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Increased adhesiveness and internalization of *Neisseria gonorrhoeae* and changes in the expression of epithelial gonococcal receptors in the Fallopian tube of copper T and Norplant® users*

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Interaction of *Neisseria gonorrhoeae* with the oviductal epithelium *in vitro* was examined in 2 cm length segments obtained after surgical sterilization from users of copper T intrauterine device (IUD) or Norplant® and control women. Segments perfused with *N.gonorrhoeae* suspensions were incubated from 30 min up to 4 h, fixed, frozen and cut in 6–10 μm sections. Bacteria were detected immunohistochemically with rabbit anti-gonococcal serum followed by light and confocal microscopy. Adhesion and internalization of gonococci by epithelial cells were observed at all incubation times, and both were higher in explants from users of copper T IUD or Norplant implants than controls. The epithelium of controls expressed CD66 and syndecan-1; but CD46 was found in only one out of six cases. The epithelium of copper T IUD users expressed CD66 but not syndecan-1 or CD46. Users of Norplant® exhibited expression of CD46, CD66 and syndecan-1. Label was always found along the luminal border of the epithelium. There were more intraepithelial lymphocytes in users of contraceptive methods than in controls. Results indicate that (i) *N.gonorrhoeae* invade the oviductal epithelium from the first minutes of exposure, (ii) the epithelium is constitutively endowed with two known receptors for the gonococcus, CD66 and syndecan-1, (iii) copper T IUD and Norplant users exhibit higher rates of attachment and internalization of the gonococcus into the oviductal epithelium associated with changes in expression of gonococcal receptors.

Key words: copper T/gonococcal receptors/*Neisseria gonorrhoeae*/Norplant®/oviduct

Introduction

Increased risk of salpingitis and pelvic inflammatory disease have been reported in intrauterine device (IUD) users (Westrom *et al*., 1976; Paavonen and Vesterinen, 1980; Beerthuizen *et al*., 1982; Ovalle *et al*., 1993; Wollen *et al*., 1994). However, the mechanisms underlying this effect remain unknown. Recently, it was reported that the epithelium of the human oviduct exhibits endocytic properties *in vitro* which are unrelated with the stage of the menstrual cycle of women at the time of salpingectomy (Imarai *et al*., 1998). Endocytosis of luminal particles was detected at incubation times as short as 2 h. This suggested that bacteria reaching the lumen of the organ might also be internalized within a short time. Previous studies of the invasion of the epithelium of the human oviduct by *Neisseria gonorrhoeae* detected internalization after 24 h of in-vitro co-incubation with no reference to shorter intervals (McGee *et al*., 1976, 1981). Thus, the initial stages of the interaction between mucosal explants of the human oviduct and *N.gonorrhoeae* were examined.

On the other hand, because several receptors for *N.gonorrhoeae* have been identified in cell lines, including CD46, CD66, syndecan and αVβ5 integrin (Nassif *et al*., 1999; Naumann *et al*., 1999), the presence of those receptors in the epithelium of the same oviductal samples was also examined.

Materials and methods

Bacteria

Clinical isolates of *N.gonorrhoeae* were obtained from patients with gonococcal infection from the Instituto de Salud Pública de Chile. Gonococci were transported to the laboratory on supplemented

*Preliminary results of this research were previously communicated at the 32nd Meeting of the Society for the Study of Reproduction (Cardenas *et al*., 1999).

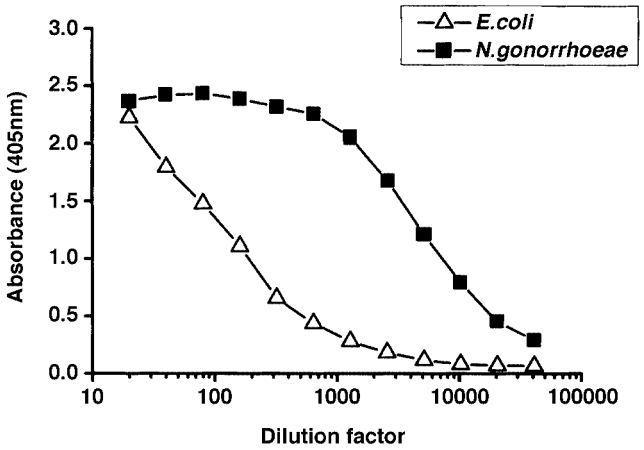

Figure 1. Titration of the anti-gonococcal activity of the rabbit antiserum. Enzyme-linked immunosorbent assay was used to verify the activity against *Neisseria gonorrhoeae* (wild type from clinical isolates) and *Escherischia coli* JM109.

Thayer-Martin agar plates (Merck, Darmstadt, Germany) and incubated at 37°C, 5% CO_2-air. After 48 h, bacteria were suspended in 8% glycerol, 10 mg/ml peptone in LB medium (10 g/l triptone, 5 g/l yeast extract, 0.17 mol/l NaCl) (Difco Laboratories, Detroit, MI, USA), frozen and stored in liquid nitrogen as described in Ward and Watt (1971). For the in-vitro infection assays, bacteria were thawed and grown on supplemented Thayer-Martin agar plates 2 days before the experiment.

Rabbit anti-gonococcal serum production

Two female rabbits were immunized by s.c. injection (Harlow and Lane, 1988) of heat-cold (–180/60°C)-inactivated *N.gonorrhoeae* suspended in complete Freund's adjuvant (Sigma, St Louis, MO, USA). After 2 weeks, the animals were boosted with the antigen with incomplete Freund's adjuvant (Sigma). Two weeks later the rabbits were killed, their blood collected and titrated for anti-gonococcal activity by enzyme-linked immunosorbent assay (ELISA). *Escherichia coli* JM109 was used to verify non-specific cross-reaction of the antiserum. For titration, a 96-well ELISA plate was incubated overnight with *N.gonorrhoeae* or *E.coli* suspensions, then incubated with the serum. The anti-gonococcal antibodies were detected with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (Ig)G (whole molecule; Sigma). The titration curves with *N.gonorrhoeae* and *E.coli* revealed that the anti-gonococcal serum displayed no cross-reactivity with *E.coli* over a range of dilutions appropriate for the purposes of this study (Figure 1). Based on these results the 1:1000 dilution was used for the histochemical detection and verified upon smears of bacteria.

Tissue donors and oviducts

Tissue samples were obtained from women seeking surgical sterilization who at the time of surgery had been using a copper T IUD for at least 3 months or Norplant subdermal implants for at least 1 year. The control group consisted of women who were not using any contraceptive method other than natural methods, for at least 1 year at the time of surgery. None of the donors was affected by gynaecological pathologies at the time of recruitment and all of them provided informed consent. The occurrence of sexually transmitted disease during the last year was an exclusion criterion, and there was no evidence in their clinical records that they had ever had gonococcal infection or pelvic inflammatory disease. Their mean age ± SD was 38.3 ± 6.9 years (n = 21). Protocols were approved by the Ethics Committee of the Universidad de Santiago de Chile. The oviducts were transported to the laboratory within 2 h of excision in sterile conditions. The muscle layer was dissected and discarded. The oviducts from five users of copper-T IUD, five users of Norplant® and 11 cyclic controls were processed as described elsewhere (Imarai *et al*., 1998). Briefly, the organs were cut into 2 cm segments which were perfused with 100–300 µl suspensions of *N.gonorrhoeae* (300 000 colony-forming units/ml) in Dulbecco's modified eagle medium containing L-glucose, L-glutamine, and pyridoxine hydrochloride (DMEM; Gibco BRL Life Technologies Inc., Gaithersburg, MD, USA). Control experiments in which gonococci were not perfused were always performed. The oviductal segments were incubated at 37°C in 5% CO_2-air for 30 min, 1, 2, 4 and 6 h. After the incubation, the lumen of the segments was gently flushed with 200 µl phosphate-buffered saline (PBS) and fixed in paraformaldehyde 4% in PBS for 1 h at 4°C, before sequential transfer to 10% sucrose in PBS for 1 h at 4°C, and 30% sucrose in PBS overnight at 4°C. The oviducts were mounted in embedding compound (Cryo-M-Bed; Bright Instruments Co. Ltd, Huntingdon, UK) and frozen at –20°C. Slices of 5–10 µm were cut using a Bright Starlet Cryostat at –20°C and mounted on gelatin-coated slides.

Immunohistochemistry for detection of N.gonorrhoeae

Gonococci attached to, and internalized into, the oviductal epithelium were detected immunohistochemically with rabbit anti-gonococcal serum and alkaline phosphatase (AP) reaction or fluorescein isothiocyanate (FITC)-conjugated antibodies as follows. After overnight incubation in 1% bovine serum albumin (BSA; Sigma)-PBS at 4°C, oviductal slices were incubated during 2 h at room temperature with rabbit anti-gonococcal serum (1:1000). When AP-conjugated antibodies were used, the endogenous AP was inactivated by incubation with 0.074% HCl in ethanol for 10 min at 4°C (Bulman and Heyderman, 1981). Slices were rinsed using PBS and incubated with AP-conjugated anti-rabbit IgG (Sigma; 1:2000) for 1 h at room temperature. After being rinsed, slices were incubated for an additional 1 h at room temperature with the AP substrate nitro-blue tetrazolium chloride (NBT; 0.35 mg/ml; Amresco Solon, Ohio, USA)/5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (BCIP; 0.175 mg/ml; Boehringer, Mannheim, Germany). Slices were stained with haematoxylin and mounted with Kaiser's gelatin (Sigma). When FITC-conjugated antibodies were used, slices were first incubated overnight in 1% BSA-PBS, and then incubated for 2 h with the rabbit anti-gonococcal serum followed by 1 h incubation with FITC-conjugated anti-rabbit Ig (gamma and light chains; Biosource, Nivelles, Belgium; 1:1000). All incubations were at room temperature. After being rinsed, slices were stained with propidium iodide (0.5 µg/ml) and mounted in 10% diazabicyclo[2.2.2]octane (DABCO; Sigma) in 90% glycerol (w/v). Gonococcal smears were used as positive controls. Negative controls in which the first antibody was omitted were routinely run in all the experiments.

Inmunohistochemistry for detection of N.gonorrhoeae receptors and quantification of mononuclear cells

The antibodies used were goat anti-human CD46, CD66, or syndecan-1 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal anti-human integrin αVβ5 (clone P1F6) from Gibco (1:200). For detection of CD46, CD66 and syndecan-1, 4–6 µm cryostat sections were placed onto gelatin-coated slides and fixed in acetone at –20°C for 10 min. After blocking with PBS-BSA 1% for 2 h at room temperature, the sections were incubated overnight with the primary antibody at 4°C. After three PBS rinses, the slices were incubated during 1 h at room temperature with FITC-conjugated rabbit anti-goat IgG (1:750; Sigma). After three PBS rinses, samples were counterstained with propidium iodide (1 µg/ml; Sigma). The slices were mounted with Dabco glycerol as indicated above. Detection of αVβ5

integrin was carried out as described by Sülz *et al*. (1998). Briefly, cryostat sections were fixed in acetone at –20°C for 10 min. After blocking with PBS-BSA 1% for 2 h at room temperature, the sections were incubated overnight at 4°C with the mouse monoclonal antibody anti-human integrin αVβ5. After three PBS rinses the sections were incubated for 30 min at room temperature with biotinylated goat anti-mouse immunoglobulin (1:500). After three rinses, the avidin-FITC complex was incubated on the sections for 1 h at room temperature. Samples were washed with water, counterstained with propidium iodide and mounted in DABCO glycerol.

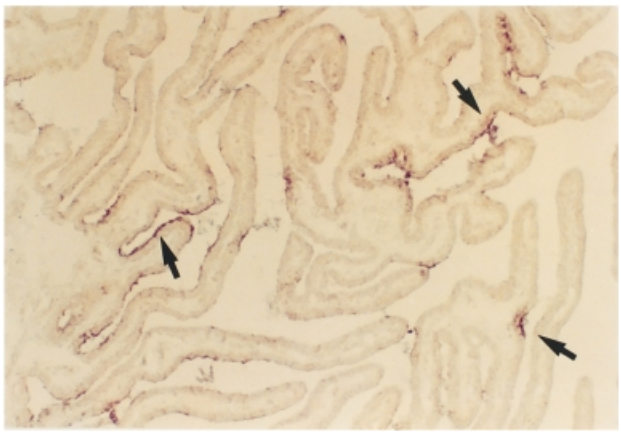

Figure 2. Panoramic view by light microscopy (original magnification ×200) of a mucosal segment incubated for 2 h after intraluminal infusion of a *Neisseria gonorrhoeae* suspension. Bacteria revealed by the dark staining of the alkaline phosphatase reaction are indicated by arrows. The patchy pattern of bacterial attachment to the luminal border is apparent.

The tissue sections used to examine the expression of gonococcal receptors allowed the quantification of intraepithelial mononuclear cells, which was done by counting all the cells with a round lymphocyte-like nucleus in an epithelial segment comprising 100–200 nuclei. These cells were interpreted as lymphocytes, as reported by others (Wollen *et al*., 1994). The confocal microscope made it possible to examine the propidium iodide-stained nuclei throughout so that misinterpretation due to inappropriately oriented cells was excluded.

Analysis of gonoccocal attachment and internalization

Slices were analysed by light and confocal microscopy. Attachment and incorporation was quantified by counting the total number of gonococci adhered to, and incorporated into, the oviductal epithelium. Three different slices per time point were examined in each case, and the average of the three measurements was used. Examination was done at ×1000 under immersion oil using a BX40 Olympus microscope. The presence of bacteria inside the epithelium (internalization) was always confirmed by confocal microscopy using an Axiovert 100 M Zeiss laser scanning microscope.

Hormone assays

A blood sample was obtained on the day of surgery to measure serum oestradiol and progesterone concentrations by radioimmunoassay using the reagents and methodology of the World Health Organization (WHO) Programme for the Provision of Matched Assay Reagents for the RIA of Hormones in Reproductive Physiology (WHO, 1987). The phase of the menstrual cycle at the time of surgery was determined by the serum hormone concentration according to WHO guidelines (1987) and the clinical records.

Statistical analysis

The number of gonoccoci were compared between groups by analysis of variance (ANOVA) and contingency tables were analysed by the

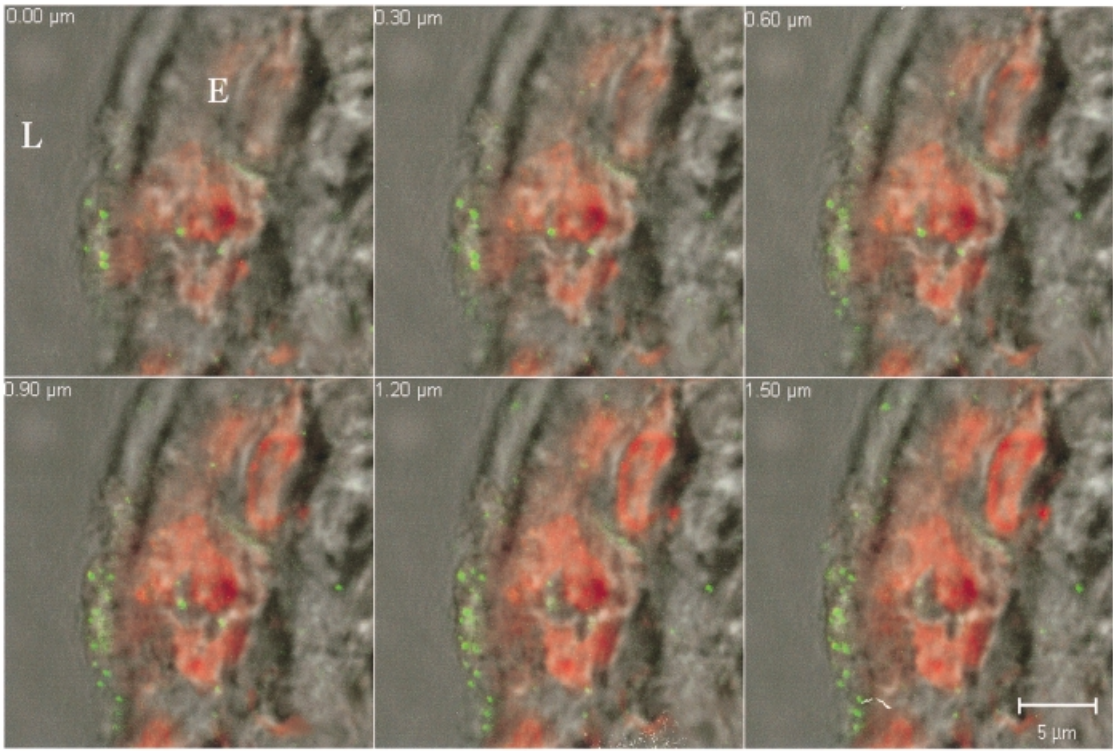

Figure 3. Confocal microscopy analysis of attachment and internalization *of Neisseria gonorrhoeae* by the oviductal epithelium after 4 h incubation. Bacteria are stained with fluorescein isothiocyanate-conjugated antibody. Successive focal planes at 0.3 µm depths show a large number of bacteria (green dots) attached to the luminal border and individual gonococci inside the epithelium. Nuclei appear red because of propidium iodide staining. Phase contrast allows the lumen to be distinguished. L = lumen; E = epithelium. Original magnification ×630.

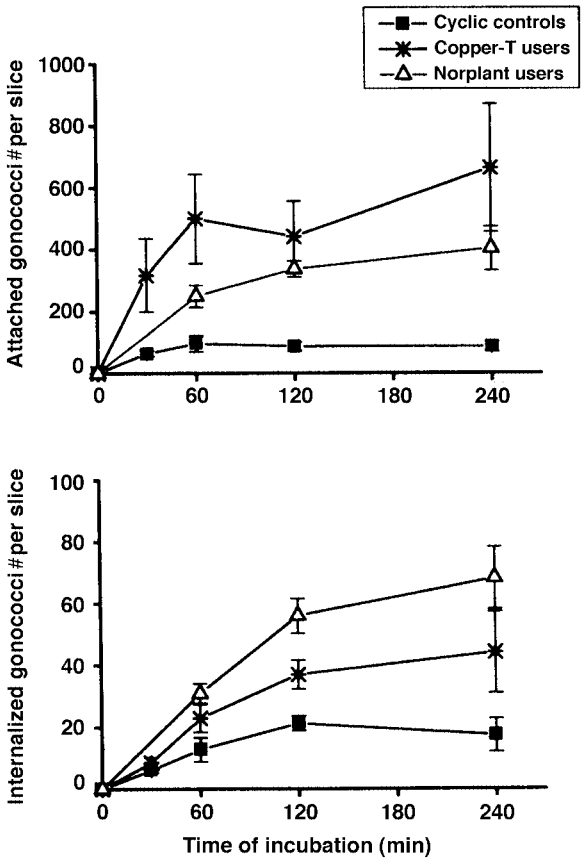

Figure 4. Time course of attachment and internalization of *Neisseria gonorrhoeae* by the oviductal epithelium after in-vitro incubation of mucosal explants from oviducts of control women and users of copper T intrauterine device or Norplant®.

Fisher exact test using the statistical package Quick Statistica (Statsoft Inc., Tulsa, OK, USA).

Results

Gonococci were found attached to the luminal border of the oviductal epithelium at all times examined (0.5, 1, 2 and 4 h), in all cases in the three groups. Attachment occurred to ciliated and non-ciliated cells with a patchy pattern when viewed under low power magnification (Figure 2). At all times there were more bacteria attached to the luminal border than internalized into the epithelium, and when bacteria had invaded the cells they were located mostly in the apical region, between the luminal surface and the nuclei of the epithelial cells (Figure 3). No association with the stage of the menstrual cycle was found. In a few cases examined at 6 h of co-incubation, gonococci were found reaching the basal region of the epithelium and invading the subepithelial tissue.

Attachment reached a maximum at 60 min of co-incubation in all groups (Figure 4). Gonococci were internalized into the epithelium from 30 min of co-incubation in all groups, but the maximum internalization was reached at 120 min and the number of internalized bacteria was about 10 times lower than those attached. The pattern or time course of attachment and internalization did not differ in users of copper T or Norplant, but their oviducts exhibited statistically significant increases in the number of gonococci attached and internalized (Figure 4).

Three of the control cases were sampled in the follicular and three in the luteal phase. The epithelium of the oviducts from these controls expressed CD66 and syndecan-1 in all the six cases examined, but αVβ5 integrin was absent and CD46 was present in only one of them. These molecules appeared along the luminal border of the epithelial cells with a patchy distribution that closely resembled the pattern of attachment of gonococci. Figure 5 illustrates the expression of the gonococcal receptors in selected cases viewed under confocal microscopy. No further attempts to compare the follicular and luteal phases were done. The pattern of expression of gonococcal receptors was different in copper T IUD users in that they did not have syndecan-1 in their oviductal epithelium, and in Norplant users who exhibited epithelial CD46 expression in all the five cases examined (Table I).

Intraepithelial lymphocytes were found in all cases examined. The percentage of intraepithelial lymphocytes in control oviducts was 1.7 ± 1 (mean ± SE of number of lymphocytes per 100 cells; $n = 5$), which was statistically different from 4.4 ± 1 ($P = 0.028$; $n = 5$) found in oviducts of users of copper T IUD and from 9.1 ± 0.7 ($P = 0.009$; $n = 5$) in oviducts of users of Norplant implants (Kruskal-Wallis ANOVA followed by Mann-Whitney *U*-test for multiple comparisons). The difference between both contraceptive methods did not reach statistical significance.

Discussion

Previous studies of the interaction between *N.gonorrhoeae* and the mucosa of the human oviduct *in vitro* focused on longer incubation times than those examined in this paper, providing a model in which the initial invasion of the mucosa needed 24 h of exposure to occur (McGee *et al.*, 1976, 1981; see reviews by Cohen and Sparling, 1992; Cooper and Moticka, 1996). Our previous work reporting that the endocytic capabilities of the oviductal epithelium allowed fast internalization of formaldehyde-fixed bacteria and proteins introduced into the lumen of mucosal segments (Imarai *et al.*, 1998), led us to examine the initial stages of the interaction between the gonococcus and the oviductal mucosa, during the first hours of exposure. The evidence provided here demonstrates that the gonococcus is able to bind to the luminal face of the epithelium almost immediately after reaching the lumen. Because the lumen of the mucosal segments was flushed after the incubation and before fixing the tissue, bacteria found attached to the epithelium can be assumed to have established a firm bond required for the ensuing invasion of the cells. Both the time course of attachment and differences between groups confirm this interpretation. Internalization of gonococci reached a peak 1 h later than attachment, but always remaining about 10 times lower, which is consistent with a receptor-mediated process that was probably saturated in the experimental conditions. Until dose-response relationships of this initial interaction are examined, this remains a matter of speculation.

Receptors to which *N.gonorrhoeae* binds to enter the cell have been described in cell lines, but it can at this stage be assumed that, if present in the oviductal epithelium, those

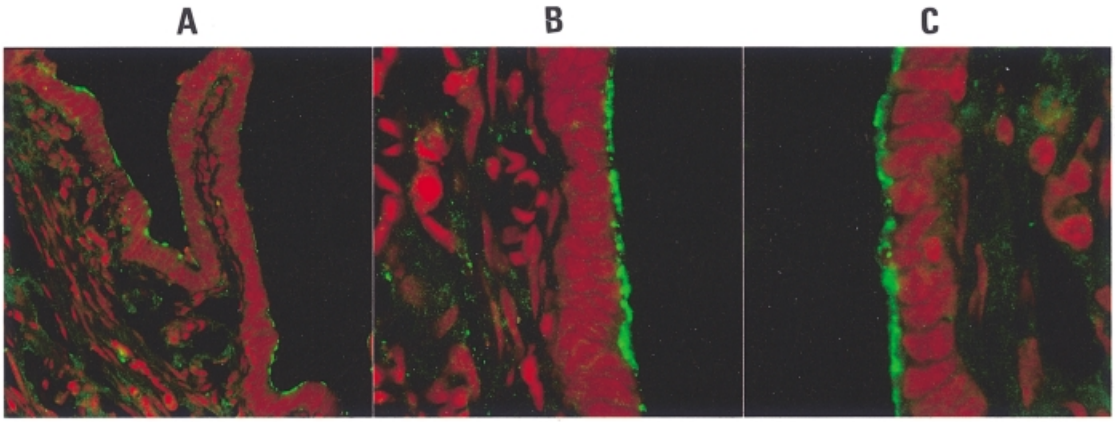

Figure 5. Examples of the expression of CD46 (**A**), CD66 (**B**) and syndecan-1 (**C**) in the oviductal mucosa of cycling donors. Label revealed by fluorescein isothiocyanate-conjugated antibodies. Nuclei stained red with propidium iodide. Total magnification for (**A**), (**B**) and (**C**) was ×1240, ×1440 and ×2120 respectively.

Table I. Immunohistochemical detection of known ligands for gonococcal adhesins in the human oviductal epithelium of cyclic women not using any contraceptive method and users of copper-T intrauterine device or Norplant® subdermal implants

Ligand	Cyclic controls	Copper-T users	Norplant® users
CD46	1/6	0/5	5/5*
CD66	6/6	5/5	5/5
Syndecan-1	6/6	0/5**	5/5
αVβ5 integrin	0/6	2/5	0/5

The values are the number of positive cases over total cases analysed. Fisher exact probability test: $*P = 0.013$, $**P = 0.002$.

molecules will have binding functions toward the gonococcus similar to the functions described in cell lines. So, the presence of CD66 and syndecan-1 in the epithelium of all the cyclic controls examined strongly suggests that these molecules are important mediators of the initial binding of the gonococcus in the human oviduct. The control subjects were three in the follicular and three in the luteal phase, and there was no obvious relationship between the expression of these molecules in the oviductal epithelium and the stage of the menstrual cycle. However, further exploration of this point may be necessary since αVβ3 integrin has been found to be expressed in this epithelium only during the implantation window (Sülz *et al*., 1998).

As previously reported for the endocytic properties of the epithelium in oviductal explants *in vitro* (Imarai *et al*., 1998), in this study there was no association between the internalization of *N.gonorrhoeae* and the phases of the menstrual cycle. This is to be expected because the entry of the gonococcus into the epithelium is regarded as endocytosis, induced by the binding of pili to gonococcal receptors in the infected cells (reviewed by Cohen and Sparling, 1992; Cooper and Moticka, 1996), in addition to several specific outer membrane proteins (Weel *et al*., 1991; Cohen and Sparling, 1992; Gorby *et al*., 1994; Naumann *et al*., 1999), and *a priori* it does not appear probable that a decrease or increase might simultaneously occur in all of those receptors during the menstrual cycle that would lead to differences in the rate of attachment of gonococci. In fact, it has previously been reported that the onset of acute salpingitis, chlamydial or gonococcus-related, is not correlated with the menstrual cycle (Sweet *et al*., 1986).

The mechanisms underlying the increased risk of salpingitis associated with copper T IUD have not been disclosed, probably because of the lack of appropriate experimental models. The results of the current study suggest that at least one of those mechanisms is increased adhesiveness and invasiveness of the epithelium by *N.gonorrhoeae* that is in turn related with changes in the epithelial expression of surface molecules. The OpaA gonococcal proteins are recognized by a heparan sulphate proteoglycan that is a member of the syndecan family (Nassif *et al*., 1999; Naumann *et al*., 1999). So, the disappearance of syndecan-1 in the oviductal epithelium of copper T users is not entirely consistent with the increased attachment of *Neisseria* in those same organs, but it is evidence of the multiplicity of mechanisms involved in the initial interaction of the gonococcus and the epithelium.

Because of its progestin-like effects, Norplant renders the cervical mucus essentially impenetrable by spermatozoa (Sivin, 1993) and probably also by bacteria ascending towards the upper segments of the reproductive tract. This would explain why Norplant is not associated with higher risk of salpingitis or pelvic inflammatory disease (Sivin, 1993; Fraser *et al*., 1998) despite the findings presented here indicating increased attachment and internalization of bacteria into the oviductal epithelium. The changes found in this study in the pattern of expression of gonococcal receptors in Norplant users can explain the increased attachment because CD46, which appeared in the luminal side of their oviductal epithelium, is a pilus receptor for pathogenic *Neisseria* (Nassif *et al*., 1999; Naumann *et al*., 1999). The evidence suggests that gonococcal salpingitis is less probable in Norplant users but it might have an aggressive course in the event that gonococci manage to reach the upper segments of the reproductive tract.

Because, in our population, histological evidence of salpingitis is usually found in ~40% of oviducts from asymptomatic cyclic women requesting sterilization, and the copper T IUD is associated with salpingitis, we decided to look at the number of intraepithelial lymphocytes to verify the presence

of inflammation. As expected, a lower percentage of intraepithelial lymphocytes were found in the control group than in users of the copper T IUD group. There are previous reports of intraepithelial lymphocytes in the human oviduct (Cardenas *et al*., 1998), and the data presented here are similar to those reported by VanBogaert *et al*. (VanBogaert *et al*., 1978). One explanation for the presence of intraepithelial lymphocytes in the human oviduct is chemotaxis by *Neisseria*, as demonstrated in organ culture by Cooper *et al*. (Cooper *et al*., 1987), but the occurrence of sexually transmitted diseases during the last year was an exclusion criterion, and samples were carefully checked and no evidence was found of *N.gonorrhoeae* in the oviductal samples not incubated with bacteria.

Totally unexpected was the increased lymphocyte infiltration of the oviductal epithelium of Norplant users. Interestingly, CD46, also known as membrane cofactor protein, that appeared in the oviductal epithelium of Norplant users, has been reported to be associated with glomerular infiltration by immune cells in immunoglobulin A nephropathy (Ootaka *et al*., 1996). The relationship between the increased gonococcal attachment and internalization, the increased CD46 expression and lymphocyte infiltration in this group is intriguing and merits further experimental studies.

In summary, this paper reports several novel aspects of the initial interaction between *N.gonorrhoeae* and the epithelium of the human oviduct. First, internalization of the gonococcus into the epithelial cells occurs during the first hours of exposure, which is possible because the oviductal epithelium appears to constitutively express at least two known ligands for the gonococcus, CD66 and syndecan-1. Second, this interaction is modified by chronic treatment with copper T IUD and Norplant subdermal implants. Both contraceptive methods increase the attachment and internalization of gonococci but through different modifications of surface molecules known to act as gonococcal receptors. Copper T leads to disappearance of syndecan-1 and Norplant to appearance of CD46, both in the luminal border of the epithelium. This effect of Norplant is associated with epithelial lymphocyte infiltration which is probably related to the expression of ligands that recruit lymphocytes into the epithelium and lumen of the organ.

Acknowledgements

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