

## Expression of Proinflammatory Cytokines and Receptors by Human Fallopian Tubes in Organ Culture following Challenge with *Neisseria gonorrhoeae*

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**Infection of the Fallopian tubes (FT) by *Neisseria gonorrhoeae* can lead to acute salpingitis, an inflammatory condition, which is a major cause of infertility. Challenge of explants of human FT with gonococci induced mRNA expression and protein secretion for the proinflammatory cytokines interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , and tumor necrosis factor alpha (TNF- $\alpha$ ) but not for granulocyte-macrophage colony-stimulating factor. In contrast, FT expression of IL-6 and of the cytokine receptors IL-6R, TNF receptor I (TNF-RI), and TNF-RII was constitutive and was not increased by gonococcal challenge. These studies suggest that several proinflammatory cytokines are likely to contribute to the cell and tissue damage observed in gonococcal salpingitis.**

*Neisseria gonorrhoeae* is the etiologic agent of gonorrhea, which is typically manifested as a mucosal infection of the male urethra and the lower genital tract of women. Localized infection with gonococci is frequently asymptomatic in women, although in 10 to 25% (3, 13, 37) of untreated individuals, infection may ascend into the upper reproductive tract to cause pelvic inflammatory disease, which encompasses a range of inflammatory conditions, including endometritis, pelvic (tubal, ovarian) peritonitis, tubal abscess, and salpingitis. The chronic sequelae, i.e., pelvic pain, tubal damage, ectopic pregnancy, and infertility, associated with pelvic inflammatory disease are recognized as important public health problems (17, 25).

Little is known of the mechanisms involved in the early stages of the inflammatory response that occurs in the Fallopian tube (FT) following ascending gonococcal infection. However, epithelial cells at mucosal surfaces have been reported to secrete chemoattractant and proinflammatory cytokines in response to infection by pathogenic bacteria (1, 16, 39). Limited studies of the experimental infection of the urethrae of human male volunteers with gonococci have demonstrated the sequential secretion of several cytokines (interleukin [IL]-6, IL-8, IL-1 $\beta$ , and tumor necrosis factor alpha [TNF- $\alpha$ ]), which culminated in the influx of leukocytes (34). However, a similar challenge model is inappropriate for women, and consequently studies of the inflammatory response have been carried out in vitro with organ cultures and more commonly with both transformed and primary cells cultured from the female reproductive tract. Despite many studies demonstrating that gonococcal infection of cultured human cells induces the production of proinflammatory and chemoattractant cytokines (4, 7, 10, 9, 23,

24, 31, 32, 33), the nature of the inflammatory response induced in FT tissue has received very little attention. The classical studies of Ward et al. (43) and McGee and colleagues (28) established a human FT organ culture model to study the pathogenesis of acute gonococcal salpingitis in vitro. Subsequent studies demonstrated that gonococci attached specifically to nonciliated cells and that attachment was mediated by both pili and Opa protein (11). Gonococcal infection resulted in damage primarily to ciliated cells, leading to sloughing of cells from the epithelium and a loss of ciliary activity (29, 38). The bacterial components suggested to mediate this damage were lipopolysaccharide and fragments of peptidoglycan (12, 30, 44). However, the nature of the inflammatory mediators induced following gonococcal interactions is poorly characterized. The studies of McGee and colleagues demonstrated that gonococci up-regulated the production of TNF- $\alpha$  by FT epithelium and that secretion of this cytokine correlated with sloughing of ciliated cells (26, 27). However, the production of other cytokines by FT mucosal epithelium was not explored. In the present study, FTs in organ culture were challenged with gonococci and the expression of a panel of proinflammatory cytokines (IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, granulocyte-macrophage colony-stimulating factor [GM-CSF], and TNF- $\alpha$ ) and cytokine receptors was investigated.

FTs were obtained, after informed consent, from fertile donors undergoing hysterectomy for reasons unrelated to this study. An exclusion criterion was the occurrence of sexually transmitted disease during the last year and a history of pelvic inflammatory disease. The Ethics Committee of the Universidad de Santiago de Chile approved all protocols. FTs (from five donors) were processed immediately after removal as described previously (18). Briefly, the muscle layer was dissected and discarded and the remaining mucosa was cut into segments of 1 cm<sup>2</sup> and placed in Dulbecco's modified Eagle's medium

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(Gibco) in 24-well tissue culture plates. After 3 h, each segment of FT was infected intraluminally with 100  $\mu$ l ( $3 \times 10^6$  CFU/ml) of an isolate of *N. gonorrhoeae* in Dulbecco's modified Eagle's medium. The organism, which was designated *N. gonorrhoeae* strain G387-98, was isolated in 1998 from a female patient presenting with cervicitis at the Instituto de Salud Pública de Chile, Santiago, Chile. After initial isolation, the strain was cultured in modified Thayer Martin agar with several passages and stored in liquid nitrogen. The phenotype of strain G387-98 was confirmed as Pil<sup>-</sup> Opa<sup>+</sup> by examining colony morphology with a stereomicroscope and with sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis and immunoblotting with specific antibodies (14). By use of anti-Opa monoclonal antibody 4B12 (a gift from M. Blake, NAV Inc., Beltsville, Md.), the strain was found to express a single Opa only. In addition, through use of monoclonal antibodies SM101 (anti-P.IA) (41) and SM198 (anti-P.IB) (2), the strain was identified as expressing the P.IA porin. Following infection, the oviductal segments were incubated at 37°C in 5% (vol/vol) CO<sub>2</sub> for intervals up to 24 h. Control segments were maintained with culture medium alone.

For confocal microscopy, oviductal tissues were placed in cold 4% (vol/vol) (Sigma) paraformaldehyde in phosphate-buffered saline (PBS) (Winkler, Santiago, Chile) pH 7.4, for 1 h at 4°C, before sequential transfer to 10% (wt/vol) sucrose in PBS for 1 h at 4°C and to 30% (wt/vol) sucrose in PBS overnight at 4°C. The oviducts were mounted in embedding compound (Cryo-M-Bed; Bright Instruments Co. Ltd., Huntingdon, United Kingdom) and frozen at -20°C. Slices (4 to 6  $\mu$ m) were cut using a Bright Starlet Cryostat at -20°C and were mounted on gelatin-coated slides. They were then blocked in PBS containing 1% (wt/vol) bovine serum albumin and were incubated at room temperature for 2 h with a rabbit antigonococcal antiserum (1/1,000 dilution) (8). Following washing in PBS, the samples were incubated for 60 min at room temperature with a fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibody (1/1,000 dilution) (Biosource). The samples were washed, counterstained with propidium iodide (1  $\mu$ g/ml) (Santa Cruz), washed again, and then mounted in a solution of PBS containing 10% (vol/vol) 1,4-diazobicyclo[2.2.2]octane (Dabco; Sigma) and 90% (vol/vol) glycerol (Gibco). A negative control consisting of oviductal sample without primary antibody was also included. The samples were examined using laser scanning confocal microscopy on an Axiovert 100 M Microscope (Zeiss). Optical sections ( $n = 25$  to 40) were usually taken at intervals of 0.1 to 1.0  $\mu$ m and used to reconstruct three-dimensional images.

Total RNA was isolated from the segments of FT at 0, 30, and 240 min after bacterial challenge, by using Trizol reagent (Gibco) according to the manufacturer's instructions. After purification, the RNA samples were incubated with DNase I (2 U/ $\mu$ l) (Gibco) to eliminate the chromosomal DNA, and total RNA (1  $\mu$ g) was reverse transcribed into single-stranded cDNA using Superscript II reverse transcriptase (200 U/ $\mu$ l) (Gibco), dithiothreitol (10 mM final concentration), and each deoxynucleoside triphosphate (Gibco) at a final concentration of 0.5 mM. Control cDNA preparations (without reverse transcriptase) were also prepared. To remove RNA complementary to the cDNA, the samples were incubated with RNase H (2 U/ $\mu$ l) (Gibco). Amplification of cytokine cDNA was carried

out with Multiplex PCR (Biosource) by using specific primers for TNF- $\alpha$ , TNF receptor I (TNF-RI), TNF-RII, IL-6, IL-6 receptor, IL-1 $\beta$ , GM-CSF, and GM-CSF receptor. Amplification of the "housekeeping" gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also carried out in the PCR. The PCRs were carried out in the presence of *Taq* DNA polymerase enzyme (2.5 U) (Gibco), with each deoxynucleoside triphosphate at a concentration of 0.8 mM and MgCl<sub>2</sub> (2 mM). In order to compare the results from different experiments, optimal cycle conditions for linear amplification were determined by a semiquantitative assay of the amplified products over a range of cycles (the number of cycles ranged from 25 to 34). Products from 32 cycles were within the linear logarithmic phase of the amplification curve and were therefore chosen for comparative analysis. The reaction mixtures were heated at 96°C for 1 min and were immediately carried through 2 cycles of PCR consisting of a denaturing step of 1 min at 96°C and an extension step of 4 min at 58°C and then through 30 cycles, each with denaturing step of 1 min at 94°C and extension step of 2.5 min at 58°C. The final extension was performed at 70°C for 10 min. As negative controls, reactions were also carried out using samples without RNA and with cDNA preparations prepared in the absence of reverse transcriptase enzyme.

The amplified products were separated on a 3% (wt/vol) agarose gel (Amresco, Solon, Ohio), which was subsequently stained with ethidium bromide (Sigma) and photographed under UV illumination. Densitometric scanning was performed with a Bio-Rad model GS-700 imaging densitometer (Bio-Rad, Hercules, Calif.), and the data were analyzed with NIH Image 1.62 software and normalized to those of GAPDH. In order to confirm the identity of each fragment, a positive control (Biosource) for each mRNA species under study was included in all reverse transcriptase PCR experiments. The mRNA values are expressed as relative units calculated according to the following formula: density of the amplification product/density of the GAPDH amplification product  $\times$  100. The data from mRNA expression densitometry were statistically analyzed by using the Kruskal-Wallis test followed by the Mann-Whitney U test for multiple comparisons. Differences were considered significant when  $P < 0.05$ .

Supernatant samples were removed at intervals up to 12 h, and secretion of cytokine proteins by FT was quantified using sandwich immunoassays as described previously (4). Briefly, capture antibodies against TNF- $\alpha$ , IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, and GM-CSF (R&D Systems) were coated on enzyme-linked immunosorbent assay plates (Nunc) at 37°C for 16 h, and after washing, the wells were blocked with PBS containing 1% (wt/vol) bovine serum albumin and 5% (wt/vol) sucrose for 1 h at 37°C. Samples of culture medium were diluted in assay buffer (Delfia; Wallac), added to enzyme-linked immunosorbent assay wells, and incubated for 2 h at 37°C. Subsequently, matched biotinylated detector antibodies were added to the wells and incubated for 2 h at 37°C. After washing, europium-labeled streptavidin was added to each well, and after 1 h of incubation, any bound label was detected by the addition of Delfia Enhancement Solution and subsequent measurement of emitted fluorescence on a Delfia fluorometer (Wallac). The concentration of each cytokine was determined by comparison with standard solutions of the corresponding purified recom-

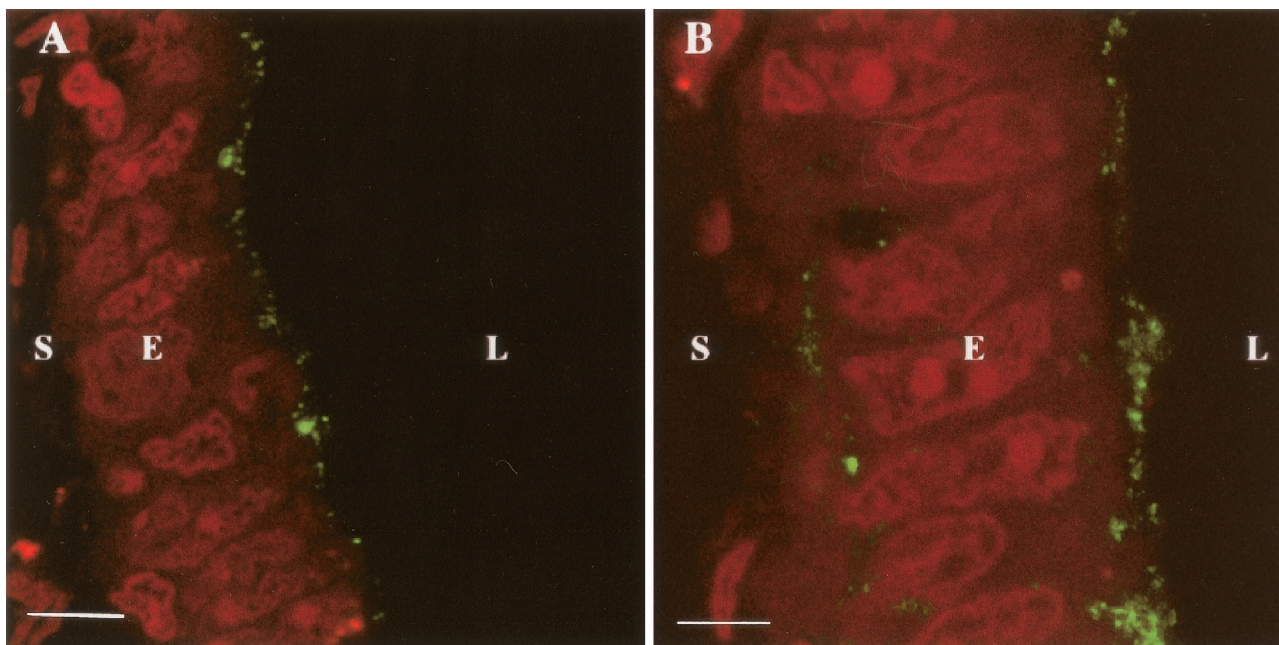


FIG. 1. Interactions of *N. gonorrhoeae* with human FT. (A)  $\text{Pil}^- \text{Opa}^+$  gonococci (green) attached to oviductal epithelial cells after 30 min of incubation (magnification,  $\times 1,344$ ). (B) Gonococci attached and internalized in FT mucosal epithelial cells after 4 h of infection (magnification,  $\times 1,722$ ). Host cell nuclei are counterstained red with propidium iodide. The scale bars in panels A and B represent 15 and 10  $\mu\text{m}$ , respectively. L, lumen; E, epithelial cells; S, stromal cells.

binant protein (Peprotech) similarly treated. A two-sample *t* test was used to compare the mean values of cytokine secretion, and a *P* of  $< 0.05$  was considered significant.

In the present study, the  $\text{Pil}^- \text{Opa}^+$  variant was found attached to the luminal border of the oviductal epithelial cells as early as at 30 min after infection, specifically to nonciliated cells. Increasing numbers of gonococci were observed attached after 4 h of infection, and there was also evidence of invasion of the epithelium (Fig. 1). In addition, several bacteria were also observed in the basal region of the epithelium and in the process of invading the subepithelial tissue after 4 h (data not shown). These observations are consistent with previous reports of gonococcal interactions with FT tissue *in vitro* (11, 20, 28, 29, 43, 44). In the present study, however, although gonococcal interactions had no effect on the ciliary activity of epithelial cells after 12 h of challenge, there appeared to be signs of apoptosis in some cells (data not shown).

Challenge of FT with the  $\text{Pil}^- \text{Opa}^+$  gonococci induced up-regulation of mRNA for the cytokines TNF- $\alpha$  and IL-1 $\beta$ , with maximal levels reached after 4 h (Fig. 2A). In contrast, there was no detectable expression of mRNA for GM-CSF or GM-CSF receptor and no GM-CSF protein secretion (data not shown). Furthermore, the cytokine receptors TNF-RI, TNF-RII, and IL-6 receptor were expressed constitutively by the FT, and challenge with gonococci did not modulate their expression (data not shown). The accumulation of individual cytokine proteins was also measured at 12 h after challenge, and there was a positive correlation between up-regulation of TNF- $\alpha$  and IL-1 $\beta$  mRNA expression and protein secretion (Fig. 2B). High levels of TNF- $\alpha$  (3 to 5 ng/ml) were detected, with lesser amounts of IL-1 $\beta$  (approximately 0.5 to 1 ng/ml) (Fig. 2B). In addition, although very low levels of IL-1- $\alpha$  (0.02 ng/ml) were

detected in culture supernatant samples after 12 h, these were still significantly greater ( $P < 0.05$ ) than control values (data not shown).

Challenge of FT with  $\text{Pil}^- \text{Opa}^+$  gonococci also up-regulated the expression of IL-6 mRNA (Fig. 2A). High levels of IL-6 protein secretion were also observed after 12 h (14 ng/ml), but these levels were not significantly greater than those observed for control FT samples (12 ng/ml [Fig. 2B]). Therefore, despite an increase in mRNA expression, challenge with gonococci did not lead to elevated IL-6 protein secretion after 12 h. Thus, at these early time points, there appeared to be no direct correlation between the levels of IL-6 mRNA expression and protein secretion. However, the measurement of secretion at 12 h is likely to be early for IL-6 protein and maximum product accumulation probably increases over time. Therefore, determination of the levels of cytokine accumulation at intervals of 24 h or more after challenge will establish whether gonococci increase IL-6 secretion during infection. It is also possible that constitutive IL-6 secretion observed in this model was due to a combination of several insults, namely, the surgical injury and interruption of blood supply and *in vitro* culture, and it remains to be seen whether constitutive IL-6 production occurs in the FT *in vivo*.

The secretion of proinflammatory cytokines by cells of the FT is likely to play a pivotal role in the host response to ascending gonococcal infection. The studies of McGee and colleagues demonstrated that piliated ( $\text{Pil}^+$ ) gonococci attached to and damaged the FT mucosal epithelium more rapidly than nonpiliated ( $\text{Pil}^-$ ) gonococci (29). In addition, it has been reported that  $\text{Pil}^+ \text{Opa}^+$  gonococci invaded FT tissue with higher frequency than a  $\text{Pil}^- \text{Opa}^-$  variant (11), and differences in the attachment of gonococci to FT mucosa and

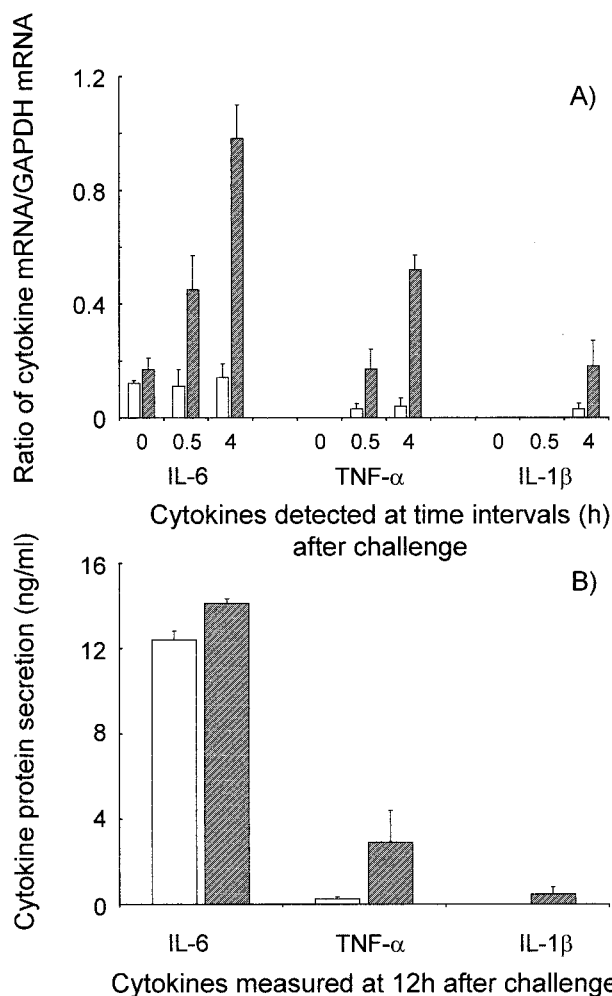


FIG. 2. *N. gonorrhoeae* induces proinflammatory cytokine production in the human FT. (A) The up-regulation of cytokine mRNA expression in human FT, following challenge with *N. gonorrhoeae* (Pil<sup>-</sup>Opa<sup>+</sup>). (B) The secretion of proinflammatory cytokines by human FT measured 12 h after challenge with gonococci. In both figures, the white bars designate cytokine production by control, unchallenged FT, and the striped bars designate cytokine production by FT following challenge with gonococci. Data are presented as the mean of five challenge experiments that used FT samples derived from different donors, and the error bars show the standard deviation.

damage to the mucosal cells have been observed with different Opa-expressing variants of gonococcal strain F62-SF (5). In the present study, a nonpiliated gonococcus was used in challenge experiments in order to avoid excessive damage to the FT epithelium, which may possibly down-regulate cytokine production. We observed that this Pil<sup>-</sup>Opa<sup>+</sup> variant was able to invade mucosal epithelial cells and induce cytokine secretion but with little cytotoxicity.

High levels of TNF-α secretion were observed following challenge of FT with Pil<sup>-</sup>Opa<sup>+</sup> gonococci in the present study, which is in agreement with similar observations of McGee and colleagues using piliated organisms to challenge FT mucosa (26). Taken together, these studies demonstrate the central role of TNF-α in the inflammatory response in the FT. The present study also demonstrated that FT tissue constitutively

expressed the receptors TNF-RI and TNF-RII, but these were not up-regulated following challenge with gonococci. Cell surface receptors for TNF-α are widely distributed among different tissues, and many cell types can express both receptors. TNF-RI is associated with cytotoxicity of TNF-α, fibroblast proliferation, and activation of the signal transduction factor NF-κB (22). In addition, the cytoplasm domain of TNF-RI has a "death domain," which has been reported to trigger an apoptotic pathway (40), and it is possible that this mechanism is induced in the mucosal epithelium of FT infected with gonococci. Therefore, cytotoxicity resulting from the effects of TNF-α, acting in concert with TNF receptors on the mucosal epithelium, may be host protective during gonococcal infection of the FT in limiting penetration of the organism to submucosal tissue.

The present study also demonstrated that proinflammatory cytokines other than TNF-α were also produced by FT tissue in response to infection. Gonococci were observed to up-regulate production of both IL-1α and IL-1β, suggesting that these cytokines also play a role(s) in salpingitis. IL-1 is an important mediator of inflammation and is likely to be a significant chemoattractant for leukocyte influx into the infected FT. In addition, up-regulated IL-1 production may also promote leukocyte adhesion to FT tissue by enhancing the expression of molecules such as intercellular adhesion molecule 1. Recent studies have shown that monolayers of cultured epithelial cells up-regulate the expression of intercellular adhesion molecule 1 in response to gonococci (19). Therefore, it is possible that up-regulation of IL-1 production is implicated in host defense against infection, by both maintaining leukocytes at the site(s) of infection and promoting their bactericidal activity (36), thereby reducing further invasion of the FT epithelium by gonococci.

The presence of IL-6 in many inflammatory diseases suggests that this cytokine has an important role in either the disease process or the host's response to disease (35). Several studies have reported that gonococci up-regulate IL-6 production by epithelial cells of the reproductive tract cultured in vitro (4, 10, 15, 33). In the present study, however, an unexpected finding was that secretion of IL-6 protein and expression of IL-6 receptor molecules were both constitutive in normal human FT tissue. Furthermore, challenge with gonococci did not increase either the levels of IL-6 protein secretion or IL-6 receptor molecules after 12 h. It is possible that constitutive expression of IL-6 may be important in the innate defense of the FT. This cytokine has been reported to prime neutrophils for enhanced oxidative respiratory burst responses (21) and also to modulate the enzymatic degradation of tissue through its controlling activities on metalloproteinases, serine proteinases, and their respective inhibitors (35). Therefore, these effects of IL-6 may also be important contributory mechanisms for limiting gonococcal penetration into submucosal tissues.

In the present study, the up-regulation of proinflammatory cytokine production following challenge with gonococci is consistent with several studies that have demonstrated that cells derived from the genitourinary tract and cultured in vitro secrete cytokines in response to infection. A model of primary human endometrial cells has demonstrated that both gonococcal pili and Opa proteins were important for inducing the

secretion of TNF- $\alpha$ , as well as of IL-6 and IL-8 (4). In addition, Pil<sup>+</sup> Opa<sup>+</sup> gonococci have been reported to up-regulate TNF- $\alpha$  production from human endometrial adenocarcinoma (Hec-1B) cells (19). In the study by Naumann et al. (33), challenge of transformed ME180, HeLa, and HaCat epithelial cells with gonococcal variants differing in their expression of pilus and Opa proteins up-regulated mRNA signals not only for TNF- $\alpha$  and IL-1 $\beta$  but also for IL-6, IL-8, GM-CSF, MCP-1, and transforming growth factor  $\beta$ . In addition, these authors also demonstrated secretion of TNF- $\alpha$ , GM-CSF, and IL-8 proteins (33). In the present study, Pil<sup>-</sup> Opa<sup>+</sup> gonococci were capable of inducing cytokine production by FT tissue, demonstrating that an inflammatory response can occur in the absence of pilus-mediated interactions. This is in accord with the observation of Naumann et al. (33) that an invasive Pil<sup>-</sup> Opa<sup>+</sup> variant and an adherent Pil<sup>+</sup> Opa<sup>-</sup> variant were equally efficient at cytokine induction. Furthermore, challenge of immortalized epithelial cell lines from the endocervix, ectocervix, and vagina with gonococci was shown to induce IL-1 $\alpha$ , IL-6, and IL-8, and significantly, both Pil<sup>+</sup> and Pil<sup>-</sup> variants induced similar levels of these cytokines (10). Taken together, all of these studies suggest that, following the interactions of gonococci, epithelial cells from all sites of genitourinary tract produce a distinct set of proinflammatory cytokines, which notably include TNF- $\alpha$ , IL-1, and IL-6. In addition, up-regulated secretion of IL-8 is a common response of these epithelial cells, and in the present study Pil<sup>-</sup> Opa<sup>+</sup> gonococci also induced secretion of this chemokine by FT tissue (data not shown). Significantly, all of these observations of elevated cytokine production in vitro are consistent with the report of elevated levels of TNF- $\alpha$ , IL-1, IL-6, and IL-8 in both the urine and plasma of men after intraurethral challenge with gonococci (34).

Although the nature of the cell types in the FT that secrete proinflammatory cytokines in response to gonococcal infection is not known, it is likely that oviductal endosalpingeal cells, resident macrophages, and infiltrating leukocytes are involved. A role for resident macrophages is supported by recent studies demonstrating in vitro secretion of TNF- $\alpha$  and IL-6 following challenge with gonococci including a Pil<sup>-</sup> Opa<sup>+</sup> variant (24). Studies with immortalized epithelial cell lines from the endocervix, ectocervix, and vagina or with primary cells cultured from the endometrium have also demonstrated a correlation between cytokine expression and gonococcal phenotype (4, 10). This is likely to be the case also with the FT, and presently we are investigating the abilities of a panel of phenotypic variants differing in their expression of pilus and different Opa proteins to induce inflammation in the explant model. In addition to the potential effects of pilus and Opa protein, bacterial components such as lipopolysaccharide, peptidoglycan, and immunoglobulin A protease have also been shown to induce cytokine and chemokine production from cultured human cell lines (6, 7, 23, 42). However, the precise nature of the gonococcal components that induce cytokine production by the FT remains to be elucidated.

In summary, infection of the FT by *N. gonorrhoeae* can lead to acute salpingitis, an inflammatory condition that is a major cause of infertility. This study has demonstrated that the human FT expresses several proinflammatory cytokines and receptors, some of which are stimulated in response to infection

with gonococci. The primary function of up-regulated production of TNF- $\alpha$ , IL-1 $\alpha$ , and IL-1 $\beta$ , in concert with constitutive expression of IL-6, may be host protective, in limiting bacterial penetration into submucosal tissues. However, this mechanism of pathogen containment is likely to be increasingly at the expense of damage to the epithelium itself. Understanding the pathogenesis of gonococcal salpingitis and the inflammatory and immune defense mechanisms involved could suggest new strategies for the prevention and treatment of this disease.

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