Morphology of human Fallopian tubes after infection with *Mycoplasma genitalium* and *Mycoplasma hominis—in vitro* organ culture study

Agata Baczynska[1,5], Peter Funch[2], Jens Fedder[3], Hans Jørgen Knudsen[4], Svend Birkelund[1] and Gunna Christiansen[1]

[1]Institute of Medical Microbiology and Immunology and [2]Department of Biological Sciences, University of Aarhus, Aarhus C, Denmark, [3]The Fertility Clinic and Scientific Unit, Horsens-Braedstrup Hospital, Horsens, Denmark and [4]Department of Gynecology and Obstetrics Horsens Hospital, Horsens, Denmark

[5]To whom correspondece should be addressed at: Institute of Medical Microbiology and Immunology, University of Aarhus, The Bartholin Building, 8000 Aarhus C, Denmark. Tel: +4589421747; E-mail: agata@medmicro.au.dk

BACKGROUND: Female infertility can be caused by scarring and occlusion of the Fallopian tubes. Sexually transmitted bacteria can damage the delicate epithelial layer of human Fallopian tubes (HFT). Genital mycoplasmas are associated with human reproductive failure. Yet, there is not enough evidence that mycoplasmas can cause tubal factor infertility. We analysed the effects of infections with *Mycoplasma hominis* and *Mycoplasma genitalium* on the HFT epithelium and compared them with the effects of infections with genital pathogens: *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. METHODS: We used an *in vitro* model in which pieces of normal HFT were infected with different bacteria, and the outcome of the infections was analysed by scanning electron microscopy (SEM) and confocal microscopy. RESULTS: The presence of *M. hominis* did not cause any morphological changes of the epithelium of HFT. Noticeable changes in the morphology of the ciliated cells were observed in *M. genitalium*-infected tissue. Five days post-infection, the cilia were abnormally swollen and some of the ciliated cells fell off the epithelium. These effects could be inhibited by pre-incubation of *M. genitalium* with antibody directed against the C-terminal part of the adhesion protein MgPa before infection of HFT organ culture. CONCLUSION: We have shown that the presence of *M. genitalium*, but not *M. hominis*, in the HFT organ culture affected the epithelium and resulted in cilia damage. The effect of infection with *M. genitalium* on the HFT was, however, very moderate when compared with the extensive damage of the epithelium caused by *N. gonorrhoeae* or *C. trachomatis*.

Key words: *Chlamydia trachomatis*/*Mycoplasma hominis*/*Mycoplasma genitalium*/*Neisseria gonorrhoeae*/SEM/Fallopian tubes/organ culture

Introduction

Damage of Fallopian tubes involving tubal occlusion or tubal fibrosis is one of the major causes of female infertility, denoted tubal factor infertility (TFI). The damage is most commonly due to genital tract infection ascending from the lower genital tract to the upper genital tract. Such infection can cause pelvic inflammatory disease (PID) (Akande, 2002). PID is a microbial infection of the upper genital tract and can include endometritis, parametritis, salpingitis and tubo-ovarian abscess. The bacterial causes are mainly *Chlamydia trachomatis, Neisseria gonorrhoeae* and aerobic and anaerobic vaginal flora including bacterial vaginosis (BV) flora (Crossman, 2006). In the developed world, the most common cause of tubal damage is *C. trachomatis* (World Health Organization, 1995; ESHRE, 1996).

The epithelium of the Fallopian tubes is composed mainly of ciliated columnar cells and non-ciliated secretory cells. For the proper function of the tube, ciliated cells need to push, by beating, the secretion from secretory cells together with an oocyte from one of the ovaries towards the uterus. Physiological ciliation and deciliation of the tubes take place all the time during the menstrual cycle, with estrogen enhancing ciliation and progesterone deciliation (Donnez *et al*., 1985). However, when the delicate epithelium layer of tubes is damaged by infection and inflammation during salpingitis, ciliated cells permanently reduce cilia in number and quality (Donnez *et al*., 1984). A study made by Patton *et al*. (1989) showed that two groups of infertile women, one with a history of overt salpingitis and the other with silent salpingitis without medical history, had abnormal epithelium of the Fallopian

tubes, including flattened mucosal folds, extensive deciliation and degeneration of secretory cells (Patton *et al.*, 1989). The patients had no signs of active disease at the time of specimen removal, which confirms that the damage done to the tubal epithelium during salpingitis was irreversible. Such damage may lead to TFI and increase the risk of ectopic pregnancy.

Not all salpingitis cases can be explained by infection with *N. gonorrhoeae* or *C. trachomatis*; therefore, many studies have been undertaken to verify whether other bacteria can be found in the tubes of salpingitis patients. The recovery of *Mycoplasma hominis* direct from the Fallopian tubes of the women with acute salpingitis (Mårdh and Weström, 1970a), followed by the generation of an antibody response to *M. hominis* in the same patient (Mårdh and Weström, 1970b), is a major evidence associating this organism with tubal infections. *M. hominis* is strongly associated with BV. It was found in at least two-third of women with BV, in comparison to just 10% of healthy women (Rosenstein *et al.*, 1996). The retrospective serological analyses for the presence of antibodies against *M. hominis* in the PID patients (Møller *et al.*, 1985) and in the infertile women (Baczynska *et al.*, 2005) showed that women with PID or tubal infertility had antibodies to *M. hominis* considerably more often than the controls. Surprisingly, different studies showed a very low prevalence (2.1 and 2.4%) of *M. hominis* in the lower genital tract of infertile patients (Witkin *et al.*, 1995; Baczynska *et al.*, 2004). Thus, there is a possibility that *M. hominis* is rather an 'accompanying' bacterium that follows the pathogenic species to the upper genital tract.

Less is known about the possible implications for human reproduction by infections with *Mycoplasma genitalium*. The micro-organism was first isolated in 1981 (Tully *et al.*, 1981), and it has been studied very extensively during the last few years. In a study by Cohen *et al.* (2005), it was found that *M. genitalium* can ascend into human Fallopian tubes (HFT), and its DNA was detected direct from the Fallopian tube and endometrium specimens of a patient suffering from mild salpingitis (Cohen *et al.*, 2005). A serological study made by Clausen *et al.* (2001) on serum samples from infertile women showed association between the presence of antibodies against *M. genitalium* and TFI. In its structure and morphology, *M. genitalium* is very similar to another human pathogen *Mycoplasma pneumoniae*, which exists on the epithelium in the respiratory tract and causes pneumonia. Both organisms have a specific attachment organelle, known as 'tip-structure', which mediates contact with host cell membranes (Razin and Jacobs, 1992). As surface parasites, they require an effective attachment apparatus including adhesion proteins such as MgPa in *M. genitalium* and P1 in *M. pneumoniae*. Upon attachment to the hamster tracheal epithelium, *M. pneumoniae* was shown to cause ciliostasis as well as cilia loss (Chandler and Barile, 1980).

Both *M. hominis* and *M. genitalium* have been associated with genital tract disorders, yet the exact mechanism of pathogenesis and the influence of genital mycoplasmas on the epithelium of the Fallopian tubes have not been revealed. *M. hominis* was previously studied in two different organ culture studies; however, there is a disagreement between the results

of these studies (Taylor-Robinson and Carney, 1974; Mårdh *et al.*, 1976). Therefore, we aimed to study the effect of infection with *M. genitalium* and *M. hominis* in an *in vitro* organ culture model. The pieces of HFT were infected with genital mycoplasmas and, for comparison, with *C. trachomatis* and *N. gonorrhoeae*. The results of the infections were analysed by SEM and confocal microscopy. The damage done to the epithelium was compared among the different bacteria.

Materials and methods

Fallopian tube tissue samples
HFT pieces were obtained with consent from five non-pregnant, premenopausal women during the course of a hysterectomy for surgical indications. The operations were planned in advance and performed after the menstrual bleeding. Thus, epithelial cells were in the estrogenic phase. The surgeons performing operations in Horsens Hospital were asked specifically to include only patients without salpingitis. The tubes were examined visually upon operation and removal from each individual patient. After the HFT tissue was transported to the University of Aarhus, the epithelium of the tubes was examined for the second time under a dissecting microscope (Leica Stereozoom, Leica Microsystems, Heerbrugg, Switzerland). There was no occlusion of the Fallopian tubes, and there were no signs of salpingitis. The project was accepted by the local Ethical Committee Journal no. VF20050074.

Culture of the pieces of HFT
Directly after the operation, the parts of salpinges were transported from Horsens Hospital to the University of Aarhus, where the culture, infection and analyses of HFT were performed. The salpinx samples were placed in a 50 ml tube (NUNC, Roskilde, Denmark) with the transporting medium Dulbecco modified Eagle's medium (DMEM) mixed 1:1 with Ham's F12 medium (Invitrogen, Grand Island, USA) substituted with 10% of fetal bovine serum and antibiotics: 10 μg/ml of gentamycin (Schering-Plough, Kenilworth, US) and 0.25 μg/ml of amphotericin B (Fungizone®, Invitrogen). Upon arrival, the HFT samples were trimmed of the adventitial tissue, opened longitudinally and cut into pieces of ~4 mm². The tissues were observed under the magnification of a dissecting microscope Leica Stereozoom (Leica Microsystems). The tissue pieces were placed in Nunclon cell culture dishes 60 × 15 mm in size (NUNC), two or three pieces in each dish, and incubated with 12 ml of the DMEM:F12 medium with antibiotics to reduce the risk of contamination during transport and tissue manipulation. All incubations of the tissue were performed in the CO_2 incubator at 35°C. After 24 h, one or two pieces (controls at day 1) were fixed and processed for SEM; the remaining tissue pieces were ready for infection with different micro-organisms.

Micro-organisms used in the study
Four different micro-organisms were used in the study: *M. hominis*, *M. genitalium*, *C. trachomatis* and *N. gonorrhoeae.* Two *M. hominis* isolates were selected and incubated in the BEa medium (Freundt *et al.*, 1979): old clinical isolate 4195 (Lin and Kass, 1974) with the high number of passages and a recent clinical isolate p.139 (unpublished) with few passages. After 48 h of incubation, the medium changed colour from orange to pink and *M. hominis* was harvested. *M. genitalium* isolate G37 American Type Culture Collection (ATCC) was used in the study and cultured in 10 ml SP-4 (Tully *et al.*, 1979) medium in TTP tissue culture flasks (MediCult, Jyllinge, Denmark). After 48 h, the SP-4 medium changed from red to orange,

and the cells were scraped off in phosphate-buffered saline (PBS). By centrifugation at 20 000×g for 30 min, 1 ml of both *Mycoplasma* suspensions (approximate concentration of 1×10^7 Colour Changing Units/ml, calculated from titration assay) was harvested. The pellets were washed in PBS and resuspended in 1 ml of DMEM:F12 antibiotics-free medium.

A clinical isolate of *N. gonorrhoeae* was cultured on the chocolate agar plates (Statens Serum Institut, Copenhagen, Denmark) from a culture frozen in trypticase soy broth with 20% glycerol (BBL Microbiology Systems, Cockeysville, USA). Additional passages on new plates were avoided. Prior to infection, fresh colonies between 24 and 48 h old were scraped off the plates with 1 μl inoculation loops and suspended in 1 ml DMEM:F12 antibiotics-free medium. The approximate concentration of *N. gonorrhoeae* undiluted suspension was 10^7 CFU/ml, calculated from previous titration experiments.

Finally, *C. trachomatis* D (UW-3/Cx) was used and suspended in DMEM:F12 to the approximate concentration of 7×10^4 inclusion forming unit (IFU)/ml.

Infection procedure

After the pieces of HFT had been maintained in the DMEM:F12 medium with antibiotics for 24 h, the tissues were transferred into new dishes. Only antibiotic-free DMEM:F12 medium was used for all infection experiments. In all experiments, 12 ml of medium was used. Two HFT pieces were left uninfected and kept in culture for another 5 days. They served as controls at the end of the experiment.

Infections with genital mycoplasmas were performed by adding 200 μl of *M. hominis* or *M. genitalium* suspension to 12 ml of fresh medium. At least two HFT pieces were used for each infection with genital mycoplasmas: one of the pieces was designated for SEM and the second one for immunofluorescence (IMF) analysis of the HFT 5 μm sections.

Experiments designated to inhibit the adhesion of *M. genitalium* to HFT tissue by pre-incubation of *M. genitalium* with antibodies directed against the adhesion protein MgPa were performed as described elsewhere (Svenstrup *et al.*, 2002). Briefly, 200 μl of *M. genitalium* suspension was pre-incubated for 30 min with 200 μl of antibodies against the C-terminal part of MgPa, rMgPa-III, diluted 1:50. The HFT pieces were then infected with the mixture of bacteria and antibodies.

Infection of the tissue with *N. gonorrhoeae* was performed by adding 60 μl of undiluted *N. gonorrhoeae* suspension to 12 ml of medium (dilution 200×). *C. trachomatis* D was diluted in 12 ml of fresh medium to the concentration of 7×10^4 IFU/ml. Both bacteria were used in our study as controls for their ability to cause tubal damage.

The infectious medium was removed and fresh medium was added to all the pieces 24 h post-infection with different bacteria. The medium was changed daily during the entire culture. For adherence inhibition assay of *M. genitalium*, each new medium contained a fresh dilution of rMgPa-III antibody. Each day, 1 ml of the medium removed from the tissue pieces infected with genital mycoplasmas was collected and kept for analysis of viability of bacteria. At day 5 post-infection, the pieces of HFT tissue were processed for SEM and IMF.

Sample preparation for SEM

Prior to fixation, the tissues were washed gently in PBS and in 0.1 M sodium cacodylate buffer (pH 7.2). Washing steps performed prior to fixation were necessary to remove contaminating materials such as mucus, secretions, red blood cells, non-adherent bacteria, broken cell debris and salt (Bozzola and Russell, 1999). All the fixatives were diluted in the sodium cacodylate buffer. The pieces were incubated for 24 h with 2% of glutaraldehyde (Sigma, St Louis, USA) at 4°C, followed by rinsing in cacodylate buffer for 10 min at least and

additional post-fixation in 2% of OsO$_4$ (Merck, Darmstadt, Germany) for 1.5 h at room temperature. After fixation, the tissues were rinsed in double-distilled H$_2$O and dehydrated in increasing concentrations of ethanol: 20, 40, 60, 70, 80 and 90, each step for 15–20 min, finishing with twice in 96% for 15–20 min. The tissues were kept in 96% ethanol at -20°C prior to the critical point drying CPD, using carbon dioxide. Thus, prepared samples were then coated with gold by Pirani 10 Coater sputter (BOC Edwards, Crawley, UK) and examined with SEM MaXim (CamScan, Waterbeach, UK) at 20–30 kV using a secondary electron detector.

IMF analysis of HFT sections

The pieces of HFT after culture with or without micro-organisms were prepared for IMF examination by confocal microscopy. Each tissue piece was quick-frozen on dry ice, embedded in Tissue-Tek O.C.T Compound (Sakura Finetek, Zoeterwoude, Netherlands) and stored at -70°C prior to cutting. Sections of 5 μm were cut on the cryomicrotomy Microm HM 500 M (Microm, Walldorf, Germany) at -20°C. The sections were placed on the microscope slides, Super Frost Plus (Menzel-Glaser, Braunschweig, Germany), and after 1 h at room temperature, they were frozen at -20°C avoiding humidity. The sections were fixed with acetone for 5 min and the borders of each sample were marked with Pap-pen (Dako, Glostrup, Denmark). Fixed samples were washed with PBS for 5 min and blocked for 20 min with normal goat serum (Dako) diluted 1:10 in PBS. The samples were washed four times with PBS and reacted with rabbit polyclonal antibodies against whole cells of *M. hominis* or *M. genitalium* for 30 min at room temperature (both diluted 1:1000). This was followed by washing steps and incubation with goat anti-rabbit antibody conjugated with fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch Laboratories, West Grove, USA) for 30 min. Secondary antibodies were diluted 1:100.

To evaluate the specificity of the primary antibodies, we used controls where HFT pieces infected with mycoplasmas were incubated with normal serum of pre-immunized rabbit instead of the primary antibody. Ewans Blue dye was used to stain the HFT tissue. Antifade solution was used for mounting glass cover slips onto the slides. Confocal images of the tissue sections were obtained using a Leica SP2 laser confocal microscope (Leica Microsystems) with an oil lens Leica HCX PL APO 100× and aperture 1.40.

Survival of mycoplasmas in the DMEM:F12 medium

The ability of mycoplasmas to survive in the DMEM:F12 medium in the presence or absence of HFT tissue was examined. At each day of organ culture, 1 ml of medium from organ culture infected with *M. hominis* or *M. genitalium* was collected.

To test whether mycoplasmas could survive in the pure DMEM:F12 medium in the absence of the HFT tissue, we used the same dilutions of mycoplasmas as were used for infection of the organ culture.

We also examined the medium from *M. genitalium*-infected tissue incubated in the presence of antibodies against MgPa at each day of culture. Aliquots of different media, containing mycoplasmas, were collected every 24 h.

These aliquots were then incubated in the BEa or SP-4 medium at 37°C for 48–72 h. Change of colours in the BEa and SP-4 media indicated the presence of live bacteria.

Results

Influence of different micro-organisms on the Fallopian tube tissue examined by SEM

We infected the pieces of HFT with different bacteria for a period of 5 days. The tissues were then fixed and prepared

for SEM analysis, which revealed differences in destruction of epithelial cells, cell types being affected and toxic effect on the entire tissue.

We used two types of uninfected controls: the first control was fixed after primary incubation for 24 h in the medium containing antibiotics and the second control after 5 additional days of incubation in antibiotic-free medium. The first control was prepared to observe the condition of the arriving tissue from each individual patient before the infections were carried out. The second control was kept until the end of all experiments and was prepared in order to check whether the organ culture kept in the pure medium for 6 days showed any damage to the HFT epithelium. Figure 1 shows examples of the two uninfected HFT controls after 1 (Figure 1A and B) and 6 days of incubation (Figure 1C and D). The mucosal surface of the controls remained undisrupted, with many

ciliated cells in the preparation. The secretory cells were covered by microvilli. The cilia of the ciliated cells were morphologically normal, thin, long and smooth and did not adhere to each other. The ciliated cells had a high number of cilia per cell. The controls showed that salpinx pieces from all patients were normal and in good condition upon arrival, and the 6 days organ culture introduced no abnormal changes or harm to the HFT epithelium.

We used *C. trachomatis* and *N. gonorrhoeae* as positive controls in our model because their ability to cause tubal damage is well known. *C. trachomatis*-infected tissue is shown in Figure 2A and B. The micro-organisms affected both ciliated and secretory cells. Many cells in the preparations had broken membranes, and 'chlamydia-like'-structures were seen inside the disrupted cells (Figure 2B). Similar observations were shown in other organ culture studies where HFT tissue
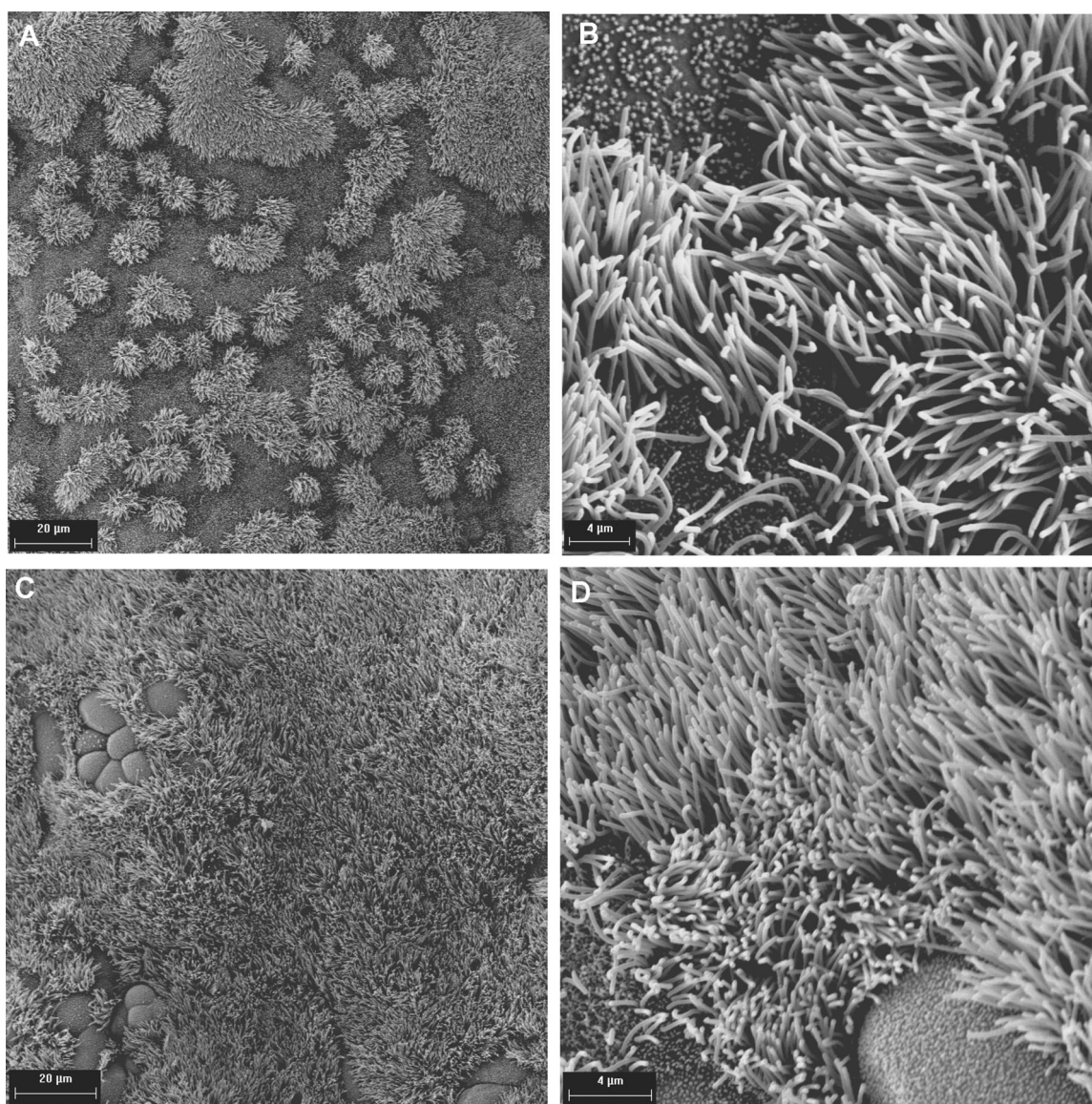

Figure 1. SEM of the human Fallopian tubes (HFT) epithelium—uninfected controls. (**A** and **B**) Uninfected HFT after 1 day of incubation in the medium containing antibiotic. (**C** and **D**) Present tissue at the end of experiment after 6 days of organ culture. All four pictures show undisrupted mucosal surface, with many ciliated and secretory cells. Normal morphology of the ciliated and secretory cells can be observed. Ciliated cells carry a large number of cilia per single cell.

was infected with *C. trachomatis* (Cooper *et al.*, 1990). Holes were observed mainly in secretory cells, rather than in ciliated cells. *C. trachomatis* appeared to have a toxic effect on the ciliated cells, as in many places almost an entire group of observed cells was dead and sloughed from the tissue (Figure 2A).

N. gonorrhoeae attached mainly to the microvilli of the secretory cells (Figure 2C). The mucosal damage was evident. Many ciliated cells sloughed from the epithelium. The tight junctions between cells also appeared loose. Heavily degenerated tissue was seen. A majority of cells were damaged after 5 days of infection (Figure 2D), and *N. gonorrhoeae* was seen inside the damaged epithelial cells (Figure 2D). The 5-day infection with *N. gonorrhoeae*

damaged the HFT tissue most heavily. It is clear that *N. gonorrhoeae* had a toxic effect on the HFT tissue culture.

Similar to the controls, *M. hominis*-infected HFT tissue was unaffected. Normal morphology of the tissue is shown in Figure 3A and B. We did not observe any deciliation; all cilia in the specimen were normal and unaffected. Because of the great variability of *M. hominis*, we used two different isolates of *M. hominis* in our studies: isolate 4195 (Figure 3A) and a recent clinical isolate p.139 (Figure 3B) isolated from a cervical swab sample of an infertile patient. By ELISA (Baczynska *et al.*, 2005), this infertile patient had also IgG antibodies against *M. hominis*. The isolates showed, however, similarity in the pattern of infection, and both appeared to be harmless to the epithelium of Fallopian tubes.
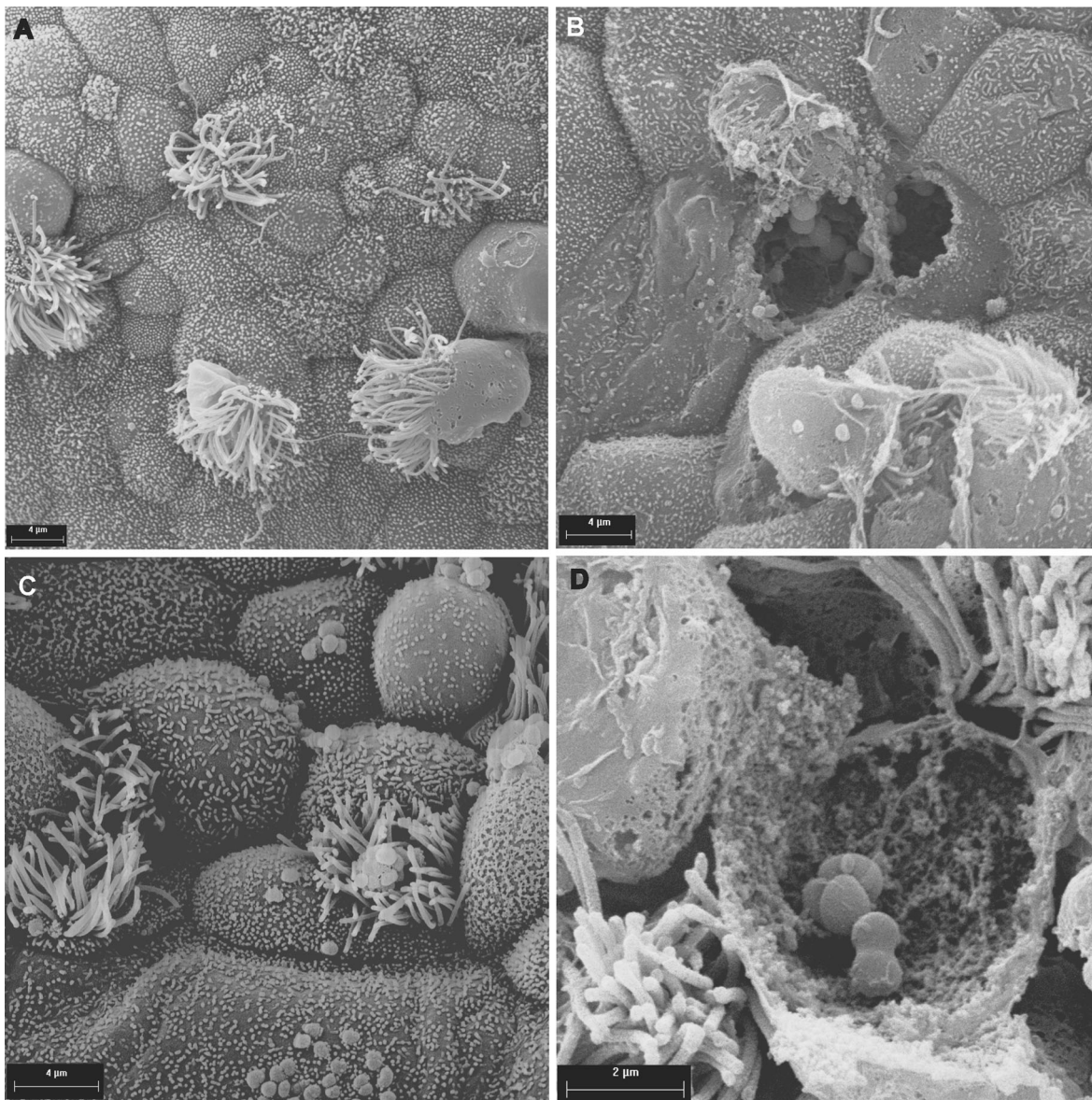

Figure 2. SEM of the HFT epithelium during infection with *C. trachomatis* or *N. gonorrhoeae*. (**A** and **B**) Tissues infected with *C. trachomatis*. The micro-organisms affected both ciliated and secretory cells. (A) A local direct cytotoxic effect of *C. trachomatis* on the ciliated epithelium. (B) Broken cell membranes and 'chlamydia-like' structures seen inside the disrupted cells. (**C** and **D**) Tissue pieces infected with *N. gonorrhoeae*. (C) Gonococci attaching mainly to the microvilli of the secretory cells. (D) Heavily degenerated tissue after 5 days of infection. Ciliated cells sloughed from the epithelium. *N. gonorrhoeae* could also be seen inside the damaged epithelial cells. A clear toxic effect of *N. gonorrhoeae* on the HFT tissue culture can be observed.

On the contrary, pieces of HFT infected with *M. genitalium* showed atypical structure of cilia after 5 days of infection (Figure 4A–C). The cilia became very large and swollen; many cilia fell apart and their number per ciliated cell also decreased dramatically (Figure 4B). We observed that many of the affected ciliated cells fell off the epithelium (Figure 4C). Very small structures, similar to *M. genitalium* cells, could be seen adhering to the tissue (Figure 4B); however, their identification remains unconfirmed. However, the tissue did not show the extensive damage, nor were the secretory cells damaged or influenced by the presence of *M. genitalium*. Neither did it seem as if *M. genitalium* had a toxic effect on the entire tissue. Overall, the changes in the morphology of the HFT epithelium due to *M. genitalium* infection were moderate.

Survival of mycoplasmas in the DMEM:F12 medium

We examined how long mycoplasmas could survive in the DMEM:F12 medium in the presence and absence of HFT tissue. Three different experiments were done. The first one was performed on the medium from *Mycoplasma*-infected HFT tissue pieces. In the second experiment, the medium from *M. genitalium*-infected tissues that were incubated with the MgPa antibodies was examined. Finally, *M. hominis* and *M. genitalium* cultures in the pure DMEM:F12 medium without addition of HFT tissue pieces were examined. The aliquots of different DMEM:F12 media were collected at each day of culture and incubated in the BEa or SP-4 *Mycoplasma* growth media. We could monitor the presence of live microorganisms throughout the incubation by observing the change in colour of the media, indicating growth of mycoplasmas. The results of these investigations are presented in Table I. Mycoplasmas which were added to the organ culture in the presence of the HFT tissue could survive the entire incubation time and were alive at day 5 post-infection. *M. genitalium* used for infection of the tissue which was incubated together with the antibodies against MgPa protein was also alive throughout the experiment up to day 5 post-infection. On the contrary, the culture of aliquots of DMEM:F12 medium containing *M. hominis* or *M. genitalium* showed that, in the absence of HFT tissue, mycoplasmas could survive only up to 3 days. The last change of *Mycoplasma* growth media was observed when the aliquots of DMEM:F12 media of day 3 culture were incubated. At days 4 and 5, DMEM:F12 contained most probably only dead mycoplasmas. Thus, the presence of HFT tissue was critical for the survival of mycoplasmas in the DMEM:F12 medium for >3 days.

IMF of the infected organ cultures

Because of their very small size, mycoplasmas were difficult to identify in SEM analyses. Even though structures comparable to mycoplasmas were seen in the preparations, similarity to dead cell debris, or even microvilli of the secretory cells, could not be excluded (Figure 4B). Neither could mycoplasmas be observed in between cilia of the ciliated cells. To visualize adhering mycoplasmas, we used IMF of the HFT sections. Tissues infected with each of the isolates of *M. hominis* and with *M. genitalium* were prepared for staining with antibodies. The adhering mycoplasmas were detected with polyclonal rabbit antibodies against whole cells of *M. genitalium* or *M. hominis*, and then they were visualized by the secondary FITC-conjugated goat anti-rabbit IgG.

Both *M. hominis* isolates showed poor adherence properties to the HFT tissue. Adhering cells were seen in clusters rather than separately (Figure 5A and B). A very low percentage of *M. hominis*-adhering cells was seen in the specimens of the HFT tissue after 5 days of infection with *M. hominis*.
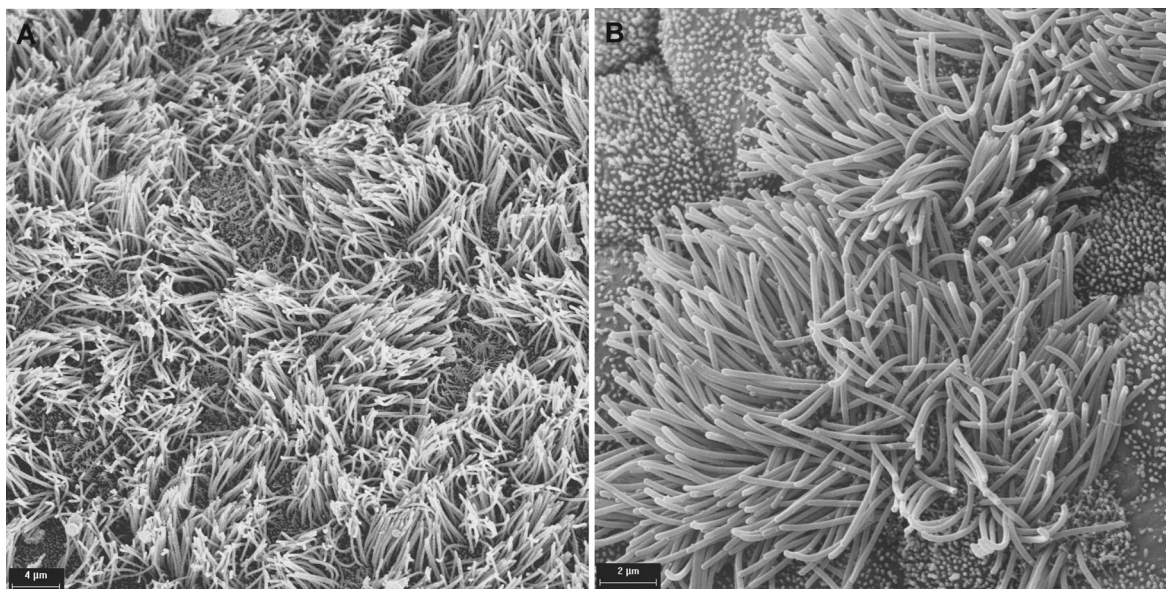
Figure 3. SEM of the HFT tissue infected with *M. hominis*. (**A**) Tissue pieces infected with *M. hominis*, isolate 4195 and (**B**) recent clinical isolate from infertile patient no. 139. The infected tissue shows a normal morphology of both ciliated and secretory cells. The cilia are not affected by the presence of *M. hominis* in the culture for 5 days. Both isolates seem harmless to the HFT epithelium.
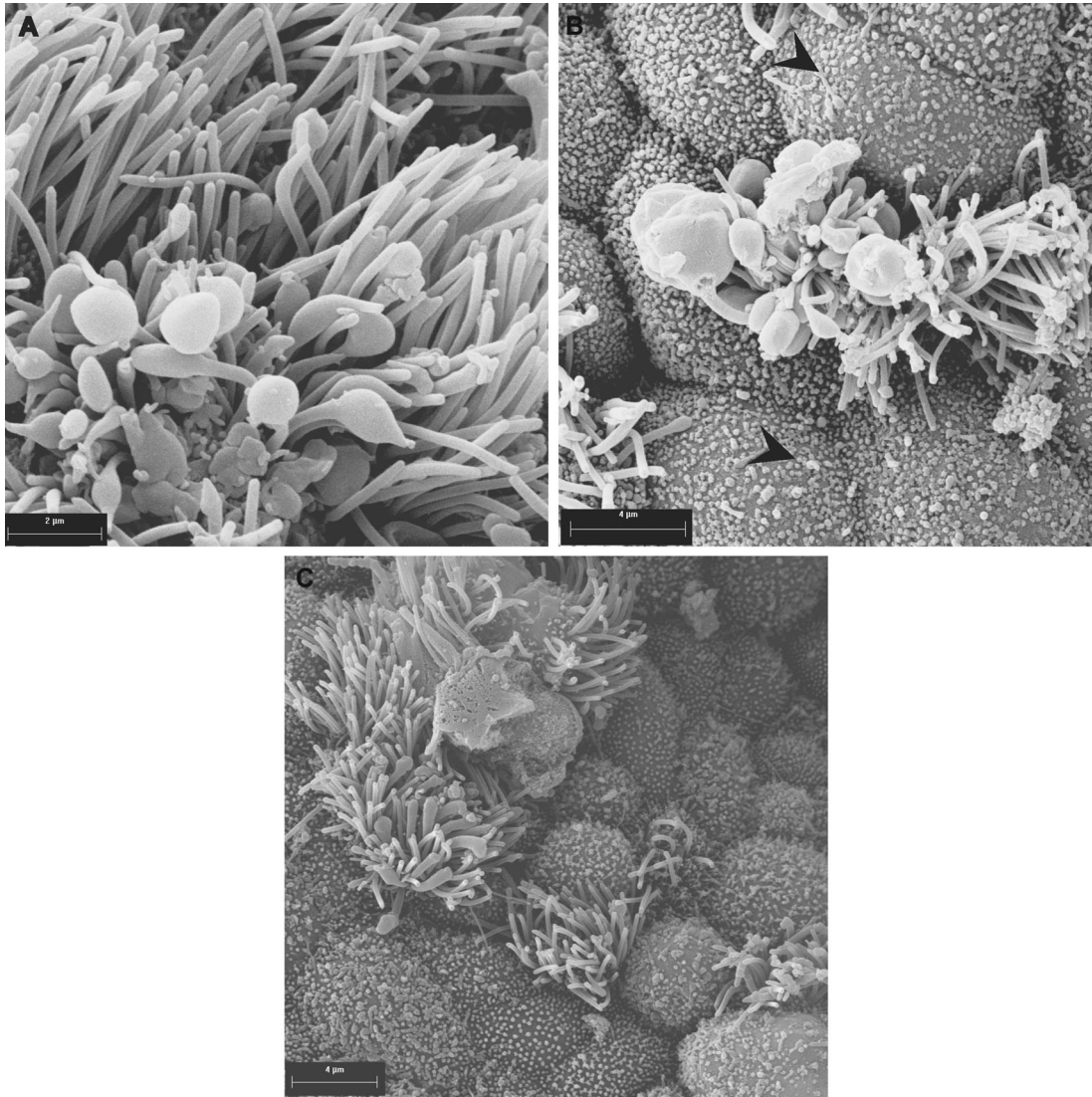
Figure 4. The effect of infection with *M. genitalium* on the ciliated cells. (**A**–**C**) The effect of infection with *M. genitalium* on the HFT epithelium. Notice that in all the pictures, the cilia of the ciliated cells are harmed by the infection. The close-up (A) shows that swelled cilia may have many shapes and swelling can appear at different places of the cilia: at the basal, middle or distal part of the cilium. (B) Cilia which literally fall apart, many of them are shortened and the number of cilia per cell is markedly reduced. Structures similar to *M. genitalium* cells can be seen in the preparation, adhering to the secretory cells and indicated with arrows. Secretory cells are not affected by *M. genitalium* infection. (C) Affected ciliated cells with two dead cells which are still partly attaching to the HFT tissue.

On the contrary, *M. genitalium* covered the epithelium in many layers and was adhering to the tissue mainly as single cells (Figure 5C and D). After 5 days of infection with *M. genitalium*, large areas of the HFT epithelium were densely covered by bacterial cells. A noticeable difference between adherence properties of *M. hominis* and *M. genitalium* could be seen when comparing Figure 5A–D.

Specificity of binding by the polyclonal rabbit antibodies was tested in tissues infected with *M. genitalium* and *M. hominis*. Samples were incubated with normal pre-immunized rabbit serum instead of primary polyclonal antibodies and then with FITC-conjugated goat anti-rabbit IgG. There was no reaction to the *Mycoplasma* cells in these controls, indicated by the complete lack of FITC staining (Figure 5E and F). Thus, the primary antibodies used were specific for detection of mycoplasmas.

Inhibition of the effects of M. genitalium on the HFT by antibodies against MgPa

Svenstrup *et al.* (2002) performed a cell culture study in which a HeLa cell line was infected with *M. genitalium*. They generated three monospecific antibodies against different parts of MgPa membrane protein, the protein that is believed to take the major role in adhesion. Antibodies against the C-terminal part of MgPa inhibited the adhesion of bacteria to HeLa cells when pre-incubated with *M. genitalium* suspension before the infection of the HeLa cells. We examined whether this monospecific antibody rMgPa-III could reduce the damage of the ciliated epithelium of the HFT organ culture by blocking *M. genitalium* from binding to the HFT tissue. The monospecific antibody was incubated with the *M. genitalium* suspension before the infection of the HFT. During 5 days of infection, the medium was changed each day for a fresh one

Table I. Survival of genital mycoplasmas during culture in the DMEM:F12 medium

Medium	Micro-organisms		
	M. genitalium	*M. hominis* 4195	*M. hominis* p. 139
DMEM:F12 with the HFT tissue	5 days	5 days	5 days
DMEM:F12 with HFT tissue and rMgPa-III antibody	5 days	N/A	N/A
DMEM:F12 without the HFT tissue	3 days	3 days	3 days

p. 139, recent clinical isolate; DMEM:F12, organ culture growth medium; HFT, human Fallopian tubes; rMgPa-III, antibodies generated against the C-terminal part of MgPa protein.

containing a new dilution of rMgPa-III antibody. Adhering mycoplasmas were analysed by IMF of the HFT sections by confocal microscopy. *M. genitalium* was detected by antibodies directed against the whole cell (PAb G37) followed by the secondary FITC-conjugated goat anti-rabbit IgG. Examples of this reaction can be seen in Figure 6A and B. Only few single cells were still attached to the tissue when MgPa antibodies were added to the organ culture. The tissue which was not incubated with antibodies against MgPa (Figure 5C and D) differed significantly as regards the amount of *Mycoplasma* cells attaching to the epithelium with several layers of adhering bacteria. Thus, rMgPa-III antibodies inhibited the adhesion of *M. genitalium* cells to the HFT epithelium.

The tissues incubated with the mixture of *M. genitalium* and antibodies were also analysed by SEM. Only occasional, very rare swelling of cilia was observed in these samples. Two examples are seen in Figure 7A and B. The epithelium had ciliated and secretory cells of normal morphology, and the majority of cilia were long and thin. Deciliation was reduced so that only few deciliated cells were observed. Therefore, the addition of antibodies markedly reduced the damage by *M. genitalium* on the HFT epithelium by reducing the amount of bacteria that could attach to the epithelial cells.

Discussion

The use of Fallopian tube organ culture to visualize the effect of infection with *M. hominis* on the delicate mucosal layer is not a new approach, although there is a disagreement between two former studies. Mårdh *et al*. (1976) showed that *M. hominis* infection of normal HFT for the period of 3 or more days resulted in abnormal swelling of cilia. In contrast, a histological study by Taylor-Robinson and Carney (1974) described that a normal HFT was unaffected by infection with *M. hominis*. Similar to Taylor-Robinson and Carney (1974), we did not observe damage to tissue samples infected with *M. hominis* (Figure 3A and B). This disagreement could result from different *M. hominis* isolates being used in the studies and from the potential differences in the virulence of these isolates. Mårdh *et al*. (1976) used a fresh isolate, obtained from the salpinx of a woman suffering from acute salpingitis, and in the study by Taylor-Robinson and Carney (1974), a

genital isolate of *M. hominis* was used, but with no specification of the isolate. Our strain 4195 was isolated from the vagina (Lin and Kass, 1974) and a recent clinical isolate p. 139 was obtained from an endocervical swab (unpublished). We cannot exclude that the isolates existing in the lower genital tract may be of commensal nature, whereas isolates from salpinges could be pathogenic to the epithelium of HFT. Both previous organ culture studies showed that addition of HFT tissue was critical for the survival of *M. hominis* in the organ culture medium and that infection of HFT tissue with *M. hominis* was persistent despite changes of the organ culture medium. This is in agreement with our observations. Both genital mycoplasmas used in our studies died in the DMEM:F12 medium after 3 days of culture when the HFT tissue was not added (Table I). Our IMF analyses of tissues infected with *M. hominis* and *M. genitalium* for 5 days showed mycoplasmas present on the epithelial layers of HFT. Even though *M. genitalium* was adhering much better than *M. hominis* (Figure 5A–D), both organisms were seen attaching to the HFT epithelium. Even pre-incubation of *M. genitalium* with antibodies against membrane protein MgPa and addition of these antibodies during each day of organ culture did not completely inhibit the attachment of bacteria to the epithelium, but the amount of adhering *M. genitalium* was considerably lower (Figure 6A and B). Collier *et al*. (1990) described that in the HFT organ culture infected with *M. genitalium*, the micro-organisms attached to the epithelium of HFT, and they also indicated that this attachment could be inhibited either by treatment with trypsin or by pre-incubation of *M. genitalium* cells with antibodies to MgPa, but data from these analyses were not shown. In our study, tissue infected with *M. genitalium* showed swelling of cilia and moderate deciliation in every SEM preparation (Figure 4A–C). This swelling was a prominent feature, which was not observed in any of the uninfected controls tested (Figure 1A–D). This effect was reversed only when the adhesion inhibition assay was done by the use of antibodies against MgPa adhesion protein (Figure 7A and B). We found that the addition of MgPa antibodies inhibited the adherence of *M. genitalium* to the HFT tissue (Figure 6A and B). We do not know what could cause the swelling of the cilia because, up to now, no classical toxin was identified in any of the mycoplasmas. Recently, Kannan and Baseman (2006) reported the existence of the virulence factor MPN372 that may be responsible for the damage observed in the human respiratory tract during infection with *M. pneumoniae*. The virulence factor, similar to the S1 subunit of *Bordetella pertussis* toxin in its N-terminus, induced vacuolization and cell death in the Baboon tracheal rings. BLAST search of the amino acid sequence of MPN372 showed no homology to any of the *M. genitalium* or *M. hominis* proteins, indicating the absence of this potential virulence factor in the two studied genital mycoplasmas. This may also be the reason why we did not observe such a great cilia loss as was seen in the tracheal cultures infected with *M. pneumoniae*. Using hamster tracheal rings infected with *M. pneumoniae* Muse *et al*. (1976) showed loss of cilia already after 48 h post-infection. At 72 h post-infection, further loss of cilia was observed; however, the remaining cilia appeared morphologically
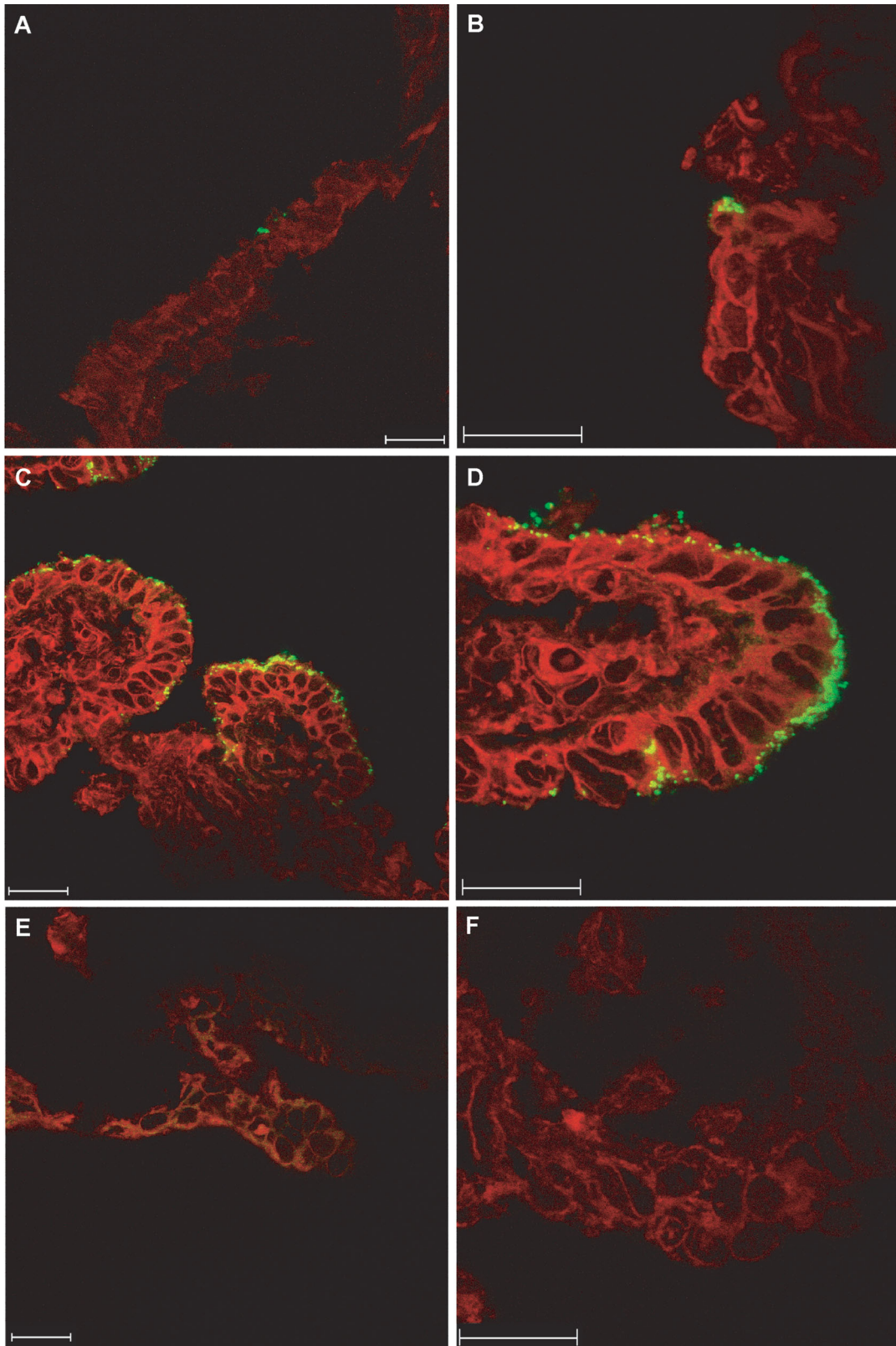
Figure 5. Immunofluorescence (IMF) of the HFT sections. (**A–F**) IMF of the sections of tissues infected with *M. hominis* (A and B) and *M. genitalium* (C–D). *Mycoplasma* cells were detected with polyclonal rabbit antibodies against whole cells and then with the secondary fluor-escein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG. Mycoplasmas are stained in green and the tissue in red. (A) A laboratory isolate 4195 adhering to the HFT tissue can be seen. (B) Staining of the clinical isolate. (C and D) Staining of *M. genitalium* attaching to the HFT tissue. *M. genitalium* adheres to the tissue much better than any isolate of *M. hominis*. (E and F) The tissue infected with *M. hominis* (E) and *M. genitalium* (F) and incubated with normal serum of pre-immunized rabbit as primary antibody. Tissues are visualized in red with Ewans Blue, and secondary antibody FITC-conjugated goat anti-rabbit IgG was used. There was no reaction between adhering mycoplasmas and normal rabbit pre-sera indicated by the lack of FITC stain. In each figure, the bar represents 20 μm.
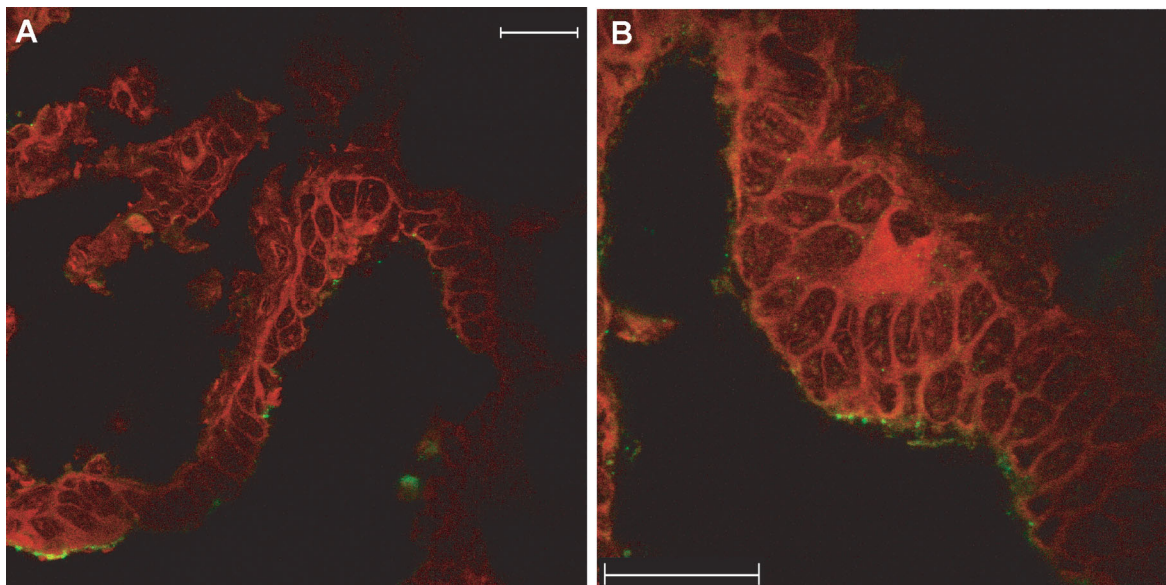
Figure 6. IMF of adhesion inhibition assay. (**A** and **B**) The adhesion inhibition assay of *M. genitalium*. The monospecific antibody rMgPa-III was used for pre-incubation of *M. genitalium* before the tissue was infected and also during each day of the organ culture. A clear reduction in the number of *Mycoplasma* cells adhering to the HFT epithelium was observed. The bar represents 20 μm.

normal, similar to those seen in uninfected tissues. Toxic by-products of metabolism of mycoplasmas, such as hydrogen peroxide and superoxide radicals, were suggested to cause oxidative damage to host cell membranes (Razin *et al.*, 1998), and such by-products could have a damaging effect on the cilia and possibly cause their swelling. Therefore, we suggest that it is rather a by-product of metabolism of *M. genitalium*, which causes swelling of the cilia than any toxin or virulence factor. To compare the effects of infection

with the two genital mycoplasmas on the HFT epithelium, the inoculation dose of *M. genitalium* was the same as the inoculation dose of *M. hominis* and based on the study of Mårdh *et al.* (1976). It would be highly relevant to infect the HFT tissues with the dose that would result in a similar infection rate as the one present in Fallopian tubes exposed *in vivo*. However, even though there are some indications about *M. genitalium* load in the female urine and cervical or urethral swabs samples (Jensen *et al.*, 2004), direct
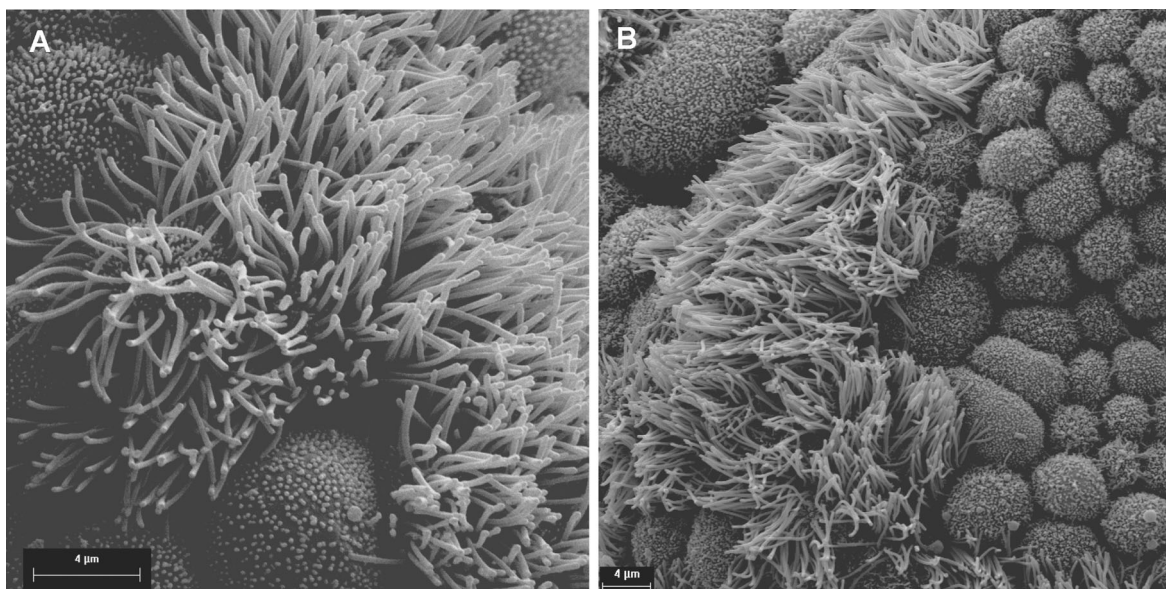

Figure 7. Reduction of *M. genitalium* effect on the HFT by adhesion inhibition. (**A** and **B**) The tissues incubated with the mixture of *M. genitalium* and antibodies analysed by SEM. Notice that the addition of antibodies markedly reduced the negative impact of *M. genitalium* on the HFT epithelium. Swelling of the cilia was not observed. Normal morphology of the epithelium cells was observed. Deciliation was also greatly reduced.

comparison to biological exposure present in the Fallopian tubes is not possible. This applies to all genital bacteria we tested in our *in vitro* model.

The results of the infection with *C. trachomatis* and *N. gonorrhoeae* used in this study as 'positive' controls were in accordance with previous studies. Both pathogens were studied in detail using Fallopian tubes organ culture. Cooper *et al.* (1990) observed a cytotoxic effect characterized by loss of microvilli and disruption of cell junctions when multiple chlamydial elementary bodies (EBs) attached to mucosal cells. They also observed ruptured epithelial cells releasing EBs. Chlamydial inclusions were seen 72 h post-infection in both ciliated and non-ciliated epithelial cells. Inclusions contained all forms of the *C. trachomatis* developmental cycle. We observed ruptured cell membranes with 'chlamydia-like' structures inside (Figure 2B) and a local cytotoxic effect on the ciliated cells (Figure 2A).

Recently, the cytotoxicity of *C. trachomatis* was associated with the presence of cytotoxin gene that encodes proteins with significant homology to large clostridial cytotoxin (Belland *et al.*, 2001). Such a toxin may be able to cause the tissue damage observed. The results of infection with *C. trachomatis* were, however, not as dramatic as results of infection with *N. gonorrhoeae*. The major feature of the gonococcal infection on organ culture is the attachment of bacteria to the microvilli of the non-ciliated cells, followed by slough of ciliated cells and invasion (McGee and Woods, 1987). There are two components that are likely to cause the damage: lipopolysaccharide (LPS), as even filter-sterilized medium from organ culture infected with *N. gonorrhoeae* could reproduce deciliation in another organ culture in the absence of bacteria (Melly *et al.*, 1981) and monomers of peptidoglycan generated and released by *N. gonorrhoeae*. A major fragment of this peptidoglycan is identical to the tracheal cytotoxin of *Bordetella pertussis* (Melly *et al.*, 1984). *N. gonorrhoeae* had the most toxic effect on the HFT organ culture in our experiments (Figure 2C and D). Incubated with the HFT tissue pieces for 5 days, *N. gonorrhoeae* caused extensive damage of the entire epithelium. A number of previous publications suggested that *N. gonorrhoeae* can invade human primary endocervical, ectocervical and urethral epithelial cells (Edwards *et al.*, 2000; Harvey *et al.*, 1997). In our model, *N. gonorrhoeae* could be observed by SEM inside damaged cells. Whether *N. gonorrhoeae* was present also inside undamaged cells could not be revealed with SEM technology. *N. gonorrhoeae* could also survive and replicate in the DMEM:F12 medium in the absence of HFT tissue. There is no doubt that in our model *N. gonorrhoeae* is the most pathogenic among all genital bacteria tested.

The results of our study demonstrate the impact of genital mycoplasmas on the HFT epithelium. Only *M. genitalium* showed a damaging effect on the epithelium in our model. So far, DNA of *M. genitalium* was detected in the Fallopian tubes only in the study of Cohen *et al.* (2005) and only from one patient, indicating that the presence of this micro-organism in the Fallopian tubes is very rare; however, more studies of the prevalence of *M. genitalium* in the Fallopian tubes would be of great value. We suggest that the organism has the potential to damage the cilia of the ciliated cells and cause a moderate

deciliation *in vivo*. Although *M. genitalium* did not show such damage to the tissue as did both positive controls *C. trachomatis* and *N. gonorrhoeae*, we believe that if left untreated, it may have serious consequences on the pathology of the Fallopian tubes.

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