Infection of human urethral epithelium with *Neisseria gonorrhoeae* elicits an upregulation of host anti-apoptotic factors and protects cells from staurosporine-induced apoptosis

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Summary

In order to better understand the host response to an infection with *Neisseria gonorrhoeae*, microarray technology was used to analyse the gene expression profile between uninfected and infected human urethral epithelium. The anti-apoptotic genes bfl-1, Cox-2 and c-IAP-2 were identified to be upregulated approximately eight-, four- or twofold, respectively, following infection. Subsequent assays including RT-PCR, real time RT-PCR and RNase protection confirmed the increased expression of these apoptotic regulators, and identified that a fourth anti-apoptotic factor, mcl-1, is also upregulated. RT-PCR and RNase protection also showed that key pro-apoptotic factors including bax, bad and bak do not change in expression. Furthermore, our studies demonstrated that infection with the gonococcus partially protects urethral epithelium from apoptosis induced by the protein kinase inhibitor, staurosporine (STS). This work shows that inhibition of programmed cell death, or apoptosis, by viral and bacterial pathogens has been previously described (Antoni *et al*., 1995; Shen and Shenk, 1995; Shirin *et al*., 2000; Nakagawa *et al*., 2001). Several bac-

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ticular pathogens including Chlamydia (Fan et al., 1998; Geng et al., 2000; Fischer et al., 2001) and Porphyromonas gingivalis (Hiroi et al., 1998; Nakhrji et al., 2001) have been identified to modulate the host apoptotic programme in such a way to inhibit apoptosis. This is significant because an efficient mechanism whereby the host can clear invading pathogens is through the initiation of the apoptotic cascade. Therefore, by disrupting the initiation of apoptosis bacterial pathogens such as Chlamydia prolong the integrity of an important intracellular niche. This modulation of programmed cell death enhances the intracellular survival of these bacterial pathogens, and contributes significantly to the disease associated with their infection.

In contrast to necrotic death, which results from direct damage or physical insult imparted on a cell, apoptotic death ensues following a systematic activation of a cell’s death programme (Hengartner, 2000). This form of ‘cell suicide’ is highly regulated, involving the interplay of numerous host factors. Regulation can be achieved at multiple levels, but in large part occurs as a result of the expression levels and the co-ordinated interactions of a group of proteins belonging to the Bcl-2 family (Tsujimoto and Shimizu, 2000). Although the exact mechanisms of regulating apoptosis are still in question, it is believed that the members of the Bcl-2 family play a critical role in determining the life-or-death decision of the host cell.

Based on these observations, we analysed the possibility that N. gonorrhoeae may modulate the expression of host genes critical in the regulation of UEC death. In order to monitor the response of the UEC to an infection with the gonococcus, we first analysed the host global gene expression profile in uninfected and infected cells through the use of microarray technology. Following a detailed analysis of the changes in gene expression, we attempted to correlate the altered expression levels with an enhanced ability of the gonococcus to survive intracellularly. Therefore, the purpose of this study was to gain insight into the response of the urethral epithelium to a gonococcal infection, and determine whether that response would potentiate the ability of N. gonorrhoeae to proliferate in host epithelium.

Results

Anti-apoptotic regulators are increased in expression in urethral epithelium following infection with N. gonorrhoeae

To determine the transcriptional response of the urethral epithelium to an infection with the gonococcus, we performed microarray analysis on HPV E6/E7 transformed human urethral epithelial cells (Harvey et al., 2002), comparing expression levels in uninfected and infected cells. Analysis of the changes in expression between three inde-

| Gene       | Signal log ratio | Fold change | P-value |
|------------|-----------------|-------------|---------|
| bfl-1      | 3.01            | 8.06        | 9 × 10⁻⁶ |
| cox-2      | 2.15            | 4.44        | 9 × 10⁻⁵ |
| c-IAP-2    | 1.40            | 2.64        | 2 × 10⁻⁶ |
| bax        | 0.13            | 1.09        | 0.5     |
| 18S rRNA   | -0.15           | 0.90        | 0.5     |

Data given are the mean values from three independent comparisons of gene expression in uninfected and infected UECs utilizing Affymetrix® microarray technology. The signal log ratio, determined using a system of statistical algorithms, estimates the magnitude and direction of the change in expression between the experimental (infected) and control (uninfected) samples. The average fold change is determined as a function of the signal log ratio, where a log₂ = 1 corresponds to a twofold increase. Change P-values were determined using a Wilcoxon’s signed rank statistical analysis comparing expression intensities between uninfected and infected samples.

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Infection of urethral epithelium with *N. gonorrhoeae* upregulates anti-apoptotic factors

**Fig. 1.** Upregulation of anti-apoptotic regulators in infected urethral epithelium.

A. UECs and Chang cells were infected for 4 h with *N. gonorrhoeae* (1291) (MOI ~ 10:1 or 100:1), or received only media (uninfected) as a baseline control. Following the infection, total RNA was harvested and cDNA synthesis performed as described in the text. RT-PCR analysis then demonstrated the upregulation of anti-apoptotic genes in UECs infected with the gonococcus, whereas no change in expression occurred in gonococcal infected Chang cells. Panel (1) 18S rRNA internal control; (2) bfl-1; (3) cox-2; (4) c-IAP-2.

B. The expression level of *bfl-1* in uninfected and infected UECs was monitored by quantitative real time RT-PCR as described in the text. 18S rRNA (white bars) was included as an internal control. Analysis of *bfl-1* expression (shaded bars) demonstrated an approximate 10-fold induction in infected cells. Data given are the mean values calculated from the results of three independent real time reactions for each condition. Data do not represent absolute values, but rather the relative abundance of transcript present in each condition.

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Gen known to induce apoptosis. Invasion of epithelial tissue by group A *Streptococcus pyogenes* (GAS) has been shown to induce apoptosis by initiating the release of cytochrome c (Cyt c) from the mitochondria and subsequently activating the caspase cascade (Tsai *et al*., 1999; Nakagawa *et al*., 2001). Infection of the urethral epithelium with GAS for 4 or 24 h (MOI ~ 100:1) led to a significant level of UEC apoptotic cell death as measured by DNA fragmentation analysis (data not shown). Furthermore, RT-PCR analysis clearly demonstrated that whereas bfl-1 is upregulated in UECs infected with the gonococcus, no increase is observed in those cells infected with GAS (data not shown). In each of the RT-PCR analyses carried out, the level of 18S rRNA was monitored in both uninfected and infected cells as an internal control, and the product abundance corresponding to 18S rRNA did not change between conditions (Fig. 1A; panel 1). As a negative control, reverse transcriptase was excluded from the cDNA synthesis reaction to control for possible DNA contamination, and no amplification was observed (data not shown).

We then wished to obtain a quantitative representation of the change in *bfl-1* expression in UECs infected with *N. gonorrhoeae*, in order to further confirm, and correlate the results with those from the microarray analyses. Therefore, real time RT-PCR was carried out to specifically analyse the changes in expression of *bfl-1* in a quantitative manner. The 18S rRNA again served as an internal control, and its expression was not altered between conditions (Fig. 1B; white bars). However, real time RT-PCR indicated an approximate 10-fold increase in *bfl-1* in infected cells (Fig. 1B; shaded bars). These results confirmed the increased transcription of *bfl-1*, and correlated very well with the approximate eightfold induction of this apoptotic regulator as indicated in the microarray experiments.

**Pro-apoptotic Bcl-2 family members are not increased in expression following a gonococcal infection**

It is believed that the interactions of members of the Bcl-2 family play an important role in the regulation of apop-
tosis. Whether or not a cell commits to die is therefore often determined by the expression status and the co-ordinated activity of both anti- and pro-apoptotic Bcl-2 family proteins (Tsujimoto and Shimizu, 2000). To gain a more comprehensive understanding of the expression levels of the Bcl-2 family in uninfected and infected cells, ribonuclease (RNase) protection assays were performed. Utilizing a probe set (hAP0–2c – Pharmingen) specific for the major pro- and anti-apoptotic members of the Bcl-2 family, we again demonstrated by RNase protection the upregulation of bfl-1 in infected UECs (Fig. 2A; arrow 3). In addition, the anti-apoptotic member, mcl-1, was increased in expression in cells infected with N. gonorrhoeae (Fig. 2A; arrow 8). However, while these anti-apoptotic regulators are upregulated, key pro-apoptotic members of the Bcl-2 family such as bad, bak, and bax were not altered in expression between conditions (Fig. 2A; arrows 4–6). Of interest, the major anti-apoptotic regulator, bcl-2, did not appear to be expressed in either uninfected or infected cells (Fig. 2A; arrow 7). These results were confirmed by RT-PCR (data not shown). This may indicate an enhanced role of bfl-1 and mcl-1 in the urethral epithelium, which may act to regulate apoptosis in the absence of bcl-2.

The results of the RNase protection assay were confirmed by designing specific primers to the major Bcl-2 family members and subsequently carrying out RT-PCR analysis. RT-PCR confirmed the increased expression of the anti-apoptotic factor, mcl-1 (Fig. 2B; panel 3), and again demonstrated that the pro-apoptotic factors bax, bak and bcl-xL are not upregulated following infection (Fig. 2B; panels 5–7). In co-ordination with the data from our microarray analyses, these experiments provide evidence that infection of the urethral epithelium with N. gonorrhoeae increases the transcription of several anti-apoptotic genes, but does not effect the expression status of key factors that act to promote host cell apoptosis.

Infection of the urethral epithelium with N. gonorrhoeae protects host cells from staurosporine-induced apoptosis

The ability of the protein kinase inhibitor, STS, to induce apoptosis has been previously described (Vander Heiden et al., 1997; Bossy-Wetzel et al., 1998). Cells treated with STS exhibit many of the hallmarks of apoptosis, including Cyt c release, caspase activation and late stage DNA fragmentation (Bossy-Wetzel et al., 1998). Overexpression of bfl-1 and mcl-1 has been shown to inhibit STS-
mediated apoptosis (Reynolds et al., 1996; Shim et al., 2000). Therefore, we examined whether infection of the urethral epithelium with *N. gonorrhoeae* could protect host cells from STS-induced death. We first monitored the extent of DNA fragmentation by measuring the hypodiploid DNA content by propidium iodide (PI) staining and FACS analysis. UECs treated for 24 h with 1 μM STS exhibited a marked increase in the percentage of hypodiploid DNA, indicating that STS was able to induce apoptosis in our cell system (Fig. 3A; panel 5). UECs treated

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**Fig. 3.** Measurement of DNA content by PI staining and FACS analysis.

A. DNA fragmentation was analysed by monitoring the extent of DNA hypodiploidy as outlined in the text. FACS profiles representative of at least three independent experiments are shown. Gates were drawn using data from uninfected cells as a means of normalization (not shown). Panel (1) Uninfected UECs; (2) UECs infected for 4 h with 1291; (3) UECs treated for 4 h with 1 μM STS; (4) UECs preinfected with 1291 for 4 h before addition of 1 μM STS for 4 h; (5) UECs treated for 24 h with 1 μM STS as a positive control for STS-mediated induction of apoptosis.

B. Average percentage hypodiploid staining from three independent experiments in UECs that received media alone (Uninfected), were infected for 4 h with *N. gonorrhoeae* (1291 wt), were treated with 1 μM STS for 4 h (4 h STS), or were preinfected with the gonococcus for 4 h before treatment with STS (1291–4 h STS). Statistical analyses were performed to determine the level of significance (*P* < 0.05 when compared to †).

C. Average percentage hypodiploid staining in Chang cells receiving identical treatments as those in B.

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for 4 h with STS exhibited an approximate twofold induction in the level of hypodiploid DNA when compared to untreated UECs (Fig. 3A; panel 3 (39.91%) compared to panel 1 (19.20%)). However, if the cells were preinfected with the gonococcus for 4 h (MOI ~ 10:1) before the addition of STS, a significant reduction ($P < 0.05$) in the level of DNA fragmentation was observed [Fig. 3A; panel 4 (25.08%)]. Nearly identical results were achieved when the cells were preinfected for 24 h (MOI ~ 10:1 or 100:1) before the addition of STS (data not shown). Infecting the urethral epithelium with the gonococcus alone did not induce DNA fragmentation above the level of uninfected cells (Fig. 3A; panel 2). The results are representative of at least three independent experiments, and $t$-test analysis was performed to demonstrate statistical significance (Fig. 3B). In contrast, preinfection of Chang cells with the gonococcus was not able to protect host cells from STS-induced apoptosis (Fig. 3C). Furthermore, infection of UECs with *E. coli* did not alter the expression status of *bfl-1*, *c-IAP-2* or *cox-2*, and did not protect UECs from STS-induced DNA fragmentation (data not shown). These results provide evidence that the observed protection from STS-induced apoptosis may be specific to a gonococcal infection of the urethral epithelium.

In addition to DNA fragmentation, we also monitored the localization of Cyt *c* as a means of analysing host cell apoptosis. The release of Cyt *c* from the mitochondria results in the activation of the caspase cascade (Bossy-Wetzel *et al.*, 1998; Jürgensmeier *et al.*, 1998), and its mitochondrial localization is in large part regulated by Bcl-2 family proteins (Yang *et al.*, 1997; Kluck *et al.*, 1997; Jürgensmeier *et al.*, 1998). Confocal microscopy was performed on cells that were labeled with 100 nM MitoTracker (Molecular Probes), a dye that selectively labels actively respiring mitochondria. Uninfected UECs, as well as cells infected with the gonococcus alone, exhibited a punctate labeling indicative of intact, active mitochondria (Fig. 4A). However, cells treated for 4 h with 1 µM STS exhibited a faint, diffuse mitochondrial labelling indicating disruption of the mitochondrial membrane potential. Interestingly, the mitochondria of cells preinfected with *N. gonorrhoeae* prior to the addition of STS exhibited labelling more like that of the uninfected cells (Fig. 4A). To analyse the localization of Cyt *c*, cells were co-labelled with an anti-Cyt *c* (6H2) monoclonal antibody (Fig. 4B). Labelling of Cyt *c* demonstrated co-localization with the mitochondria of both uninfected and infected cells when the two separate fluorophores were merged (Fig. 4C). However, both the level of Cyt *c* labeling, and its mitochondrial localization, was significantly reduced in UECs treated with STS. Labelling of the cytochrome and its localization within mitochondria was restored in cells preinfected with the gonococcus before addition of STS (Fig. 4C). Together with our analysis of DNA fragmenta-

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**Fig. 4.** Confocal analysis of cytochrome *c* localization. UECs were left uninfected, infected for 4 h with *N. gonorrhoeae* (1291), treated for 4 h with 1 µM STS, or were preinfected with 1291 before the addition of STS. Following these treatments, the cells were stained with 100 nM MitoTracker (A), fixed, permeabilized, and then co-stained with a mAb selective to cytochrome *c* (B). Representative composite y-projections depicted as individual colour images, as well as a merge of the two fluorophores (C) are shown.

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tion, these results show that infection of the urethral epithelium with *N. gonorrhoeae* protects host cells from STS-mediated apoptosis.

**Discussion**

We have demonstrated the upregulation of several anti-apoptotic genes in human urethral epithelium infected with *N. gonorrhoeae*. Analysis of infected cells by microarray technology, RT-PCR, quantitative real time RT-PCR and RNase protection confirmed the increased expression of bfl-1, cox-2, mcl-1 and c-IAP-2. These studies also identified that infection of UECs with the gonococcus does not alter the expression of key pro-apoptotic members of the Bcl-2 family. These findings were extended by the observation that infection of UECs with *N. gonorrhoeae* partially protects host cells from apoptosis induced by the protein kinase inhibitor, STS. Furthermore, the host cell response to a gonococcal infection appears to differ between different cell types, as an infection of Chang cells with *N. gonorrhoeae* did not elicit a response similar to UECs. Based on these results, we propose a model wherein gonococcal infection of the urethral epithelium alters host gene expression to inhibit apoptosis, thereby promoting the survival of this pathogen within epithelial tissue.

The regulation of apoptosis is a highly orchestrated process, involving the co-ordinated activity and interactions of multiple factors. Primary sites of control include the regulation of Cyt c localization and the activity of a group of cysteine proteases, termed caspases. The localization of Cyt c and the activation of caspases are often regulated by the activities of the Bcl-2 family. Overexpression of pro-apoptotic members of this family, including Bad or Bax, has been shown to induce apoptosis (Kelekar *et al.*, 1997; Jürgensmeier *et al.*, 1998). In contrast, overexpression of anti-apoptotic members, such as Bcl-2 and Bfl-1, has been demonstrated to abrogate the function of pro-apoptotic factors and elicit a protective effect on host cells (Yang *et al.*, 1997; D’Souza *et al.*, 2000; Werner *et al.*, 2002). Therefore, the expression levels of either pro- or anti-apoptotic factors play an important role in determining the life-or-death decision of the host cell. The anti-apoptotic genes identified to be upregulated in our studies each function to regulate cell death through distinct mechanisms. Bfl-1 has been shown to inhibit apoptosis by interacting with several pro-apoptotic factors, including Bid (Werner *et al.*, 2002). In response to CD95 engagement and caspase-8 activation, Bid transmits apoptotic signals to the mitochondria (Li *et al.*, 1998; Luo *et al.*, 1998). Bfl-1 can block these signals by interacting with Bid, preventing Bid from associating with other pro-apoptotic factors, such as Bax or Bak (Werner *et al.*, 2002). This inhibits the release of Cyt c and the subsequent initiation of the apoptotic cascade. Bfl-1 has also been shown to inhibit apoptosis through a direct interaction with the major pro-apoptotic determinant, Bax (Zhang *et al.*, 2000). These reports suggest an important and multifaceted function of Bfl-1 in regulating apoptosis.

The inducible prostaglandin H synthase, Cox-2, is an enzyme involved in the conversion of free arachidonic acid into prostaglandins (PG) (Smith *et al.*, 2000). Overexpression of cox-2 in epithelial cells has been shown to promote tumour formation, angiogenesis and inhibit apoptosis (Tsujii *et al.*, 1998; Kim *et al.*, 2000). Although the exact mechanism of Cox-2-mediated apoptosis inhibition is still in question, its role in regulating cell death appears to involve several factors. In support of our findings, overexpression of cox-2 has been shown to significantly up regulate the anti-apoptotic Bcl-2 family member, mcl-1 (Lin *et al.*, 2001). Mcl-1 was identified to have functional homology to Bcl-2, and can inhibit apoptosis induced by c-Myc overexpression (Reynolds *et al.*, 1994). Furthermore, Mcl-1 can interact directly with the pro-apoptotic factor, Bok, inhibiting its ability to initiate cell death (Hsu *et al.*, 1997). Cox-2-mediated mcl-1 upregulation and the subsequent inhibition of apoptosis appear to be linked to alterations in intracellular signalling and transcriptional activation. The PG products resulting from Cox-2 catalysis can activate G protein-linked receptors, or may influence transcription by interacting with nuclear receptors (Smith *et al.*, 2000). In addition, cox-2 overexpression activates the phosphatidylinositol 3 kinase (PI3K)/Akt pathway, which promotes the upregulation of mcl-1 (Lin *et al.*, 2001). These findings suggest that Cox-2 may directly, or indirectly through the activation of mcl-1, play an important role in the inhibition of programmed cell death.

We also identified that c-IAP-2, which encodes the human inhibitor of apoptosis protein-1 (HIAP-1), is significantly upregulated in infected cells. HIAP-1 belongs to the inhibitor of apoptosis protein (IAP) family, which functions to regulate apoptosis downstream of Cyt c release (Deveraux *et al.*, 1998). HIAP-1 has been shown to bind and inhibit the activation and/or the activity of caspase-3, -7 and -9 (Roy *et al.*, 1997; Deveraux *et al.*, 1998). These caspases can become activated following the stimulation of different apoptotic pathways (Deveraux *et al.*, 1998). Therefore, targeting of these caspases suggests that HIAP-1 can suppress apoptosis initiated by multiple pathways. Our results demonstrating the increased expression of c-IAP-2 and several Bcl-2 family genes indicate that a gonococcal infection alters the expression of several host genes that act to inhibit apoptosis by functionally distinct mechanisms.

The dramatic alterations in apoptotic regulator gene expression outlined in this report raises the following question; what benefit would a bacterial pathogen derive from modulating the host cell death programme? There are certainly examples of bacteria that benefit from the
induction of host cell apoptosis. Pathogens that induce apoptosis include S. pyogenes, S. typhimurium, Shigella flexneri and Yersinia enterolitica (Zychlinsky et al., 1992; Hersh et al., 1999; Tsai et al., 1999; Ruckdeschel et al., 2001). The initiation of macrophage apoptosis by Salmonella, Shigella and Yersinia has been attributed to an enhanced ability of these organisms to evade macrophage killing, and often allows the bacteria to gain access to the submucosa (Grassmé et al., 2001). For instance, Shigella is not able to infect the gastrointestinal epithelium when this cell layer is intact. Therefore, induction of host cell apoptosis at the Peyers’ patches allows Shigella to gain access to deeper tissue (Chen et al., 1996). In contrast, pathogens such as Chlamydia pneumoniae, Porphyromonas gingivalis and Helicobacter pylori have been shown to inhibit apoptosis (Fan et al., 1998; Hiroi et al., 1998; Kim et al., 2000; Shirin et al., 2000; Fischer et al., 2001; Nakhjiri et al., 2001). The suppression of apoptosis by Chlamydia allows this obligate intracellular bacterium to maintain an environment necessary for its replication, and may explain the ability of this pathogen to cause chronic infections (Fischer et al., 2001). In addition to maintaining a cellular environment that supports bacterial replication, blocking apoptosis might also prevent the uptake and clearance of infected cells by neighbouring phagocytes. This may be critical for bacterial viability, as a major host mechanism of clearing infected cells is through the initiation of apoptosis (Savill and Fadok, 2000). Finally, bacterial pathogens may depend on the host cell for certain components that enhance the organism’s intracellular or extracellular survival. For example, Chlamydia requires cellular-derived factors, such as host nucleotides and sphingolipids, in order for bacterial replication to occur (van Ooij et al., 2000; Fischer et al., 2001).

The results outlined in this report are in support of the findings published by Massari and co-workers in which N. meningitidis was identified to protect host cells from apoptosis through the activity of the Neisserial porin, PorB (Massari et al., 2000; 2003). However, our findings are in contrast to reports by Müller and co-workers that demonstrated N. gonorrhoeae can induce host cell apoptosis as a result of porin targeting the mitochondria (Müller et al., 1999; 2000). These studies demonstrating an induction of apoptosis by the gonococcus were performed mainly in HeLa cells (a human cervical carcinoma cell line), whereas our studies were performed in HPV E6/E7 transformed urethral epithelial cells and confirmed using primary cells derived from the male urethra. The discrepancies in the results may be due to differences in the apoptotic response to a gonococcal infection between different cell systems. Our studies demonstrating a dramatic difference in the response of Chang cells and UECs to the gonococcus would certainly provide evidence to support this.

In summary, we have described the increased expression of anti-apoptotic genes in human urethral epithelium following an infection with N. gonorrhoeae. In addition, we demonstrated that a gonococcal infection protects host cells from STS-mediated Cyt c release and DNA fragmentation. This work provides another striking example of a pathogen that is able to manipulate host cell activity in order to promote bacterial survival. Future work will certainly focus on the mechanisms responsible for the alteration of gene expression and the inhibition of apoptosis. These studies will not only provide an increased understanding of gonococcal pathogenesis, but may also further characterize additional factors and pathways involved in regulating programmed cell death.

Experimental procedures

**Epithelial cell culture**

Primary human urethral epithelial cells used in these studies were derived from membranous urethral tissue explants obtained from male patients undergoing prostate surgery. Primary human urethral epithelial cells were grown, maintained and passaged as previously described (Harvey et al., 2001). In brief, surgical tissue samples were seeded in prostate epithelial growth medium (PrEGM; Cambrex, San Diego, CA) supplemented with 5% heat-inactivated fetal calf serum (FCS) on 100 mm tissue culture-treated Petri dishes. Five days after seeding, cultures were maintained in FCS-free PrEGM for approximately 3–4 weeks. A mixture of 0.25% trypsin and 0.1% EDTA was used to lift PHUECs during passaging. Cells were incubated in trypsin solution for 2 min at 25°C, followed by removal of trypsin and incubation for 5 min at 37°C. Primary human urethral epithelial cells were suspended in 5% FCS-PrEGM, centrifuged for 2 min at 1380 g and resuspended in the desired volume of PrEGM before seeding to 60 mm tissue culture-treated Petri dishes.

Human papillomavirus (HPV) E6/E7 transformed human urethral epithelial cells (THUECs) previously described (Harvey et al., 2002), were also used in these studies based on the availability of primary tissue. Transformed human urethral epithelial cells have been characterized to express the receptor to which the gonococcus binds, and the cellular response to a gonococcal infection is similar to that of primary urethral cells (Harvey et al., 2002). Transformed human urethral epithelial cells were thawed from liquid nitrogen storage in a 37°C water bath and 1 ml cell suspensions were added to 5 ml of PrEGM supplemented with 5% FCS. Diluted THUEC suspensions were seeded to 25 cm² tissue culture-treated flasks (Corning, Cambridge, MA). Twenty-four hours after seeding, cultures were maintained in FCS-free PrEGM. Upon reaching the desired confluence (>90%) transformed human urethral epithelial cells were lifted, collected, and centrifuged as described above for PHUECs. Transformed human urethral epithelial cells were resuspended in the desired volume of PrEGM before seeding to 100 mm tissue culture-treated Petri dishes.

Chang cells used in these studies were thawed from liquid nitrogen storage in a 37°C water bath and seeded into 100 mm tissue culture-treated Petri dishes. Chang cells were grown in RPMI-1640 (Invitrogen) supplemented with 10% FCS/1%
L-glutamine, and maintained using standard tissue culture protocols.

**Bacteria and urethral cell infection**

*Neisseria gonorrhoeae* strain 1291 was used in these studies. This strain was originally isolated from a male with gonococcal urethritis, and has been characterized to express a single species of LOS (Dudas and Apicella, 1988; John et al., 1991). Strain 1291 was grown overnight (37°C/5% CO₂) on GC agar, and P + (pilus-positive), Opa + (opacity-associated protein-positive) gonococci were selected on the basis of colony morphology. The group A *Streptococcus pyogenes* strain (GAS) used in these studies is a patient isolate acquired by a pharyngeal swab. The group A *Streptococcus pyogenes* strain were grown overnight (37°C) on blood agar. Studies utilizing E. coli were performed using a urinary tract isolate, grown overnight (37°C) on LB agar. Infections of UECs were performed as previously described (Harvey et al., 2001). Bacteria were collected from an overnight plate culture, suspended in GC broth or PBS and adjusted to an OD₆₀₀ = 0.16 (~10⁶ gonococci ml⁻¹), before dilution in antibiotic-free PrEGM. Multiplicity of infection (MOI) ratios were approximately 100 bacteria/epithelial cell or 10 bacteria/epithelial cell, as indicated. Infection was allowed to occur for 4 h, after which the cell monolayers were washed 2 x 2 min in fresh antibiotic-free PrEGM and processed as described below.

**Microarray analysis**

Following infection, total RNA was harvested from uninfected and infected cells with Tri Reagent (Sigma) following standard protocols. RNA samples were then DNase treated and column purified using the RNeasy® Minikit (Qiagen). Following subsequent spectrophotometric quantification and resolution on a 1% agarose gel to ensure purity, samples were sent to the University of Iowa DNA facility (Iowa City, IA, USA) for further sample preparation. Samples were processed and hybridized on the Affymetrix Human U95A GeneChip® according to manufacturer’s protocols. Data was compiled using the Affymetrix GeneArray Scanner (Hewlett Packard) and analysed with Microarray Suite v5.0 analysis software. Experiments were carried out in triplicate for each condition, and data from the infected samples were normalized to that of the uninfected baseline control.

**RT-PCR analysis**

For RT-PCR studies, cells were confluent and challenged with the gonococcus as described above. Total RNA was purified with Tri Reagent (Sigma). cDNA synthesis was performed using the First Strand cDNA synthesis kit for RT-PCR (AMV) (Roche Biochemical). Primers specific to *bfl-1* (forward, 5'-TGG AGT GCG TCC TAC AGA TAC-3'; reverse, 5'-GCT TTT GCC TTA CCT ATT CTC-3'), *cox-2* (forward, 5'-CCT CCT GTG CCT GAT GAT TG-3'; reverse, 5'-CCG TAG ATG CTC AGG GAC TT-3'), c-IAP-2 (forward, 5'-AGT CTT CCG TCT GGT CCT GGT T-3'; reverse, 5'-TTG ATT GGA GAG GGG TAC G-3'), mcl-1 (forward, 5'-CGC ACT TTT GGC TAC GGA GAA-3'; reverse, 5'-TTA CCA GAT TCC CCG ACC AAC-3'), bcl-x (forward, 5'-GCA CAG CAG CAG TTT GGA T-3'; reverse, 5'-TGC TGC ATT GTT CCC ATA G-3'), bax (forward, 5'-ATG CGT CCA CCA AGA AGC-3'; reverse, 5'-GCG AGT GAG GCG GTG AGC-3'), bak (forward, 5'-TTA CCG CCA TCA GCA GGA A-3'; reverse, 5'-GTT AGT AGC CGA AGC CCA GAA G-3'), and bcl-xL (forward, 5'-CAC AGC AGC AGT TTG GAT G-3'; reverse, 5'-AAG AGT GAG CCC AGC AGA A-3') were designed and used for the amplification of a 363, 396, 356, 357, 391, 400, 290, or 290 bp ampiclon respectively. The constitutively expressed 18S rRNA control was analysed for comparison to the indicated apoptotic regulators mRNA levels. Quantum RNA 18S internal standards (Ambion) were used according to the manufacturer’s instructions, with amplification resulting in a 488 bp RT-PCR product. Polymerase chain reaction products (15 µl) were run on a 1% agarose gel and analysed following standard EtBr staining.

**Real time RT-PCR analysis**

The expression of *bfl-1* was monitored by real time RT-PCR using the TaqMan® One Step PCR kit (Roche) according to the manufacturer’s protocol. Primers specific for *bfl-1* (forward, 5'-GAC TAT CTG CAG TGC GTG CTA CAG-3'; reverse, 5'-AAC ATT TTG TAG CAC TCT GCA GGT T-3'; TaqMan® probe, 5'-CCA CAAC CCT GGA TCA GGT CCC AGC A-3') were designed to amplify a 77-bp ampiclon within the *bfl-1* coding region. Primers were designed for 18S rRNA (forward, 5'-CGC CGC TAG AGG TGA AAT TC-3'; reverse, TCT TGG CAA ATG TTC CGT CT-3'; Taq-Man® probe, 5'-TGG ACC GGC GCA AGA CGG AC-3') and the resulting 63 bp ampiclon was monitored as a constitutively expressed internal control. Real time RT-PCR was carried out using an ABI Prism 7700 Sequence Detector (Perkin Elmer) with ABI Prism 7700 v1.7 analysis software at the University of Iowa DNA facility.

**RNase protection assay**

The expression of the major pro- and anti-apoptotic Bcl-2 family members was analysed using the Ribonuclease protection assay system and hAPO-2c probe template set (Pharmingen, San Diego, CA). Probe synthesis, hybridization and RNase treatment were carried out according to the manufacturer’s instructions. Gel resolution of RNase protected fragments was then performed by running the samples on an 8.6% polyacrylamide gel, after which the gel was air-dried and exposed to X-ray film for 18 h. Band intensities were quantified using the Instant Imager (Packard) with Instant Imager analysis software. Bands corresponding to Bcl-2 family apoptotic regulators were normalized to that of the L32 and GAPDH housekeeping genes included as an internal control.

**Induction of host cell apoptosis**

UECs and Chang cells were incubated for 4 or 24 h in the presence of 1 µM STS (Sigma) to induce host cell apoptosis. In brief, STS was diluted in antibiotic-free PrEGM to a working concentration of 1 µM, and cells were incubated in the presence of STS (37°C/5% CO₂) for the indicated time point. Following the incubation with STS, the cells were washed and subsequently treated as described in the text and processed as described below.
Flow cytometric analysis to measure hypodiploid DNA

The DNA content of UECs was measured as follows. Following the appropriate treatment (as noted), cells were washed, collected, and resuspended in 900 μl 70% EtOH. Cells were incubated at −20°C overnight, centrifuged at 1380 g, and the pellet subsequently washed twice in 2 ml of PBS. Cells were then resuspended in 500 μl DNA extraction buffer (0.1 M citric acid/0.2 M Na2HPO4), incubated for 5 min at 25°C containing 10 μl trypsin and 0.1 ml RNaseA, incubated for 30 min at 25°C in the dark, washed and examined by flow cytometry on a FACScan flow cytometer (Becton Dickinson) using SUMMIT v3.0 acquisition and winMDI analysis software. Gates were drawn using data from the uninfected samples as a means of normalization.

Confocal microscopy

For microscopy studies we used the Zeiss LSM 510 laser scanning confocal microscope at the University of Iowa Central Microscopy Research Facility (Iowa City, IA, USA). Cells were seeded on glass coverslips as described previously (Harvey et al., 1997) and treated as indicated in the figure legend. Before fixation, samples were incubated with 100 nM Mitotracker Red 580 (Molecular Probes) for 30 min at 37°C. The samples were then fixed in 2% paraformaldehyde-PBS, and stored at 4°C overnight. Following fixation, the samples were washed 2×2 min with PBS, permeabilized with 0.2% Triton X-100, blocked for 30 min in 5% normal goat serum-PBS, and then incubated in the presence of a 1:50 dilution of anti-Cyt c mouse monoclonal antibody 6H2 (Santa Cruz Biotechnology). Next, the samples were incubated in the presence of a 1:100 dilution of goat anti-mouse IgG FITC (Jackson ImmunoResearch). The coverslips were then mounted, overlayed with Vectashield (Vector Laboratories, Burlingame, CA), and analysed by LSCM.

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