The Molecular Basis of Nonoxynol-9–Induced Vaginal Inflammation and Its Possible Relevance to Human Immunodeficiency Virus Type 1 Transmission

Raina N. Fichorova, Lynne D. Tucker, and Deborah J. Anderson

An international effort is underway to develop user-controlled topical vaginal formulations that provide protection against human immunodeficiency virus type 1 (HIV-1) and other sexually transmitted pathogens through direct microbicidal action and/or enhancement of natural defense mechanisms in the cervicovaginal environment [1]. Serious public health concerns were raised by a recent phase 3 clinical trial that showed that nonoxynol-9 (N-9), a leading microbicide candidate widely used as an over-the-counter spermicide, may actually increase human immunodeficiency virus type 1 (HIV-1) transmission. The present study links N-9–induced vaginal inflammation to increased risk of HIV-1 infection. Analysis of molecular and cellular components in cervicovaginal secretions, as well as results from in vitro activation of cervicovaginal epithelial cells and U1/HIV promonocytic cells, showed that multiple N-9 use can promote HIV-1 transmission through interleukin-1–mediated NF-κB activation, which leads to chemokine-induced recruitment of HIV-1 host cells and increased HIV-1 replication in infected cells. Furthermore, this study identifies in vitro and in vivo model systems for monitoring undesirable proinflammatory effects of microbicides and other vaginal products.

Topical microbicides are being sought to prevent sexually transmitted diseases by inactivating pathogens while preserving or enhancing the natural mucosal barrier. Serious public health concerns were raised by a recent phase 3 clinical trial that showed that nonoxynol-9 (N-9), a leading microbicide candidate widely used as an over-the-counter spermicide, may actually increase human immunodeficiency virus type 1 (HIV-1) transmission. The present study links N-9–induced vaginal inflammation to increased risk of HIV-1 infection. Analysis of molecular and cellular components in cervicovaginal secretions, as well as results from in vitro activation of cervicovaginal epithelial cells and U1/HIV promonocytic cells, showed that multiple N-9 use can promote HIV-1 transmission through interleukin-1–mediated NF-κB activation, which leads to chemokine-induced recruitment of HIV-1 host cells and increased HIV-1 replication in infected cells. Furthermore, this study identifies in vitro and in vivo model systems for monitoring undesirable proinflammatory effects of microbicides and other vaginal products.

An international effort is underway to develop user-controlled topical vaginal formulations that provide protection against human immunodeficiency virus type 1 (HIV-1) and other sexually transmitted pathogens through direct microbicidal action and/or enhancement of natural defense mechanisms in the cervicovaginal environment [1]. Serious public health concerns were raised by a recent phase 3 clinical trial that showed that a product (COL-1492) containing nonoxynol-9 (N-9), a leading vaginal microbicide candidate, may increase HIV-1 transmission [2]. N-9 has been used for >30 years, in the United States and other countries, it continues to be included in numerous spermicidal and lubricant products. It is a nonionic surfactant with demonstrable in vitro toxicity to a number of species of bacteria and enveloped viruses, including HIV-1 [3]. Although its frequent use has been associated with vaginal irritation and ulceration [4–7], various N-9 products, including COL-1492, have been found generally safe by colposcopic examination in phase 1 and 2 clinical trials [8–10].

Although monitoring for cervicovaginal lesions has been a routine part of clinical safety microbicide trials, little is known about potential subtle changes in the cervicovaginal mucosal barrier, including induction of mucosal inflammation and interference with host defense mechanisms. Proinflammatory events are generally beneficial for clearance of bacterial vaginal infections; however, in the case of HIV-1, vaginal inflammation may enhance infection by attracting HIV-1 host cells to the vaginal mucosa and by activating HIV-1 transcription in infected cells via cytokine up-regulation of the NF-κB. The HIV proviral enhancer located in the long terminal repeat (LTR) contains 2 NF-κB binding sites upstream of the TATA box and another one in the trans-activation response region that act in synergy with other transcription factors to enhance HIV-1 expression in response to NF-κB activation stimuli [11]. Inflammatory processes at mucosal surfaces are usually down-regulated by anti-inflammatory factors, such as secretory leukocyte protease inhibitor (SLPI), interleukin-1 receptor antagonist (IL-1ra), and soluble tumor necrosis factor receptors (sTNF-Rs), which are produced by the healthy mucosal epithelium.

Although proinflammatory cytokines in the lower female genital tract have been addressed in some infection studies, little attention has been paid to counterbalancing anti-inflammatory factors. Furthermore, although vaginal inflammation in symptomatic sexually transmitted diseases has been recognized as a significant risk factor for HIV-1 transmission [12], little is
known about the impact of chemically induced inflammation on the barrier functions of the human vagina.

In the present study, we established a combination of in vitro and in vivo model systems using cellular and molecular end points to assess the link between N-9–induced proinflammatory events in the vaginal mucosa and the risk of HIV-1 infection.

Materials and Methods

Clinical treatment and assessment protocols. Ten healthy white female volunteers 24–48 years old and at low risk for HIV-1 infection participated in the study. Exclusion criteria included hormonal contraception and recent or current vaginal infections. Women were not studied during menses or within 48 h of sexual intercourse or use of any vaginal product, as these conditions may affect levels of cytokines and chemokines in cervicovaginal secretions. Two protocols were used. The first entailed sampling of cervicovaginal secretions before and at 12, 36, and 60 h after a single application of 150 mg of N-9 (Gynol II; Ortho Pharmaceuticals). The second protocol entailed 1 sampling before and 3 (12, 36, and 60 h) after applications of N-9.

Cervicovaginal samples were obtained by lavage with 10 cm³ of sterile saline directed at the posterior vaginal wall. Cervicovaginal lavage (CVL) specimens were centrifuged at 500 g within 1 h of collection. The supernatant was aliquoted and was frozen at −70°C until use in cytokine and chemokine and HIV-1 activation assays. The cell pellet was resuspended in 1 cm³ of saline. An Endtz test [13] was performed on a portion of the cell suspension to quantify polymorphonuclear neutrophils. The remaining cell suspension was smeared onto 8-well teflon-coated slides, was fixed in absolute methanol, and was stored at −70°C for immunohistologic studies of cell populations associated with N-9–induced vaginal inflammation (as described elsewhere [14]), using monoclonal antibodies (MAbs) against leukocyte phenotypic antigens (CD45, Becton-Dickinson; CD3, CD4, CD8, and CD122, Immunotech; CD22 and CD68, Dako). A MAb that recognizes an epitope overlapping the CD44 receptor of CD44+ leukocytes (Pharmingen; Max M.Abs) was used to detect the activated form of NF-κB in the CVL cell pellets [15].

Cell lines. Previously established and well-characterized human papillomavirus (HPV)-16/E6E7–immortalized vaginal (VK2/E6E7), ectocervical (Ect1/E6E7), and endocervical (End1/E6E7) epithelial cell lines were cultured in keratinocyte serum-free medium (Life Technologies Gibco BRL) supplemented with 50 µg/mL of bovine pituitary extract, 0.1 mg/mL of epidermal growth factor, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and CaCl₂, to a final calcium concentration of 0.4 mM [16, 17]. Primary ectocervical epithelial cells (CrEC-Ec; Clonetics) were cultured in the same medium but without additional CaCl₂ supplementation (final calcium concentration, 0.1 mM). The primary ectocervical cell cultures were used in their third and fourth passage, and the immortalized cell lines were used in passages 33–48. The HeLa 3S cervical adenocarcinoma cell line (American Type Culture Collection) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1% bovine serum albumin (BSA). Human neutrophil elastase (Calbiochem) was used to prepare a standard curve. Equal volumes (50 µL) of CVL, Tris assay buffer, and substrate were mixed in a 96-well plate, and optical densities were read at 405 nm.

Neutrophil elastase assay. Human neutrophil elastase was quantified by a colorimetric assay using pyroGlu-Pro-Val-pNA (Chromogenix) in 100 mM Tris (pH 8.3), 0.96 mM NaCl, and 1% bovine serum albumin [19]. Human neutrophil elastase (Calbiochem) was used to prepare a standard curve. Equal volumes (50 µL) of CVL, Tris assay buffer, and substrate were mixed in a 96-well plate, and optical densities were read at 405 nm.

NF-xB electrophoretic mobility-shift assay (EMSA). EMSA was performed on nuclear extracts of cytokine- and N-9-stimulated End1/E6E7 and VK2/E6E7 epithelial cells to screen for NF-xB ac-
activation. Cell pellets were resuspended in buffer (10 mM Tris HCl [pH 7.8], 5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 0.3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], 10 mM b-glycerol-phosphate, 0.3 M sucrose, and 1.0 µg/mL each of the protease inhibitors aprotonin, antipain, leupeptin, chymostatin, and pepstatin), were incubated on ice for 15 min, and were lysed in 0.5% Nonidet P-40. Nuclei were collected by centrifugation and were resuspended in 20 mM Tris (pH 7.8), 5 mM MgCl₂, 320 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EGTA, 0.5 mM PMSF, 10 mM b-glycerol-phosphate, 25% glycerol, and protease inhibitors. After a 15-min incubation on ice, the nuclear extracts were cleared by centrifugation and were incubated with ³²P-labeled NF-κB oligoprobes in the presence of binding buffer (Promega) for 30 min at room temperature, and then the reaction components were separated by electrophoresis on a 4% native polyacrylamide gel. The protein-DNA complexes and free oligos were detected by autoradiography using x-ray films (Eastman Kodak). The method is based on the observation that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than unbound DNA fragments or oligonucleotides [20].

Quantification of NF-κB activation in the pNF-κB–secreted alkaline phosphatase (SEAP) reporter gene system. The pNF-κB–SEAP reporter plasmid (Clontech) was transiently transfected into epithelial cells by use of the QIAGEN Effectene transfection reagent kit (Qiagen). If the NF-κB pathway is induced, endogenous NF-κB binds to the κ enhancer element located in the promoter region of the pNF-κB–SEAP vector, thus activating the transcription of the SEAP reporter gene. In addition, pSEAP basic vector, which lacks the eukaryotic promoter and enhancer sequences, was used as a negative control, and the pSEAP control vector, which contains the SEAP structural gene under transcriptional control of the SV40 promoter and enhancer, was used as a positive control for transfection and expression of exogenous DNA [21].

The transfection procedure was done on 60% confluent monolayers in 24-well plates at a DNA:transfection reagent ratio of 1:10 and with 0.1 µg of plasmid DNA/well for 6 h. After 6 h, DNA and transfection reagents were removed, fresh keratinocyte serum-free medium was added, and the cells were left in the CO₂ incubator at 37°C for 24 h, to allow for complete transfection. Parallel transfected and nontransfected cultures were treated for 90 min, 6 h, or 24 h with various concentrations of N-9 and exogenous cytokines in the presence or absence of IL-1α blocking goat antibody or unrelated goat IgG, as described above. The SEAP was detected in culture supernatants by the Great escAPE SEAP chemiluminescence assay (Clontech), which was used according to the manufacturer’s protocol, using a Dynatech MLX microplate luminometer (Dynex Technologies). The chemiluminescence emitted by a SEAP-activated substrate (CSPD) was measured in relative luminescence units. ELISA-measured cytokine concentrations in the same supernatants that were used for SEAP detection were used to correlate NF-κB activation with cytokine patterns. After samples were obtained for SEAP and cytokine assays, cell viability was assessed by use of MITT.

HIV-1 activation protocols and p24 measurement in U1/HIV-1 cells. U1/HIV-1 cells were plated at 10⁵ cells/well in 48-well culture plates and were incubated with rhIL-1α (50 ng/mL), rhTNF-α (30 ng/mL), or human CVLs diluted 1:50 at 37°C in 5% CO₂. Culture supernatants were assayed for HIV-1 p24 core protein production 48 h later by use of an ELISA kit (Life Science Products). In another set of experiments, U1/HIV cells (5 × 10⁶/100 µL per

<table>
<thead>
<tr>
<th>Mediator</th>
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<th>12 h</th>
<th>36 h</th>
<th>60 h</th>
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<tr>
<td>IL-1β</td>
<td>125 (25–164)</td>
<td>82 (27–143)</td>
<td>90 (32–142)</td>
<td>144 (115–154)</td>
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<tr>
<td>IL-1α</td>
<td>81 (&lt;1–398)</td>
<td>121 (72–179)</td>
<td>118 (58–148)</td>
<td>188 (116–232)</td>
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<tr>
<td>IL-8</td>
<td>946 (400–1328)</td>
<td>1258 (998–1452)</td>
<td>1198 (1038–1344)</td>
<td>1253 (1185–1314)</td>
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<tr>
<td>IL-1αa</td>
<td>82 (60–109)</td>
<td>120 (40–225)</td>
<td>92 (0.4–155)</td>
<td>162 (53–270)</td>
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<tr>
<td>TNF-R1</td>
<td>395 (&lt;2–906)</td>
<td>123 (99–225)</td>
<td>195 (&lt;2–47)</td>
<td>418 (&lt;2–1480)</td>
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<tr>
<td>SLPI</td>
<td>101 (78–145)</td>
<td>10 (0.4–20)</td>
<td>51 (&lt;0.0–100)</td>
<td>125 (&lt;0.0–224)</td>
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3 Exposures
<table>
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<th>Mediator</th>
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<th>12 h</th>
<th>36 h</th>
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<tr>
<td>IL-1β</td>
<td>12 (&lt;4–68)</td>
<td>796 (435–1105)</td>
<td>819 (413–1162)</td>
<td>888 (134–1339)</td>
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<tr>
<td>IL-1αa</td>
<td>94 (&lt;1–296)</td>
<td>283 (&lt;1–708)</td>
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<tr>
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<td>35 (&lt;2–68)</td>
<td>63 (&lt;10–838)</td>
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<td>IL-8</td>
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<td>1512 (194–2632)</td>
<td>1948 (1103–2641)</td>
<td>1522 (662–2310)</td>
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<tr>
<td>MIP-1α</td>
<td>&lt;7</td>
<td>127 (&lt;7–422)</td>
<td>324 (&lt;7–1919)</td>
<td>416 (&lt;7–1319)</td>
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<tr>
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<td>170 (&lt;11–1450)</td>
<td>1270 (&lt;7–2858)</td>
<td>1822 (1194–2373)</td>
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<td>IL-1αa</td>
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<td>TNF-R11</td>
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<td>TNF-R11</td>
<td>237 (&lt;1–568)</td>
<td>527 (&lt;6–930)</td>
<td>2328 (1051–4177)</td>
<td>2422 (&lt;4–4457)</td>
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<td>SLPI</td>
<td>332 (201–453)</td>
<td>23 (&lt;0.0–212)</td>
<td>38 (&lt;1–86)</td>
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<tr>
<td>NE</td>
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<td>8.2 (3.6–11.6)</td>
<td>9.3 (2.6–12.9)</td>
<td>2.4 (0.0–7.7)</td>
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Table 1. Time course of soluble mediators detectable in cervicovaginal secretions of healthy women at baseline (12 h before treatment) and after single (n = 6) or multiple (n = 4) exposures to nonoxynol-9 (N-9).

NOTE. Data are median (95% confidence interval) of picograms per milliliter of neat lavage, except for interleukin (IL)-1 receptor antagonist (IL-1ra), secretory leukocyte protease inhibitor (SLPI), and neutrophil elastase (NE), which are given in nanograms per milliliter; MIP, macrophage inflammatory protein; TNF-R, tumor necrosis factor receptor.

* Values significantly different from baseline (P < .05).
Figure 1. Immunocytochemical staining (red) of cells in cervicovaginal lavage (CVL) smears before and after vaginal application of nonoxynol-9 (N-9). A, CD68+/H11001 macrophages were observed rarely in baseline CVLs. B, CD68+/H11001 macrophages were numerous (arrowheads) in CVL specimens after 3 once-daily doses of N-9. C, Epithelial and white blood cells in baseline CVL specimens demonstrated a faint reactivity with the monoclonal antibody that recognized the activated NF-kB p65 subunit. D, In contrast, epithelial cells (open arrow) and round cells with monocytic morphology (black arrow) stained strongly positive for activated p65 after 3 once-daily doses of N-9. E, Nuclear localization (black arrow) of activated p65 in CVL cells. A–D, Original magnification, ×125; E, original magnification, ×250.

well) were incubated in 96-well plates with 2-fold dilutions of N-9 for 48 h. At the end of this time, 100 μL of 0.5% Triton X-100 was added to each well for 10 min, and the cell lysates were assessed for total (intra- and extracellular) p24.

Statistical analysis. Statistical analysis was done using Instat (version 3.0; Graphpad Software). In vitro data were analyzed using Student’s t test. Clinical data were analyzed by repeated measures analysis of variance with Dunnet’s post test, to compare treated versus control samples in each individual and linear trend post test to assess N-9–induced effects over time.
Results

Multiple vaginal applications of N-9 altered the profiles of cytokines, chemokines, and other inflammatory regulators in cervicovaginal secretions. Proinflammatory and anti-inflammatory mediators were quantified by ELISA in cervicovaginal lavages that were collected 12 h before N-9 application (baseline) and at 3 time points after discontinuation of N-9 use. Spiking studies, in which various concentrations of N-9 were added to cytokine standards, revealed that N-9 concentrations ≤5% did not affect the detection of most of the cytokines measured in this study, with the exception of IL-1α and IL-1β (70% recovery of spiked IL-1α/β standards in 5% N-9 and 100% recovery in 1.25% N-9 solution). Addition of CVL or keratinocyte culture media to cytokine standards also had no effect on cytokine measurement.

As shown in table 1, when compared with baseline values, a single application of N-9 did not induce significant changes in IL-1α, IL-1β, IL-8, sTNF-RI, sTNF-RII, or IL-1ra in CVLs obtained 12, 36, and 60 h after the N-9 application. SLPI was significantly reduced in CVLs obtained 12 h after N-9 use. TNF-α, macrophage inflammatory protein (MIP)–1α, MIP-1β, and RANTES were undetectable in CVL samples obtained before or after single N-9 applications.

In contrast, significant changes in the proinflammatory cytokine profiles were observed after 3 consecutive applications of N-9 (table 1). IL-1α and IL-1β were significantly and persistently increased in each subject after the third application of N-9. TNF-α and MIP-1α were undetectable at baseline and appeared in individual CVLs at various intervals after discontinuation of N-9 use, whereas IL-8 and MIP-1β showed progressive significant increases over time, with IL-8 reaching peak values typically at 36 h after N-9 use and MIP-1β showing a late peak at 60 h after N-9 use. At the same time, multiple exposures to N-9 caused diverse effects on anti-inflammatory factors, such as IL-1ra, SLPI, and sTNF-Rs, which were present in normal (baseline) cervicovaginal secretions at various but high concentrations.

Although SLPI levels consistently were decreased, concentrations of sTNF-Rs showed diverse kinetic patterns. TNF-RI did not change significantly, but sTNF-RII significantly at 36 h after N-9 use. Although IL-1ra levels were elevated slightly or unchanged in individual CVLs after N-9 application, because of the markedly increased release of IL-1α and IL-1β, the effective IL-1α:IL-1 (α + β) ratio decreased significantly (P < .01) from 697 ± 0.189 at baseline (ranges of 600–1200) to 130 ± 29 at 12 h, 115 ± 22 at 36 h, and 120 ± 18 at 60 h after discontinuation of N-9.

Multiple exposure to N-9 caused influx of inflammatory cells in the cervicovaginal secretions and nuclear translocation of p65 NF-κB in cervicovaginal cells Sloughed epithelial cell sheets commonly were observed in CVLs sampled after the second and third applications of N-9 (data not shown). One application of N-9 did not cause significant changes in polymorphonuclear leukocytes (PMNL), as demonstrated by the Endtz test: counts ranged from <0.04 × 10⁶ to 1.6 × 10⁶ cells/mL lavage. In contrast, a dramatic influx of PMNL was observed after the third application of N-9, typically after an elevation of IL-8, with maximum cell counts occurring 36 and/or 60 h after N-9 exposure: counts ranged from 3.8 × 10⁶ to 9 × 10⁶ cells/mL per lavage. The number of PMNL detected by the Endtz test correlated with human neutrophil elastase levels and activity, which markedly were increased over baseline levels in CVLs of all test subjects after 3 applications of N-9 (table 1).

Immunohistologic analysis revealed that macrophages (CD68⁺ cells) also were increased markedly in cervicovaginal secretions during this period (figure 1A and 1B), whereas few T or B cells were detected in CVL cell pellets before or after N-9 treatment (data not shown). Possible activation of NF-κB in the CVL cells was investigated, using a MAb specific to the nuclear localization sequence of the p65 NF-κB subunit. The epitope recognized by this antibody is exposed on activated p65
only after degradation of the inhibitory protein, I-κB. Epithelial cells present in CVLs at baseline were virtually negative for activated p65, whereas abundant positive epithelial cells and a few positive cells with monocytic morphology were observed 36 and 60 h after 3 days of N-9 use (figure 1C–1E).

N-9 caused IL-1α/β release, activation of NF-κB, and subsequent IL-8 up-regulation by cervical and vaginal epithelial cells in vitro. The cytotoxic and cytokine responses 1 h after N-9 exposure (8–500 µg/mL) were similar in the immortalized vaginal and cervical epithelial cell lines and primary cervical epithelial cell cultures (figure 2). In contrast, the human cervical adenocarcinoma cell line HeLa was much less sensitive to N-9–induced cytotoxicity. The CMC vehicle control was completely nontoxic and did not cause any cytokine alterations within a concentration range of 1–1000 µg/mL (data not shown).

Depending on dose and length of exposure, N-9 induced diverse effects on the secretion of immunobiologic mediators by the immortalized epithelial cells. TNF-α, MIP-1α, and MIP-1β were not detectable in supernatants from control or N-9–treated cervical and vaginal cultures at the selected time points and culture densities. Secretory profiles of IL-1β, IL-1α, and IL-8 were significantly altered by N-9 (figure 3). High doses
of N-9 (100% cell death within 24 h) caused rapid (within 30 min) extracellular release of IL-1β and IL-1α by lysed cells, whereas subtoxic doses (10%–30% cell death within 24 h) induced a more gradual increase in IL-1α/β levels within 6 h and a further increase over 24 h in the case of IL-1α. IL-8 production was significantly increased per number of viable cells in both primary and immortalized epithelial cells after a 24-h incubation with subtoxic and nontoxic N-9 concentrations. Although SLPI was released by lysed cells at toxic concentrations of N-9 (32 μg/mL in 24 h), SLPI levels were decreased at subtoxic and nontoxic N-9 doses, compared with levels in untreated epithelial cells (figure 3).

Although exogenous IL-1α and TNF-α caused rapid (within 30 min) NF-κB nuclear translocation in the cervical and vaginal epithelial cells (data not shown), N-9–induced nuclear translocation occurred more slowly (6 h) and followed a significant increase in IL-1α/β levels at 30 min, suggesting an indirect, cytokine-mediated mechanism of NF-κB activation (figure 4A). In the same assay, NF-κB nuclear translocation in the presence of rhIL-1α and N-9 was blocked by polyclonal anti–human IL-1α. Significantly increased IL-8 levels were measured in supernatants from the same cultures that demonstrated NF-κB translocation. The stimulatory effect of N-9 on IL-8 production was significantly inhibited partially by anti–IL-1α antibody (figure 4B).

Effective NF-κB transactivation in the presence of N-9 was confirmed in the SEAP reporter gene system and was similarly inhibited by anti–IL-1α, as demonstrated by significantly increased stimulation indices (figure 4C). IL-8 secretory profiles in the same culture supernatants mirrored the SEAP profiles (figure 4D).

Cervicovaginal lavages collected after multiple use of N-9 activated HIV-1 expression in vitro. To determine whether cervicovaginal secretions from N-9 users can induce HIV-1 expression, we treated U1/HIV cells with CVLs obtained from 3 women 12 h before N-9 use (baseline) and 12, 36, 60, and 108 h after 3 daily doses of N-9. As shown in figure 5A, HIV-1 p24 production by the reporter promonocytic cells line increased after treatment with post–N-9 CVL samples, compared with control untreated cells and cells treated with baseline CVL samples. The highest magnitude of expression (∼10-fold increase) was induced by CVLs obtained 60 h after discontinuation of N-9 (P < .05). In contrast, direct exposure of U1/HIV cells to
subtoxic and nontoxic concentrations of N-9 (TD_{50,α} 16–1 μg/mL, respectively) for the same time period did not induce a significant increase in p24 production (data not shown). Treatment of U1/HIV cells with rhTNF-α and rhIL-1α also induced significant increases in p24 production within the 48-h stimulation period (figure 5B), suggesting that these cytokines, which were both elevated in the cervicovaginal secretions after multiple N-9 use, may be involved in HIV-1 activation. A positive correlation was found between p24 values and concentrations of IL-1α in the CVL samples (r = .62).

Discussion

The combination of in vitro and in vivo findings from our study provides insight into the molecular mechanisms and cell types involved in N-9–induced inflammation, which could promote HIV-1 infection (summarized in figure 6).

We found that subtoxic doses of N-9, although perhaps not grossly disruptive of the genital tract mucosal surface, may promote accumulation of proinflammatory effects through an IL-1–induced NF-κB activation loop. Injured or irritated epithelial cells release intracellular stores of IL-1α/β, which are extremely efficient inducers of proinflammatory signal transduction pathways, including the transcription factor NF-κB, in a variety of cell types [22]. NF-κB accelerates and coordinates the expression of many proinflammatory proteins, including cytokines, chemokines, and adhesion molecules [23], which could enhance HIV-1 infection through recruitment and/or activation of HIV-1–infected target cells at the site of inflammation. Although single exposure to N-9 did not significantly affect the cervicovaginal cytokine milieu, multiple exposures caused a significant increase in IL-1α and IL-1β levels in the cervicovaginal secretions of healthy women days after discontinuation of use. At the same time, low doses of N-9, which were nontoxic from within 30 min to 6 h of exposure time, gradually induced IL-1α and IL-1β release by cervical and vaginal epithelial cells.

NF-κB activation experiments further suggested that epithelial cells could be the initiators of early proinflammatory responses via IL-1α–mediated NF-κB activation potentiated by decreased ratios of anti-inflammatory mediators, such as IL-1ra and SLPI, in cervicovaginal secretions. Moreover, nuclear translocation of the NF-κB p65 subunit, which is a component of the NF-κB dimer complexes that can activate the HIV-1 LTR [11, 24], was detectable in cervicovaginal cells in vivo 36 and 60 h following repeated N-9 use. Also at these time points, increased levels of the chemokines IL-8 and MIP-1α/β but not RANTES correlated with the marked influx of neutrophils and macrophages but not T cells in cervicovaginal secretions. Concentrations of IL-8 but not MIP-1α/β or TNF-α were also dramatically elevated (subsequent to IL-1 increase) in culture supernatants from N-9–treated cervicovaginal epithelial cells in vitro. These findings suggest that while epithelial cells may initiate the proinflammatory events via IL-1 and NF-κB up-regulated IL-8, activated neutrophils and macrophages may be the predominant sources of the elevated MIP-1α/β and TNF-α observed in the cervicovaginal secretions after discontinuation of N-9 use.

In addition to the role of NF-κB in the recruitment and activation of macrophages that can serve as HIV-1 host cells, its activation can have a direct effect on HIV-1 replication. Several viruses, including retroviruses (HIV-1), adenoviruses, and herpesviruses (herpes simplex virus–1 and human cytomegalovirus), have NF-κB binding sites in their promoter/enhancer regions [25]. The presence of NF-κB binding sites in the HIV-1 LTR renders the virus responsive to host proinflammatory signals. IL-1α and β and TNF-α have been shown to enhance the transcription of HIV-1 in infected monocytes and T cells via activation of NF-κB [26–28].

In our study, rhIL-1α and rhTNF-α induced significant HIV-1 p24 production in the chronically infected U1/HIV promonocytic cell line. In the same system, levels of endogenous IL-1α were positively correlated with increased p24 expression following exposure to CVLs obtained after repeated N-9 use. Maximum p24 production in this in vitro model was induced
Figure 6. Schematic presentation of the molecular mechanisms of nonoxynol-9 (N-9)–induced inflammation in the female genital tract, which can lead to increased risk of human immunodeficiency virus type 1 (HIV-1) transmission. N-9 causes release of interleukin (IL)–1α/β from membrane-compromised cervicovaginal cells. IL-1 binds to the IL-1 receptor type 1 (IL-1RI) on the surface of neighboring cells, which activates cytoplasmic kinases and leads to the phosphorylation (P), ubiquityness (Ub), and degradation of cytoplasmic inhibitors (IkBs) of NF-κB. The NF-κB p65 dimers then freely translocate into the nucleus to initiate the expression of a number of proinflammatory factors and increase HIV-1 expression through the HIV-1 long terminal repeat (LTR), which contains NF-κB binding sites. These molecular events potentially lead to influx/activation of HIV-1 host/target cells (macrophages, T cells, and dendritic cells) and to increased HIV-1 shedding in the genital tract. MIP, macrophage inflammatory protein; TNF, tumor necrosis factor.

by CVLs collected 60 h after discontinuation of N-9, highlighting the long-term and cumulative effects of N-9 use.

Resting T cells or cells of the monocyte-macrophage lineage can maintain a latent state of viral infection and constitute an inducible HIV-1 reservoir in infected patients [29]. The prevalence of such cells in the female genital tract mucosa has not been studied; however, latent replication-competent virus has been detected in the seminal cells of HIV-1–infected men receiving highly active antiretroviral therapy and is considered to be a major impediment to eradicating HIV [30]. It is clear that activation of HIV in latently infected monocytes/macrophages by N-9–induced inflammatory stimuli in the vaginal mucosa may accelerate the course of infection or increase the risk of HIV transmission.
To our knowledge, this study is the first to address the balance between anti-inflammatory and proinflammatory mediators in vaginal inflammation. The role of naturally occurring anti-inflammatory factors in HIV-1 infection remains obscure. SLPI, a neutrophil elastase and cathepsin G inhibitor, has been shown to inhibit HIV-1 in vitro [31], a function which may be associated with blocking of intracellular proteases and preservation of the NF-κB inhibitory protein IκBα [19].

IL-1ra is another naturally occurring anti-inflammatory protein that competitively blocks IL-1 binding to type I and type II IL-1 receptors, but it has no agonist activity [32]. Reduced IL-1ra production has been demonstrated in HIV-1-infected dendritic cells, which, along with elevated TNF-α and IL-1, may contribute to enhanced replication of HIV-1 in bystander T cells [33]. A direct blocking effect of IL-1ra on IL-1–induced HIV-1 expression in monocytes suggests a pathway for control of HIV replication and disease progression in infected individuals [28]. The effectively decreased IL-1ra–to–IL-1 ratios recorded in our study may contribute directly to increased risk of HIV-1 infection and transmission.

The presence of sTNF-Rs in cervicovaginal secretions, which, to our knowledge, is demonstrated for the first time in our study, may have a dual role in the control of inflammation in the cervicovaginal environment. On one hand, sTNF-Rs can reduce the toxic effects of TNF-α, and, on the other hand, they can serve as carriers and stabilizers of TNF-α, which has a comparatively high rate of decay in biological secretions [34]. Thus, depending on their relative concentrations, sTNF-Rs may inhibit or augment the effect of TNF-α in the female genital tract.

Our pilot clinical study suggests that monitoring proinflammatory and anti-inflammatory molecules in cervicovaginal lavage specimens during clinical trials can provide biochemical markers of vaginal irritation, whereas enumeration of macrophages and neutrophils in cervicovaginal secretions serves as a functional readout of vaginal inflammation. Our data also indicate that HPV16/E6E7 immortalized cervical and vaginal epithelial cell lines can provide an important model system for preclinical testing of vaginal microbicide products. These cell lines are highly sensitive to N-9–induced cytoxicity and have cytokine responses that resemble those of primary epithelial cell cultures and agree with our in vivo observations. Further studies are needed to fully characterize the HIV-1 infection risk associated with inflammatory processes in the vaginal environment and to determine whether anti-inflammatory agents targeted at IL-1 and/or the NF-κB network would decrease the risk of HIV-1 transmission, thereby enhancing the antimicrobial efficacy of N-9 and other vaginal products.

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