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Infection of Human Fallopian Tube Epithelial Cells with *Neisseria gonorrhoeae* Protects Cells from Tumor Necrosis Factor Alpha-Induced Apoptosis

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Following infection with *Neisseria gonorrhoeae*, bacteria may ascend into the Fallopian tubes (FT) and induce salpingitis, a major cause of infertility. In the FT, interactions between mucosal epithelial cells and gonococci are pivotal events in the pathogen's infection cycle and the inflammatory response. In the current study, primary FT epithelial cells were infected in vitro with different multiplicities of infection (MOI) of Pil^+ Opa^+ gonococci. Bacteria showed a dose-dependent association with cells and induced the secretion of tumor necrosis factor alpha (TNF-α). A significant finding was that gonococcal infection (MOI = 1) induced apoptosis in approximately 30% of cells, whereas increasing numbers of bacteria (MOI = 10 to 100) did not induce apoptosis. Apoptosis was observed in only 11% of cells with associated bacteria, whereas >84% of cells with no adherent bacteria were apoptotic. TNF-α was a key contributor to apoptosis, since (i) culture supernatants from cells infected with gonococci (MOI = 1) induced apoptosis in naïve cultures, suggesting that a soluble factor was responsible; (ii) gonococcal infection-induced apoptosis was inhibited with anti-TNF-α antibodies; and (iii) the addition of exogenous TNF-α induced apoptosis, which was inhibited by the presence of increasing numbers of bacteria (MOI = 10 to 100). These data suggest that TNF-α-mediated apoptosis of FT epithelial cells is likely a primary host defense mechanism to prevent pathogen colonization. However, epithelial cell-associated gonococci have evolved a mechanism to protect the cells from undergoing TNF-α-mediated apoptosis, and this modulation of the host innate response may contribute to establishment of infection. Understanding the antiapoptotic mechanisms used by *Neisseria gonorrhoeae* will inform the pathogenesis of salpingitis and could suggest new intervention strategies for prevention and treatment of the disease.

The female reproductive tract is an immunologically unique site which must respond to a diverse array of sexually transmitted pathogens and must also be tolerant to allogeneic sperm and to conceptuses. Pelvic inflammatory disease (PID) is an acute clinical syndrome associated with the ascending spread of microorganisms through the female reproductive tract (80). PID encompasses a multitude of inflammatory conditions of the upper reproductive tract organs, with the majority of proven cases of PID being caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (gonococcus) (32), and coinfection with both pathogens is common.

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, and the organism infects the mucosal epithelia of the male urethra and the lower genital tracts (vagina/cervix) of women. Localized infection with gonococci leads to a mucopurulent cervicitis in women, but it is also frequently asymptomatic. However, in approximately 10 to 25% (7, 26, 70) of untreated individuals, infection may ascend into the upper reproductive tract to involve the endometrium, ovaries, myometrium, parametrium, and Fallopian tubes (FT) (32, 46). The host response to this ascending infection is manifested as endometritis, pelvic (tubal or ovarian) peritonitis, tubal abscess, and salpingitis in the FT, and all of these inflammatory conditions encompass the clinical syndrome of PID. Long-term sequelae that develop in individuals presenting with PID, such as chronic pelvic pain, tubal damage, and ectopic pregnancy (7, 26, 70), are recognized as important public health problems worldwide (32, 46).

The FT is essentially a muscular organ whose lumen is lined by columnar ciliated cells and secretory cells with microvilli (68), and it plays a critical role in mammalian reproduction, functioning as a channel and storage organ for spermatozoa, a collecting vessel for oocytes released from the ovaries, the site of fertilization and zygote formation, and a means for transporting the early embryo to the uterus (54, 68). It is recognized that salpingitis induced by gonococcal infection causes significant tissue damage in the FT, which is resolved by a process of repair by infiltrating fibroblasts that leads to scarring. These events cause functional impairment of the tubes and irreversible infertility (80). However, little is known of the molecular mechanisms involved in the early stages of infection of the FT by ascending gonococci that initiate the inflammatory response. Studying the pathogenesis of gonococcus-induced salpingitis has relied on the use of ex vivo human FT organ tube

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cultures (49, 78). With this model, it has been shown that gonococci attach specifically to nonciliated cells and that this process is mediated by both pili and Opa protein adhesins (22, 51). Gonococcal infection results primarily in damage to ciliated cells, leading to a loss of ciliary activity and eventually to sloughing of cells from the epithelium (50, 71). This cell death correlates with up-regulated production of tumor necrosis factor alpha (TNF-α) by the FT epithelium (47, 48), and the key bacterial components implicated are lipopolysaccharide and fragments of peptidoglycan (25, 52, 53). More recently, Maisey et al. (40) demonstrated that gonococcal infection of human FT also up-regulates the expression of interleukin-1α (IL-1α), IL-1β, and IL-8 in addition to TNF-α. In contrast, the expression of IL-6 and the cytokine receptors IL-6R, TNF-RI, and TNF-RII is constitutive and not increased by gonococcal challenge.

The studies of McGee et al. demonstrated that gonococcal infection could induce cell death in FT epithelium. Cell death can occur through necrosis, which is accompanied by an aggressive inflammatory response, and apoptosis, which is an evolutionarily conserved, highly regulated genetic and biochemical process that is required for the development and homeostasis of multicellular organisms (15). Apoptosis has been observed as a response to infection by a wide range of pathogens (13, 23, 79), and in a recent study, Maisey et al. described the presence of an apoptotic phenotype for some cells of the FT epithelium following infection of explants with gonococci in vitro (40). The present study was therefore undertaken to investigate these observations by exploring the roles of *Neisseria gonorrhoeae* and TNF-α in the induction of apoptosis in primary epithelial cells cultured from human FT.

MATERIALS AND METHODS

Culture of primary epithelial cells from human Fallopian tubes. FT 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 or a history of pelvic inflammatory disease. The Ethics Committee of the Universidad de Santiago de Chile approved all protocols. FT were processed immediately after removal, as described previously (33). Briefly, the organ was washed with phosphate-buffered saline (PBS), pH 7.4, the lumen was exposed through a longitudinal cut, and strips of mucosal folds were dissected. Strips were washed in TC199 medium (Gibco BRL), dissected, and then digested with trypsin as described previously (33). The resulting cell suspension was centrifuged, washed, and seeded into 24-well tissue culture plates in TC199 medium containing 10% (vol/vol) fetal calf serum (Gibco BRL), insulin (5 mg/ml), glutamine (1 mM), and pyruvate (1 mM). Epithelial cells were incubated at 37°C in an atmosphere of 5% (vol/vol) CO_2 for at least 3 days to 80 to 90% confluence (in order to avoid contact inhibition of the cells) and were characterized by immunohistochemical staining with antibodies to specific cellular markers, as described previously (74).

Bacterial strain, growth conditions, and challenge of FT epithelial cell cultures. *Neisseria gonorrhoeae* strain P9, variant −17 (Pil^+ Opa^+) (75), expressing a red-shift mutant green fluorescent protein, has been described previously (9) and was used for FT epithelial cell challenge experiments. The gonococcal variant was grown routinely on GC agar containing ampicillin (5 μg ml^{-1}) overnight at 37°C in 5% (vol/vol) CO_2.

FT epithelial cells were grown in cell culture wells (60 × 15 mm; Orange Scientific) to 80 to 90% confluence. Since cells from different donors exhibited different rates of proliferation, cell numbers were counted for each experiment prior to the addition of bacteria. Epithelial cells (1.5×10^4 to 5×10^5 per well) were challenged with *Neisseria gonorrhoeae* P9-17 resuspended in Dulbecco's modified Eagle's medium (Gibco BRL) at multiplicities of infection (MOIs) of 1, 10, and 100 for 12 h. For each individual experiment, double-blind assays were carried out to determine the interaction of *Neisseria gonorrhoeae* with epithelial cells, and laser scanning confocal microscopy (Axiovert 100 M microscope; Zeiss) was used by two independent investigators to count bacteria associated with cells in six separate fields, with each field containing 200 epithelial cells.

The association of gonococci with FT epithelial cells and any subsequent internalization of bacteria were also quantified by viable counting after saponin lysis and gentamicin treatment as previously described (28, 76).

Detection of apoptosis. Human Fallopian tube epithelial cells were cultured on type 1 collagen (PAA Laboratories, Somerset, England)-coated glass coverslips (12 mm; Marienfeld, Germany) and infected with various MOIs of *Neisseria gonorrhoeae* P9-17. Apoptosis was assessed at the beginning of the experiment (0 h) and 12 h after challenge by the following two methods.

(i) In situ cell death detection fluorescein assay. The in situ cell death detection fluorescein assay (Boehringer Mannheim, Germany) is based on the labeling of DNA strand breaks (by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling [TUNEL] technology) in order to detect and quantify apoptosis at the single-cell level. DNA fragmentation was assessed according to the manufacturer's protocol, using the enzyme terminal deoxynucleotidyltransferase (TdT), which forms a polymeric tail by catalytically incorporating rodamine-12-dUTP (TMR) into free 3′-OH DNA ends (21), which can be quantified by fluorescence microscopy. Briefly, cells were washed three times with PBS, pH 7.4 (Winkler, Chile), and fixed with 2% (vol/vol) cold paraformaldehyde in PBS for 1 h at room temperature. After being washed with PBS, cells were treated with 0.1% (vol/vol) Triton X-100 in 0.1% (wt/vol) sodium citrate solution for 15 min at 37°C in order to permeabilize the cell membranes. Samples were treated twice, for 3 min each time, with PBS containing 5% (wt/vol) bovine serum albumin (BSA) and 0.1% (vol/vol) Triton X-100. The TUNEL reaction mixture, containing enzyme (TdT) and labeling solution (nucleotide mixture), was prepared according to the manufacturer's instructions, added to permeabilized samples, and incubated in a humidified atmosphere for 60 min at 37°C. For negative controls, cells were treated identically, but in the absence of TdT enzyme. As a positive control, cells were treated with DNase I for 10 min at room temperature to induce DNA strand breaks prior to labeling procedures.

(ii) Detection of caspase-3 in FT epithelial cells. Caspases are crucial mediators of apoptosis, and caspase-3 is a frequently activated death protease that catalyzes the specific cleavage of many key cellular proteins and is indispensable for apoptotic chromatin condensation and DNA fragmentation (63). The presence of caspase-3 in FT epithelial cells, with and without gonococcal challenge, was determined by fluorescence microscopy. Briefly, cells were washed with Tris-buffered saline, pH 7.4 (TBS), and fixed by treatment with 4% (vol/vol) paraformaldehyde at room temperature for 10 min. After being washed with TBS, the cells were permeabilized as described above. Detergent was removed by washing the cells with TBS, and the cell monolayers were blocked by incubation at room temperature for 1 h with PBS containing 10% (vol/vol) normal goat serum and 1% (wt/vol) BSA. Permeabilized cell monolayers were then left overnight at 4°C with a 1/50 dilution of antibody to cleaved caspase-3 (Asp175) (Cell Signaling Technology, New England Biolabs) prepared in TBS containing 1% (wt/vol) BSA. After the cells were washed, bound antibody was detected following incubation with a fluorescence-labeled secondary antibody (1/1,500 dilution of Alexa fluor 546-conjugated goat anti-rabbit immunoglobulin G [IgG]; Molecular Probes) for 45 min at room temperature in the dark.

For both assays, coverslips were mounted in DABCO (1,4-diazabicyclo-[2.2.2]octane) and then examined by 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 μm and used to reconstruct three-dimensional images. The apoptotic phenotype was characterized by the presence of nuclei stained red within epithelial cells. The percentage of apoptotic cells was determined for each concentration of gonococcus (MOI = 1, 10, or 100) tested and for control, uninfected cells by counting the number of apoptotic cells (determined by TUNEL and the presence of caspase-3) present in a field of 200 cells, and a mean percentage was determined for six independent fields. The overall mean levels of apoptosis, with standard errors, were calculated from the percent apoptosis data collected from 10 (MOI = 1), 8 (MOI = 10), and 7 (MOI = 100) experiments carried out with FT epithelial cell lines derived from 10 donors for the TUNEL assay and from 5 donors for the caspase-3 assay.

Assessment of cell viability. The viability of FT epithelial cells grown on separate coverslips was also assessed at the beginning (0 h) and end (12 h) of each experiment by exclusion of trypan blue (0.4% [wt/vol]; Sigma), as described previously (61).

Quantitation of TNF-α production by FT epithelial cells. The levels of TNF-α induced by bacterial challenge were determined with a specific immunoassay using matched pairs of antibodies, as previously described (40).

Inhibition of *Neisseria gonorrhoeae*-mediated apoptosis in FT epithelial cells by treatment with anti-TNF-α antibodies. Human FT epithelial cells were infected with *Neisseria gonorrhoeae* (MOI = 1) in the presence and absence of

anti-human TNF-α1 and -TNF-α2 antibodies according to the protocol described by Kim et al. (36). Apoptosis was determined as described above. Antibodies to TNF-α1 (monoclonal antibody 610) and -TNF-α2 (polyclonal total goat IgG [AB-210-NA]) were obtained from R&D Systems. In addition, the challenge experiment was carried out in the presence of an irrelevant antibody (sc-2028; total goat IgG) obtained from Santa Cruz Biotechnology. All antibodies were used at a final concentration of 10 μg/ml.

Inhibition of TNF-α-mediated apoptosis in FT epithelial cells by infection with *Neisseria gonorrhoeae*. Human FT epithelial cells were cultured in 24-well plates in the presence or absence of *Neisseria gonorrhoeae* (MOI = 100) for 12 h and then were treated for 5 h with human recombinant TNF-α (Pharmingen, San Diego, CA) at a concentration of 40 ng/ml. TNF-α-dependent apoptosis was induced by treatment of the cells for 5 h with the cytokine alone.

Statistics. The data for induction of apoptosis are presented as means with standard errors of the means (SEM). Overall analyses were performed with the Kruskal-Wallis test, followed by the Mann-Whitney test for pairwise comparison when overall significance was detected. *P* values of <0.05 were considered significant.

RESULTS

Apoptosis in human FT epithelial cells infected with *Neisseria gonorrhoeae*. Human Fallopian tube epithelial cells from different donors were cultured in vitro and challenged with increasing concentrations of *Neisseria gonorrhoeae* (MOI = 1, 10, and 100), and apoptosis was quantified after 12 h with a TUNEL assay and by determination of caspase-3 activity. A mean background level of apoptosis of approximately 10% was observed within populations of control, uninfected cells that were derived from different donors (Fig. 1A and B). Challenge of the cells with an equivalent MOI of bacteria induced a significant increase ($P < 0.05$) in apoptosis, with approximately 20 to 28% of the cells displaying the characteristic apoptotic phenotype, as determined by TUNEL and caspase-3 assays (Fig. 1A and B, respectively). In contrast, the levels of apoptosis induced by challenge with *Neisseria gonorrhoeae* at MOIs of 10 and 100 were significantly lower ($P < 0.05$) than the levels induced by the equivalent MOI of bacteria and similar to control values (Fig. 1A and B).

Analysis of bacterial association with human FT epithelial cells and the induction of apoptosis. Confocal microscopy was used to quantify the association of gonococci with the surfaces of FT epithelial cells. Gonococci showed a dose-dependent increase in association with FT epithelial cells, and by 12 h, significantly ($P < 0.05$) larger numbers of bacteria were associated with the cells following infection at an MOI of 100 than after infections at MOIs of 1 and 10 (Fig. 2a). The orthogonal analysis software of the confocal microscope was then used on the three-dimensional reconstruction of confocal images obtained by phase-contrast microscopy to demonstrate whether there was any correlation between the apoptotic phenotype shown by individual epithelial cells and the association of gonococci. Figure 2b shows a representative high-resolution confocal image of green fluorescent protein-labeled gonococci (MOI = 1) in association with human FT epithelial cells. No bacteria were observed in association with cells exhibiting the apoptotic phenotype (red nuclei), whereas gonococci were clearly observed in association with a live FT epithelial cell. The presence of associated bacteria correlated with the viability of epithelial cells, and data collected from 10 different experiments carried out with FT epithelial cells from 10 different donors demonstrated that apoptosis was observed in only 11% ± 1% of epithelial cells with associated gonococci, whereas 84% ± 11% of host cells without bacteria were apoptotic.

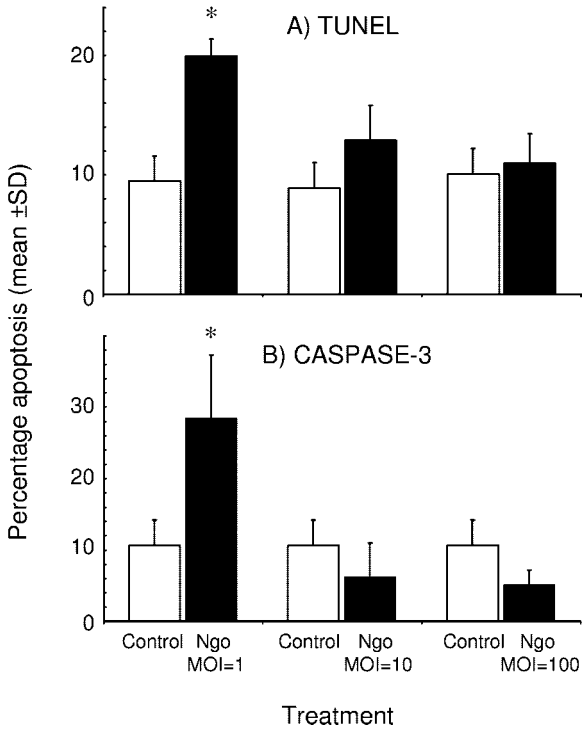

FIG. 1. Apoptosis in human Fallopian tube epithelial cells challenged in vitro with *Neisseria gonorrhoeae*. Apoptosis was detected in cultures of primary human FT epithelial cells 12 h after challenge with *Neisseria gonorrhoeae* (Ngo; MOI = 1, 10, or 100) by (A) TUNEL assay and (B) detection of caspase-3 activity. Each column denotes the mean and SEM (error bar) from 10 experiments (MOI = 1), 8 experiments (MOI = 10), or 7 experiments (MOI = 100) carried out with FT epithelial cells from 5 and 10 donors for the caspase-3 and TUNEL assays, respectively.

Correlation between apoptosis of FT epithelial cells induced by *Neisseria gonorrhoeae* and the presence of TNF-α. Several studies have demonstrated that gonococci up-regulate the production of TNF-α by FT organ culture epithelium and that secretion of this cytokine correlates with a cytopathic effect (40, 47, 48). In the current study, monolayers of FT epithelial cells also produced TNF-α in response to challenge with gonococci (Fig. 3). However, TNF-α induction was independent of the MOI of bacteria used, since no significant differences ($P > 0.05$) were observed in the high levels of cytokine secreted after 12 h of challenge with MOIs of 1 (mean, 86 ± 46 ng/ml), 10 (73 ± 36 ng/ml), and 100 (99 ± 39 ng/ml).

To determine whether TNF-α secretion correlated with the apoptotic phenotype, human FT epithelial cells were infected with *Neisseria gonorrhoeae* (MOI = 1) in the presence and absence of anti-human TNF-α1 and TNF-α2 antibodies. Significant ($P < 0.05$) apoptosis was induced above control levels following infection with gonococci (Fig. 4). This observed increase in apoptosis was significantly reduced ($P < 0.05$) in FT epithelial cell cultures challenged with gonococci in the presence of antibodies to both human TNF-α1 and TNF-α2 (Fig. 4). Moreover, the inhibitory effect was specific, since treatment with an irrelevant antibody had no significant effect on apoptosis induced by gonococcal infection (Fig. 4).

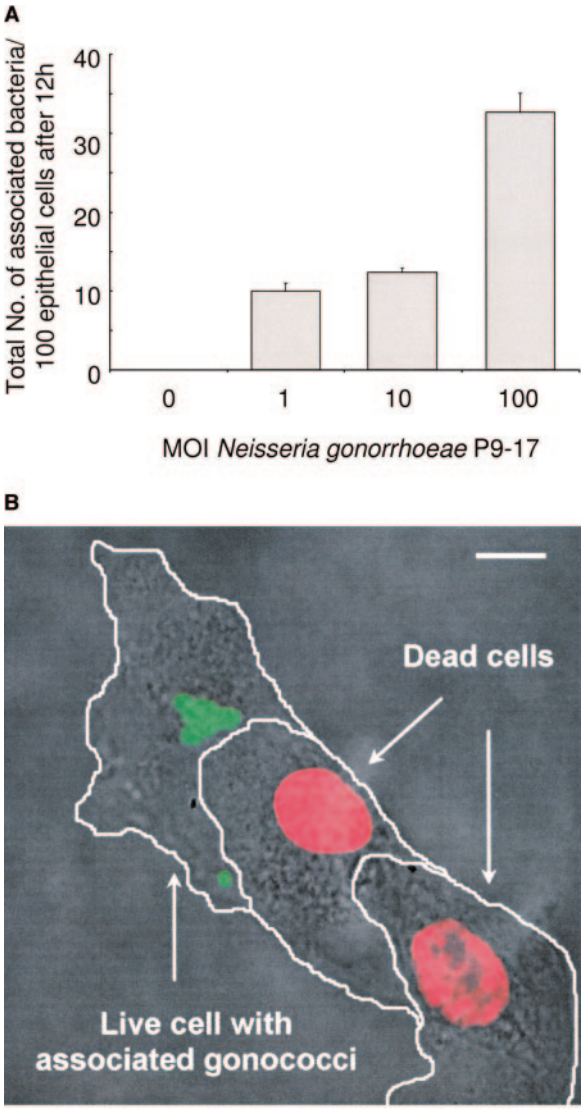

FIG. 2. (A) Association of *Neisseria gonorrhoeae* with FT epithelial cells, determined after a 12-h challenge with different MOIs of bacteria by confocal microscopy. Each column denotes the mean association and SEM (error bar) from three independent experiments. (B) Association of *Neisseria gonorrhoeae* protects human FT epithelial cells from apoptosis. Cultures of primary human FT epithelial cells were challenged with *Neisseria gonorrhoeae* (MOI = 1) for 12 h, and the confocal image, which is representative of 10 experiments carried out with FT from 10 donors, clearly shows the apoptotic phenotype (red-nucleated cells, determined by TUNEL staining) present in two cells without associated bacteria, whereas the single cell with associated bacteria is viable. Bar, 10 μm.

The ability of TNF-α to directly induce apoptosis in FT epithelial cell cultures was confirmed by treating naïve cells with exogenous cytokine (Fig. 5). In order to confirm that infection with *Neisseria gonorrhoeae* could inhibit TNF-α-induced apoptosis, the cells were infected at an MOI of 100 with gonococci for 12 h before treatment with exogenous cytokine for 5 h. As a result, apoptosis was reduced to levels similar ($P > 0.05$) to those of control, untreated cells (Fig. 5). In order to demonstrate that the inhibition of TNF-α-induced apoptosis was not due to the ability of gonococci to adsorb or degrade

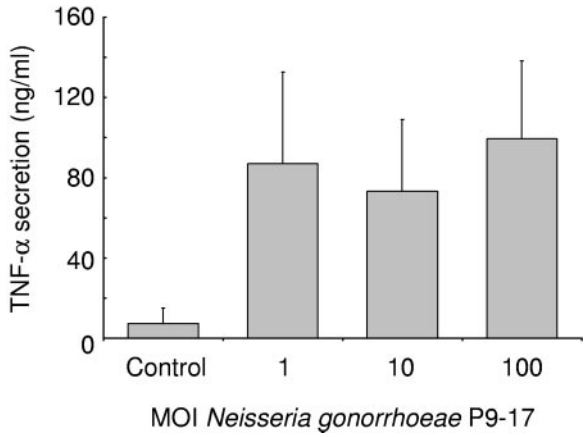

FIG. 3. Secretion of TNF-α from FT epithelial cells following challenge with different MOIs of *Neisseria gonorrhoeae* for 12 h. Each column represents the mean TNF-α level and SEM (error bar) from experiments with cells from five individual donors.

the cytokine, exogenous TNF-α was incubated for 5 h at 37°C in the presence of different MOIs (1, 10, and 100) of gonococci. A control sample of cytokine was incubated without gonococci. The bacteria were then removed by centrifugation, and the cytokine contents of the supernatants were assayed by an enzyme-linked immunosorbent assay (40). There was no significant difference ($P > 0.05$) in the levels of TNF-α recovered after incubation with gonococci at different MOIs and the level in the control sample (data not shown). Thus, gonococci did not adsorb or degrade TNF-α.

It is possible that less efficient invasion of FT epithelial cells

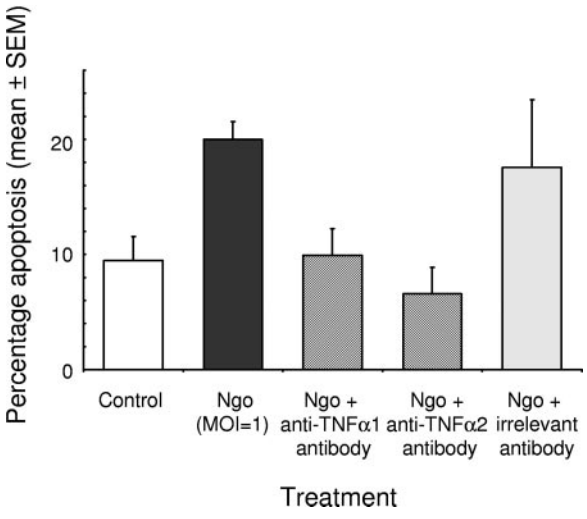

FIG. 4. Correlation between apoptosis of FT epithelial cells induced by *Neisseria gonorrhoeae* and the presence of TNF-α. Cultures of primary human FT epithelial cells were challenged with *Neisseria gonorrhoeae* (Ngo; MOI = 1) for 12 h in the presence and absence of anti-human TNF-α1 and TNF-α2 antibodies. Apoptosis induction was detected by TUNEL assay and analyzed by confocal microscopy. As a control, cells were also challenged with *Neisseria gonorrhoeae* in the presence of an irrelevant antibody. Data are from six experiments carried out with FT epithelial cells from six different donors, with the columns representing the means and the error bars representing the SEM.

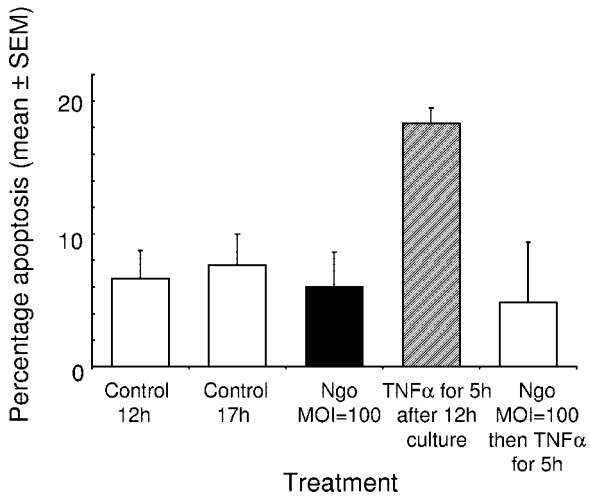

FIG. 5. Inhibition of TNF-α-induced apoptosis in FT epithelial cells by *Neisseria gonorrhoeae*. Apoptosis in cultures of primary human FT epithelial cells was induced by treatment with exogenous TNF-α for 5 h. Challenge with *Neisseria gonorrhoeae* (Ngo; MOI = 100) for 12 h did not induce apoptosis. Cells challenged with gonococci before treatment with exogenous TNF-α were protected from the proapoptotic effects of the cytokine. Data are from six experiments carried out with FT epithelial cells from six different donors, with the columns representing the means and the error bars representing the SEM.

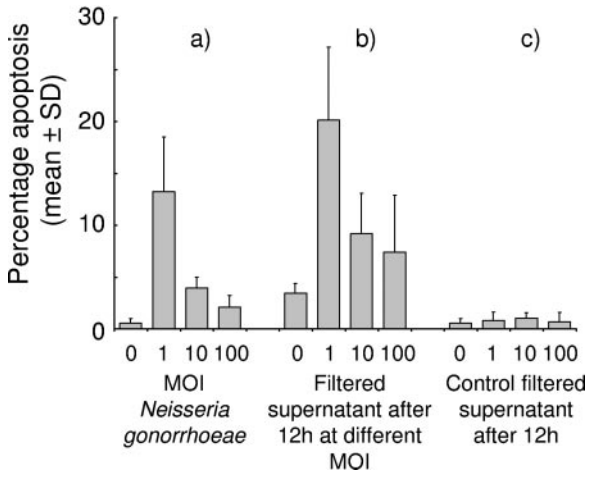

FIG. 6. Effects of supernatants from FT epithelial cell cultures infected for 12 h with gonococci on naïve cell cultures. Culture supernatants from FT epithelial cells that had been infected with gonococci (MOI = 1, 10, or 100) for 12 h were centrifuged to remove bacteria, filtered (0.2-μm pore size), and applied to fresh FT epithelial cells, and apoptosis was then determined by TUNEL assay following a further 12-h incubation. (a) Apoptosis induced by gonococci after the usual 12-h challenge. (b) Apoptosis of naïve FT epithelial cells induced by supernatants taken from cell cultures infected with different MOIs for 12 h. (c) Apoptosis of naïve FT epithelial cells induced by supernatants taken from control, uninfected cell cultures. Each column represents the mean and SEM (error bar) of nine experiments with cells from three donors.

by gonococci at increasing MOIs could account for the reduction of apoptosis. In order to investigate this, FT epithelial cell cultures were infected at MOIs of 1, 10, and 100 with Pil^+ Opa^+ gonococci, and the internalization of bacteria was quantified after 12 h by the saponin-gentamicin assay. Approximately 1 to 2% of bacteria associating with FT epithelial cells were internalized, and notably, there were no significant differences ($P > 0.05$) in the percentages of gonococci internalized, as calculated from the total number associated with each concentration tested (data not shown). Thus, the reduction in apoptosis observed in cultures challenged with bacteria at an MOI of 100 was not due to any decrease in the ability of gonococci to invade FT epithelial cells.

We next investigated whether supernatants from FT epithelial cell cultures that were infected for 12 h with gonococci (MOI = 1, 10, and 100) caused apoptosis in naïve cell cultures. Culture supernatants were centrifuged to remove bacteria and applied to fresh FT epithelial cells, and apoptosis was then determined following a further 12-h incubation. As expected, only small numbers of gonococci (MOI = 1) were able to induce apoptosis (Fig. 6a). Moreover, supernatants from cell cultures infected at a low MOI were able to induce apoptosis of naïve epithelial cells (Fig. 6b). In contrast, neither bacteria at a higher MOI nor culture supernatants from similarly infected cultures was able to induce significant apoptosis above control levels (Fig. 6a and b).

DISCUSSION

The regulation of host cell death represents a critical stage in the interaction between a pathogen and its host. While it is increasingly acknowledged that many pathogens can induce apoptosis of host cells (79), conversely, there is also evidence to suggest that some pathogens can protect infected host cells against apoptosis induced by immune cells or external stimuli (27, 73). In the current study, apoptosis of primary epithelial cells derived from human Fallopian tubes following infection with *Neisseria gonorrhoeae* was investigated. Infection with a small number of gonococci (MOI = 1) significantly increased apoptosis in FT epithelial cells. In contrast, increasing numbers of infecting bacteria (MOI = 10 to 100) inhibited apoptosis. Moreover, no gonococci were observed in association with cells that exhibited apoptosis, whereas the association of bacteria was correlated with cell viability. Thus, inhibition of apoptosis may be a mechanism used by the gonococcus to survive and proliferate in the FT epithelium. This conclusion is supported by studies with several other bacteria, including *Rickettsia rickettsii* (10), *Porphyromonas gingivalis* (58), *Bartonella* (37), *Chlamydiae pneumoniae* (6, 19, 20, 66), and *C. trachomatis* (12, 18), which have all been reported to inhibit apoptosis as a mechanism to allow host cell survival in order to promote bacterial survival at sites of infection.

Several studies have reported that pathogenic *Neisseria* species can modulate apoptosis in cell lines in vitro. Infection with whole gonococci has been shown to up-regulate the expression of the antiapoptotic genes *bfl-1*, *cox-2*, and *c-IAP-2* and to partially protect primary human urethral epithelium from apoptosis induced by the protein kinase inhibitor staurosporine (3). Although the bacterial factors responsible for protection against apoptosis are not known, several studies have suggested that neisserial outer membrane porin proteins may be involved (45). Purified gonococcal porin IB stimulated increases of the antiapoptotic genes *bfl-1*, *cox-2*, and *c-IAP-2* in human urethral epithelial cells (4), and purified PorB from

Neisseria meningitidis has been shown to prevent apoptosis of B cells, Jurkat cells, and HeLa cells (43, 44). In contrast, the studies of Muller et al. demonstrated that the gonococcal PIB porin induced calcium flux and apoptosis in HeLa and Jurkat cells (56, 57), although incubation of HeLa cells with gonococcal PIB purified according to the protocols used for meningococcal PorB did not induce cell death (43). Despite these differences, the site of action of both gonococcal and meningococcal porins appears to be a protein-protein interaction with the mitochondrial voltage-dependent anionic channel (VDAC) porin (43, 56). However, the interaction of gonococcal PIB porin with mitochondrial VDAC in FT epithelial cells and its role in inhibiting apoptosis are not known. In addition, the contributions of several other gonococcal components to modulating apoptosis cannot be excluded, given that the Opa protein, lipooligosaccharide, and pili also trigger host cell signaling events (1, 16, 24, 31, 35, 38, 55, 59, 62, 64). Indeed, during human infection, the colony phenotypes recovered from the cervixes of women with diagnosed salpingitis have been reported to be either mixtures of equal quantities of Pil^+ Opa^+ and Pil^+ Opa^- gonococci or predominantly Pil^+ Opa^+ (14). In the current study, we have shown that Pil^+ Opa^+ gonococci modulate apoptosis of FT epithelial cells. However, since the colony phenotype recovered from FT of women with diagnosed salpingitis was predominantly Pil^+ Opa^- (14), future studies will compare the effects of non-Opa-expressing bacteria on apoptosis of FT epithelial cells.

An important mechanism for inducing apoptosis is activation of the death receptor pathway by extracellular death-inducing ligands of the TNF superfamily e.g., TNF-α, which binds to the cognate cell surface receptors TNF-RI and -RII (39). A major response to infectious disease is cytotoxicity resulting from activation of this pathway, and the production of TNF-α has been shown to correlate with a cytopathic effect in FT explants (40, 48). In the current study, gonococcal interactions induced the secretion of TNF-α by isolated FT epithelial cells, and apoptosis could be induced by the addition of exogenous cytokine. In addition, culture supernatants from cells infected with small numbers of bacteria were able to induce apoptosis in naïve cultures, suggesting that a soluble factor, probably TNF-α, was responsible. Moreover, apoptosis induced by gonococci could be inhibited with anti-TNF-α antibodies. Thus, the current study demonstrates that secretion of TNF-α induced by the interactions of small numbers of infecting gonococci appears to contribute significantly to cytotoxicity in FT epithelial cells in vitro. A relationship between TNF-α secretion and apoptosis in the FT in vivo has also been suggested by studies using a mouse model of infection with a *Chlamydia trachomatis* mouse-specific pneumonitis strain (60). Infection with this pathogen led to a large increase in apoptotic cells in murine oviducts, but treatment with anti-TNF-α antibodies led to a significant decrease in the level of apoptosis in the upper genital tract.

In the current study, TNF-α-induced apoptosis was inhibited by the presence of large numbers of bacteria, and the inhibition was probably not due to adsorption or degradation of the cytokine by bacteria. Significantly, culture supernatants from cells infected with large numbers of bacteria did not induce apoptosis in naïve cultures, despite the presence of high concentrations of TNF-α, suggesting that increasing concentrations of as-yet-uncharacterized bacterial products may be responsible for protection. Although the mechanism is not known, a recent study has shown that an IgA1 protease produced by gonococci could inhibit apoptosis induced by TNF-α in the monocyte cell line U937. In those cells, inhibition of apoptosis was observed to correlate with specific cleavage of the TNF-RII molecule on the surfaces of the cells by IgA1 protease (2). Although the TNF-RII molecule does not contain the death domain, it has been reported to transduce the TNF-dependent apoptosis signal (29). We have previously reported that FT epithelial cells express mRNAs for both TNF-RI and TNF-RII (40), and it is possible that the inhibition of apoptosis mediated by increasing numbers of gonococci is due to IgA protease-mediated cleavage of host cell receptors, with attendant consequences for downstream intracellular signaling.

Although high levels of gonococci inhibited TNF-α-mediated apoptosis in the majority of donor cell cultures, an observation from our study was that no protection was observed in a minority of cultures (<20%). Possible explanations for this include the fact that the suitability of FT for quantitative studies is influenced by the endocrinologic status of the donor (49), which may also be manifested in cultures of primary epithelial cells. In addition, the physiological state of the cells, which is dependent on the stage of the menstrual cycle at which the cells are removed and cultured (33), may have an effect. These conditions may also be important factors influencing host cell-pathogen interactions in vivo.

An important question to consider for the current study is how the MOIs of gonococci used to infect FT epithelial cells correlate with the numbers of bacteria in clinical PID. To our knowledge, there are no definitive data on the numbers of gonococci present in the FT of humans diagnosed with salpingitis. Several studies on the polymicrobial etiology of PID have reported that gonococci were recovered less often from the human FT than from the cervix (5, 8, 11, 17, 42), but no comparative data were presented. Some indication of comparative bacterial numbers was afforded by a study with a murine model of long-term genital tract infection, which reported that following intravaginal infection with approximately 10^6 CFU of gonococci per mouse, a low level of gonococci (10^2 to 10^3 CFU) was recovered from the uterine horns (34). The different MOIs of gonococci used to challenge FT epithelial cells in our study (approximate range, 10^3 to 10^7 CFU), however, are similar to infecting doses of gonococci used in other in vitro and in vivo studies. For example, FT explants were infected with 2×10^5 CFU/ml, with bacterial counts rising to 10^7 CFU/ml after 20 h (50), and in a gonococcal challenge model, male volunteers were infected with 10^3 to 10^4 CFU, with numbers of viable bacteria recovered at the onset of symptomatic urethritis being in the region of 3×10^6 CFU per ml of urinary sediment (67, 69).

Despite the absence of definite correlates in vivo, data from the current study can be used nevertheless to propose a model to extend our knowledge regarding the sequence of events occurring during gonococcal infection of the FT and the development of salpingitis. *Neisseria gonorrhoeae* ascends from the lower genital tract into the Fallopian tubes, and several studies have demonstrated that gonococcal infection induces the production of inflammatory mediators (IL-6, IL-8, IL-1β,

and TNF-α) in the human genitourinary tract (67) and in ex vivo models of urethral epithelial cells (30), endometrial cells (9), FT epithelium (40, 48), and resident macrophages (41). Inflammation is required for the resolution of ascending gonococcal infection, but a chronic response promotes long-term tissue damage and the scarring process that is observed in salpingitis (65). In the initial stages of colonization of the FT, gonococci attach to microvilli of nonciliated cells (71, 72), and small numbers of bacteria induce apoptosis of uninfected, ciliated cells within the infected mucosal epithelium, which is likely mediated by TNF-α (48). Death and sloughing of adjacent uninfected cells (48) would enable the gonococcus to disseminate from the initial sites of colonization by penetrating the mucosal epithelium to infect deeper underlying tissue. However, this process would increase the likelihood of invading bacteria interacting with sentinel dendritic cells (81). Conversely, as bacterial numbers increase within the FT, apoptosis induced by TNF-α is inhibited in epithelial cells with associated bacteria, suggesting that gonococci can colonize the epithelium, possibly without alerting immune effector cells. This mechanism is consistent with previous studies demonstrating that gonococci adhere to nonciliated epithelial cells, become phagocytized by these viable cells, and are transported within phagocytic vacuoles to the base of the cell, where exocytosis into subepithelial tissues (71, 72, 77) and dissemination to the pelvic organs subsequently occur.

In conclusion, TNF-α-mediated apoptosis of FT epithelial cells in response to the invading gonococcus appears to be a host defense mechanism to prevent pathogen colonization. However, gonococci have evolved a mechanism to inhibit apoptosis, and this modulation of the host innate response is likely an intrinsic event in the pathogen's infection cycle that contributes to the establishment of infection. Understanding the antiapoptotic mechanisms used by *Neisseria gonorrhoeae* will inform the pathogenesis of salpingitis and could suggest new intervention strategies for prevention and treatment of the disease.

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