None	3	5	6	7	10
<i>E. coli</i> endotoxin (µg/ml) (n = 8):						
	0.2					
	2.0					
	0.2					
	2.0					
	0.2					
	2.0					
	2.0					

* Mean of six determinations on each specimen at the four (or five) indicated readings

† Only experiments in which the control organ cultures had frequencies within the reference range are reported

NR = normal range; n = no. of experiments

Fig. 2 Mean differences between the MCW frequencies of non-infected Fallopian tube organ cultures (○) and simultaneously performed cultures experimentally infected with a laboratory-adapted strain (strain 5) of *Neisseria gonorrhoeae* (●). (The straight lines indicate mean $\pm$ 2 SD in non-infected cultures.)

in medium containing cell-free filtrates from other cultures infected with freshly isolated gonococci producing T1/T2 colonies the ciliary activity in nine out of 10 organ cultures ceased within 42 hours (Table). Thus the MCW frequencies in eight out of 17 recordings with detectable activity were below 600 c/min—that is, 300, 365, 380, 415, 420, 425, 516, and 581 c/min.

In those organ cultures to which cell-free filtrates from non-infected cultures had been added, the

MCW frequencies remained within normal range throughout the period of culture maintenance.

Challenged with gonococcal endotoxin

In none of the 14 tubal organ cultures challenged with gonococcal endotoxin did the MCW frequency remain within the reference range for more than 18 hours (Table). In these specimens the decrease in ciliary activity was gradual. In 29 out of 39 recordings with detectable activity, the MCW frequencies were below the lower limit for the reference range. The subnormal MCW activity ranged between the lower limit for the reference curve and 403 c/min. After 54 hours of culture maintenance, no ciliary activity could be detected in 10 of the 14 organ cultures whereas in all non-infected control cultures the MCW activity was still within the reference range.

Challenged with *E. coli* endotoxin

In all eight organ cultures challenged with *E. coli* endotoxin at a concentration of 0.2 µg/ml medium, the ciliary activity ceased within 42 hours (Table). Similar results were obtained when a concentration of 1.0 µg/ml of the same endotoxin was used. In all but four of the 64 recordings the ciliary activity was either within normal range or entirely absent (Table). The lag before ciliary activity stopped was the same in the experiments with the two different concentrations of endotoxin used.

SCANNING ELECTRON MICROSCOPY

In non-infected Fallopian tube organ cultures, scanning electron microscopy showed generally normal surface structures throughout the culture maintenance period—that is, 54 hours.

In the organ cultures infected with *N. gonorrhoeae*, the mucosal surface structures did not show any changes as regards the non-infected cultures during at least 48 hours of incubation.

No morphological differences between the cilia of infected and non-infected organ cultures could be demonstrated. Scanning electron micrographs showed an unchanged morphology of the cilia throughout the maintenance period of the organ cultures (Fig. 3).

During the first 48 hours of incubation of the organ cultures, the gonococci generally formed micro-colonies on the surface of the secretory cells but never of ciliated cells (Fig. 3). After 48 hours the number of gonococci on the mucosal surface decreased.

When the organ cultures were infected with gonococci forming T1/T2 colonies their diplococcal structure was clearly visible. The gonococci in the micro-colonies were interspersed by fine threads (Fig.

4a). The surface of the gonococci was ragged and, on some, small buds or protrusions could be seen (Fig. 4b).

When gonococci producing T3/T4 colonies were used to infect the organ cultures micro-colonies were also seen. Threads or protrusions did not occur, and the surfaces of the bacteria seemed 'naked' compared with those of gonococci producing T1/T2 colonies (Fig. 5).

RECOVERY OF GONOCOCCI FROM INFECTED ORGAN CULTURES

The numbers of cfu of gonococci which were re-isolated from the media of the organ cultures infected with gonococci producing T1/T2 colonies are given in Figure 6. In 11 out of 17 samples collected during the first 30 hours of incubation, the number of cfu per ml medium was 1-3 log higher than the infective dose, that is, approximately 5×10^4 cfu per ml. At 48 hours the mean number of cfu per ml medium was the same as the infective dose. At 72 hours—that is, 24 hours after changing the tissue culture medium—the samples contained 10^2 - 10^5 cfu per ml (Fig. 6).

Fig. 3. Scanning electron micrograph of micro-colony of gonococci (producing T4 colonies) on the surface of secretory cells of Fallopian tube epithelium maintained as organ culture for 48 hours. (Experimental infection.) ($\times 6000$).

Fig. 4 Scanning electron micrographs of: (a) gonococci (producing T2 colonies) showing diplococcal structure with ragged surface and 'threads' between bacterial cells ($\times 24\,000$ original magnification); (b) buds or protrusions on gonococcal cells producing T2 colonies ($\times 24\,000$ original magnification). (Experimentally challenged organ cultures of Fallopian tube epithelium maintained for 24 hours.)

In organ cultures infected with gonococci of colonial type T3/T4, no notable increase in the number of organisms was observed during the first 30 hours of incubation (Fig. 6).

No living gonococci could be reisolated after 24 hours of incubation of pure organ culture medium when an inoculum containing the same number of organisms was used to infect the organ cultures (Fig. 6).

In all cultures infected with gonococci producing T1/T2 or T3/T4 colonies, the gonococci reisolated from the organ culture media invariably produced colonies on the primary isolation plate which were of the same morphological type as the organisms used to infect the cultures. The aerobic and anaerobic control cultures performed daily on blood agar plates for detection of contaminants in the organ culture media all gave negative results, except for two

Fig. 5. Scanning electron micrograph of micro-colony of gonococci producing T4 colonies on the surface of Fallopian tube epithelium maintained as organ culture for 24 hours showing 'naked' appearance of the bacterial cells. ($\times 12\,000$).

Fig. 6. Number of colony-forming units of gonococci reisolated from the maintenance medium of Fallopian tube organ cultures experimentally infected with approximately 5×10^4 organisms per ml medium of gonococci producing T1/T2 and T3/T4 colonies. Arrow denotes time of replacement of organ culture medium by fresh sterile medium. The broken line (---) shows the recovery of *N. gonorrhoeae* from pure maintenance medium inoculated with an equal number of T2 colony-producing gonococci.

experiments, the results of which are not reported here.

Discussion

The light beam reflex method reproduces mucociliary wave movements accurately (Toremalm *et al.*, 1974). Variations in the ciliary beat frequency produce corresponding changes in the MCW pattern (Håkansson and Toremalm, 1965). The method has been used for quantitative determination of the ciliary activity in human Fallopian tubes maintained as organ cultures (Weström *et al.*, 1977). In premenopausal women the MCW frequency did not vary during the menstrual cycle and in different parts of the tubes.

Compared to earlier methods used to assess the ciliary activity in tubal organ culture specimens (McGee *et al.*, 1976), the light beam reflex method has several advantages. It offers reproducible numerical values of the MCW activity. Thus the measurements are not dependent on subjective estimations. It also allows rapid registrations and can be performed on any part of the tubal epithelium.

In the present study only specimens from the ampullary portion of the tubes were used, mainly

because they were more easily dissected out than other parts of the tube. Registrations were made from six different areas of the epithelium on each specimen. In each registration, the equipment used covered about 50 ciliated cells. The values of the MCW frequencies were the mean of these six determinations. The deviation from the mean in each of the six registrations never exceeded 50 cycles/min.

The reference values for the MCW frequencies were derived from about 1500 registrations. Only tubal specimens in which the non-challenged controls showed frequencies within the reference range were used. As previously evaluated (unpublished data), the presence of the two antimicrobials added to the culture medium did not affect the MCW frequencies.

In the non-challenged Fallopian tube organ cultures the MCW pattern showed a slow decrease from a mean of 1161 to 894 c/min during the first 54 hours of culture maintenance. The decrease was most probably not due to a reduction in the number of beating cilia, since MCW frequencies in areas of tubal epithelium with a low ciliary density (isthmus) and maintained as organ cultures for an equally long period of time did not differ from those of areas with a high ciliary density (infundibulum) (Weström *et al.*, 1977). The decrease in the MCW frequencies in the non-challenged specimens during culture maintenance corresponded approximately to observations by McGee *et al.*, (1976), who determined the ciliary activity in the same type of organ cultures under a light microscope. The gradual decrease in ciliary activity might be explained by rheological changes in the secretion layer covering the ciliated surface. The sudden cessation of ciliary activity at frequency of about 600 c/min after more than 54 hours of culture maintenance might be due to a depletion of mitochondrial ATP in the ciliary cells.

In organ cultures infected with gonococci producing T1/T2 colonies, the reisolation studies showed a rapid multiplication of the organisms during the first 30 hours of culture maintenance. Corresponding studies on the laboratory-adapted strains producing T3/T4 colonies showed that in no case did the number of cfu/ml exceed that present in the medium at the start of the experiment. Using light microscopy with oblique illumination, the colonial morphology of reisolated gonococci was the same as that of the organisms used to infect the cultures.

SEM showed that gonococci of all the strains studied seemed to become attached to the epithelial surface to form micro-colonies, although they never attached to ciliated cells. Gonococci producing T1/T2 colonies had buds or protrusions on the cell surface and fine threads interspersed between the organisms, as seen in scanning electron micrographs. Such structures were not demonstrable in laboratory-

adapted gonococci. The nature of the structures is not known, but whether or not the threads represent pili is worth consideration.

In this study, no morphological changes of the surface epithelium, including the ciliated cells, were shown by SEM during the 54 hours of culture maintenance. When using Fallopian tube organ cultures experimentally infected with *N. gonorrhoeae* (producing T4 colonies), Taylor-Robinson *et al.* (1974) noted that loss of ciliary activity preceded any damage to the ciliary cells.

In the present study, gonococci producing T1/T2 colonies caused a decrease and subsequent cessation of ciliary activity of the tubal epithelium whereas the laboratory-adapted strains did not. McGee *et al.* (1978) recently reported similar findings. These workers also found that cell-free filtrates (Johnson *et al.*, 1977) and gonococcal endotoxin (McGee *et al.*, 1978) affected the ciliary activity of tubal epithelium maintained as organ culture.

In this study, cell-free filtrates of medium from Fallopian tube organ cultures experimentally infected with gonococci producing T1/T2 colonies as well as endotoxin from a laboratory-adapted strain of gonococci had an effect on the ciliary activity similar to that of gonococcal cells producing T1/T2 colonies. The difference in the effects on the MCW frequencies in organ cultures challenged with whole gonococcal cells and those challenged with endotoxin from the same T4 colony-producing strain may be due to only small amounts of endotoxin being released from the bacterial cells. In contrast to the T1/T2 colony-producing gonococci, this laboratory-adapted strain did not multiply as indicated by the reisolation studies, which suggests a slow cell turnover and subsequent small release of endotoxin.

Reimar *et al.* (1978) were unable to document any effect of *E. coli* endotoxin on the MCW activity in organ cultures of rabbit tracheal epithelium, even when a concentration as high as 1 mg/ml was used. Their period of observation was only five hours. In our study, *E. coli* endotoxin produced cessation of ciliary activity within six hours in half of the experiments irrespective of whether 0.2 or 2.0 µg/ml was used. In 15 of 16 experiments, no MCW activity had been detected after the organ cultures had been maintained for 30 hours. No gradual decrease in MCW activity was observed, which contrasted with the experiments using *N. gonorrhoeae* endotoxin. In the 40 determinations at various times using gonococcal endotoxin, 25 showed an MCW frequency below the normal range, which was in contrast to only four of 64 determinations using *E. coli* endotoxin. The difference in the effect of the two endotoxins might be dose-dependent but could also be due to a differential activity.

Whether the ciliostatic effect shown by gonococcal and *E. coli* endotoxins in our *in-vitro* study also occurs *in vivo* in the naturally diseased host is not known, but, if so, the endotoxins might promote the adherence of invading organisms to the mucosal lining of the Fallopian tubes.

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