A Critical Gap in Microbicide Development Is the Absence of Surrogate Safety Markers. The Objective of the Present Study Was to Develop a Murine Model to Examine the Mucosal Response to Microbicides and to Assess the Functional Implication of Observed Changes. Mice Received 14 Daily Intravaginal Doses of Nonoxynol-9, PRO 2000, or Placebo Gel. Nonoxynol-9 Induced an Inflammatory Response Characterized by Increases in Levels of Cytokines and Chemokines, Recruitment of Neutrophils and Monocytes into the Genital Tract, and Activation of the Transcription Factors NF-κB and Activator Protein–1. Minimal Inflammation Was Observed in Response to 2% PRO 2000. Nonoxynol-9–Treated Mice Were Significantly More Susceptible to Challenge with a Low Dose of Herpes Simplex Virus Type 2; the Response of PRO 2000–Treated Mice Was Similar to the Response to Placebo. These Findings Suggest That PRO 2000 Has Little Deleterious Effect on Mucosal Immunity and, If Validated by Clinical Experiences, Support the Inclusion of This Model in the Preclinical Evaluation of Future Candidate Microbicides.
which was described and standardized in the 1960s, is recommended by the Food and Drug Administration to assess the toxicity of vaginal products [10]. The model consists of 10 daily doses of agent administered vaginally. Histological sections are scored for epithelial disruption, leukocyte infiltration, edema, and vascular changes. However, the RVI model does not provide information at a molecular or immunological level. Recently, the rabbit model was modified to include characterization of interleukin (IL)-1, IL-6, and IL-8 responses in vaginal washings after a single or 3 daily applications of spermicides, including nonoxynol-9 (N-9) and benzalkonium chloride (BZC) [11]. BZC was the most damaging and triggered IL-1 release and IL-6 secretion; the biological significance of these findings is unknown.

Microbicidal agents may trigger changes in the genital tract, including the induction of inflammation or loss of host defenses, which are not reflected by histological analysis or colposcopy. Genital tract secretions obtained by cervicovaginal lavage (CVL) of healthy women provide protection against HSV infection in vitro and reduce viral yields by ~90% [12]. Although less pronounced, vaginal secretions also provide some protection against HIV infection [13], and we recently demonstrated significant antibacterial activity of CVL samples [14]. Loss of these defenses could enhance susceptibility to infection, particularly in the setting of intermittent application or as the local concentration of microbicide wanes.

There is extensive experience with murine models studying mucosal responses to pathogens. This experience, combined with the low cost and the availability of a wide array of reagents, suggests that the mouse may provide an optimal model to study mucosal responses to microbicides, despite the limitation that mice are not susceptible to HIV. A few studies have examined the acute effects of a single dose of microbicide in the mouse. After a single intravaginal administration of N-9, infiltration of macrophages into the genital tract was observed [15]. Another study examined the impact of a single dose of N-9 and several other detergents in a mouse model [16]. Colposcopy failed to detect signs of toxicity, but histological examination detected epithelial cell disruption, recruitment of macrophages, and release of inflammatory chemokines [16].

No studies have addressed the murine mucosal response to repeated applications of microbicides. To address this gap, we developed a comprehensive murine model to determine whether repeated exposure to candidate microbicides alters the genital tract environment and increases susceptibility to HSV infection. Compounds tested were Advantage-S, a 3.5% N-9 product; 2% PRO 2000 gel; and hydroxyethylcellulose (HEC) gel. Advantage-S was selected because it has been extensively studied in human clinical trials; 2% PRO 2000, a naphthalene sulfonic acid polymer that blocks HSV and HIV entry [17–19], was selected because it is currently in phase 2 and 3 clinical trials; and HEC was selected because it is being used in several clinical trials as a placebo gel.

**MATERIALS AND METHODS**

**Microbicides.** Vaginal gels tested were Advantage-S (3.5% N-9; Columbia Laboratories), 2% PRO 2000 (Indevus Pharmaceuticals), and a placebo gel, HEC, which was obtained from the International Partnership for Microbicides.

**Murine model.** Murine studies were conducted with the approval of the Mount Sinai School of Medicine Institutional Animal Care and Use Committee. Female BALB/c mice (8–10 weeks old) were pretreated subcutaneously with 2 mg of medroxyprogesterone acetate (Sicor Pharmaceuticals) 5 days before gel application. Medroxyprogesterone increases susceptibility to HSV infection by thinning the epithelium and increasing expression of the HSV coreceptor nectin-1 [20]; for control purposes, medroxyprogesterone treatment was also included in the mucosal immune studies. Forty microliters of gel was delivered intravaginally daily for 14 days. Vaginal washes were collected from groups of 5 mice by washing with 100 μL of sterile normal saline, delivered and recovered 5 consecutive times, at baseline and on days 3, 7, 14, and 21. Mice from each treatment group were killed on days 7, 14, and 21, and the cervix and vagina were excised either for histological, reverse-transcriptase polymerase chain reaction (RT-PCR), or fluorescence-activated cell sorter (FACS) analysis.

**Cytokine and chemokine analysis.** Protease inhibitors (Complete Protease Inhibitor Cocktail; Roche Applied Science) were added to each sample before centrifugation at 210 g for 10 min at 4°C, to remove mucous and cellular debris without triggering lysis. The supernatants were stored at −80°C. Pooled vaginal washes were assayed for cytokines and chemokines by use of the Fluorokine MAP MultiAnalyte profiling system (R&D Systems), were measured by use of the LumineX 100 system, and were analyzed by use of StarStation (version 2.0; Applied Cytometry Systems). Prior studies from our laboratory have demonstrated that residual drug does not interfere with cytokine recovery [21].

**RT-PCR.** For cytokine and chemokine mRNA determination, vaginal tissue was homogenized and total RNA was extracted using the Absolutely RNA Miniprep Kit (Stratagene). Reverse transcription was performed using 1 μg of RNA and the StrataScript cDNA Synthesis Kit (Stratagene). Quantitative real-time PCR was conducted in duplicate with 25 ng of cDNA (pooled from 4 mice/group) and with 0.4 μmol/L of each primer in a 30-μL final reaction volume of 1× SYBR Green PCR Master Mix (Applied Biosystems). PCR cycling conditions on an ABI PRISM 7700 were as follows: 1 cycle, 50°C for 2 min; 1 cycle, 95°C for 10 min; 40 cycles, 95°C for 15 s; and 1 cycle, 60°C for 1 min. Relative expression levels were calculated.
Figure 1. Increase in cytokine and chemokine levels in response to microbicides. The concentrations of cytokines and chemokines in vaginal washes pooled from at least 10 mice at each time point were measured at baseline (day 0) and 3, 7, 14, and 21 days after 14 daily applications of nonoxynol-9 (N-9), PRO 2000, or hydroxyethylcellulose (HEC). The sensitivities for each analyte (in picograms per milliliter) are as follows: interleukin (IL)–1β, 3.3; tumor necrosis factor (TNF)–α, 0.42; IL-2, 1.99; IL-4, 1.89; IL-6, 0.71; IL-10, 0.59; IL-12, 7.61; interferon (IFN)–γ, 5.25; monocyte chemoattractant protein (MCP)–1, 0.95; and macrophage inflammatory protein (MIP)–2, 2.2. Little or no IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-12, or TNF-α was detected in any of the vaginal washes. Results for the detectable mediators are presented as values obtained from at least 2 independent experiments (A). mRNA levels were quantified from tissue on day 3 by quantitative real-time polymerase chain reaction (Q-RT-PCR). Results represent ubiquitin-normalized values relative to HEC obtained from pooled samples (n = 4 mice/group) and are mean ± SE values obtained from at least 2 independent experiments (B).

as $2^{-\Delta C_{t}}$ ubiquitin – Ct gene (where “Ct” indicates cycle threshold), using ubiquitin RNA as endogenous control.

FACS analysis. Mouse cervical and vaginal tissue was excised on necropsy and stored on ice in RPMI 1640. Intact tissue was digested at room temperature for 15 min in 0.5% EDTA and then digested in 10 mL of freshly prepared collagenase solution (clotriodipeptidase A prepared from Clostridium histolyticum; Invitrogen) at a concentration of 150 U/mL in RPMI 1640 (10% fetal bovine serum, 1% pen-strep, and 2 mmol/L L-glutamine) at 37°C for 1.5 h [22]. Enzyme-digested samples were triturated through a 70-µm filter into medium and filtered (40-µm filter). Cells were washed in 0.5% bovine serum albumin (BSA) in PBS, and viable cells were counted by trypan blue exclusion. Cells were blocked with Fe block (anti-CD16/32; eBioscience; 1:200 dilution) and subdivided (5 × 10^5 cells), to permit staining with different antibodies; an unstained control sample was included in each assay. Cells were incubated with fluorochrome-conjugated antibodies against mouse CD3, CD19, CD45, F4/80, CD11b, CD11c, and Gr1 (all from e-Bioscience) at a final concentration of 1:200 for 45 min at 4°C. Samples were washed thrice with 0.5% BSA in PBS and were resuspended in 300 µL for acquisition by color FACS analysis. Propidium iodide (1.5 µg/mL; Sigma-Aldrich) was added before acquisition. At least 100,000 events were acquired per sample and were analyzed by use of FlowJo software (Tree Star, S.A.L.).

Histological analysis. Formalin-fixed vaginas were processed and embedded in paraffin, tissue was transversely sec-
Figure 2. Recruitment of immune cells into the genital tract, as determined by fluorescence-activated cell sorter (FACS) analysis. In 2 replicate experiments, vaginas from mice treated with nonoxynol-9 (N-9), PRO 2000, or hydroxyethylcellulose (HEC) gel (n = 5 per group) were harvested and stained for FACS analysis after 7 and 14 days of treatment as well as 7 days after treatment ceased (day 21). Results are shown as the mean ± SE value at each time point.

Transcription factor assay. Nuclear extracts from frozen vaginas were prepared using Active Motif’s nuclear extract kit. Nuclear protein was quantified by the Quick Start Bradford Protein Assay from Bio-Rad, and nuclear extracts were stored at −80°C. These samples were analyzed for activator protein (AP)–1 (cFos) and NF-kB (p65) levels by use of Active Motif’s TransAm ELISA-based assay kits.

HSV susceptibility. Female BALB/c mice were pretreated with 2 mg of subcutaneous medroxyprogesterone acetate 5 days before intravaginal instillation of 3.5% N-9, 2% PRO 2000, or HEC. The mice were treated daily for 7 days. Twelve hours after the final drug treatment, the mice were inoculated with 0.1 LD₉₀ of HSV-2 (strain G) (15 μL/mouse), which is equivalent to 1 × 10⁴ pfu/mouse. Mice were evaluated daily for evidence of disease [23]. Fifteen mice per group were evaluated in 3 independent experiments.

Statistical analysis. GraphPad Prism (version 4; GraphPad Software) was used for statistical analysis. Cytokine, FACS, RT-PCR, and transcription factor results were analyzed by 1-way analysis of variance with Tukey’s post test, to compare groups. Kaplan-Meier survival curves were assessed by log-rank test. P < .05 was considered to be significant.

RESULTS

Impact of microbicides on vaginal cytokines and chemokines. Inflammatory responses may limit the beneficial effect of microbicides. The concentrations of total protein, cytokines, and chemokines in vaginal washes pooled from at least 10 mice at each time point were measured at baseline (day 0) and 3, 7, 14, and 21 days after 14 daily applications of N-9, PRO 2000, or HEC gel. The total protein concentration did not change and was comparable in all groups (data not shown). There was a significant increase in levels of monocyte chemoattractant protein (MCP)–1, macrophage inflammatory protein (MIP)–2, and IL-1β in vaginal washes at days 3 and 7 (P < .01) for N-9–treated mice, compared with those at baseline (figure 1A). PRO 2000–treated mice also demonstrated a significant increase in MIP-2 levels, but only on day 3 (P < .001). Little or no interferon-γ, IL-2, IL-4, IL-6, IL-10, IL-12, or tumor necrosis factor (TNF)–α was detected in any of the vaginal washes.

Parallel results were obtained by examining mRNA by RT-PCR on mouse vaginal tissue at days 3 and 7. Figure 1B shows day 3 mRNA levels (as a fold increase over HEC placebo–treated animals) for MCP-1/CCL2, RANTES/CCL5, MIP-2, IL-1α, IL-1β, IL-6, and TNF-α in mice treated with N-9 and PRO 2000. N-9 induced a significant increase relative to HEC in levels of all of the mediators except IL-6 (P < .05). The inflammatory response to N-9 persisted on day 7 (data not shown). PRO 2000 induced modest increases in gene expression on day 3; only the increase in TNF-α was significant (P < .05) (figure 1B).

Leukocyte infiltration. To determine whether the increase in chemokine levels was associated with recruitment of leukocytes into the genital tract, the cellular composition in vaginal tissue was examined by FACS analysis. In 2 independent experiments, vaginas from mice treated with N-9, PRO 2000, and...
HEC (n = 5/group) were harvested on days 7, 14, and 21 and stained for FACS analysis. No background staining was observed with isotype control antibodies (data not shown). There was a significant increase in the total number of leukocytes (CD45+) after 7 days of N-9, but not PRO 2000, treatment (P < .05) (figure 2), which is consistent with the increase in MCP-1 level in vaginal washes (figure 1). Although there was a trend toward increased numbers of neutrophils (CD11b+Gr1int), monocytes/macrophages (F4/80 and CD11b+Gr1low), and dendritic cells (CD11c+) in mice treated with N-9 and PRO 2000 compared with mice that received HEC, no statistically significant changes in subpopulations were detected.

**Effects of microbicide treatment on vaginal epithelium.** Histological sections of vaginal tissue obtained from mice that had received no gel and mice treated with N-9, PRO 2000, or HEC gel were reviewed blindly by a pathologist (D.D.P.). The vaginal epithelium was relatively thin, which is consistent with medroxyprogesterone treatment. On day 7, untreated and HEC-treated mice showed no histological changes (figure 3). In contrast, mice that received N-9 showed evidence of disrupted epithelium with some necrosis. Infiltration with neutrophils and lymphocytes was observed in 4 of 5 examined slides. In contrast, mice that received PRO 2000 gel showed only scattered neutrophils but no epithelial disruption or necrosis. Similar findings were observed on day 14. However, by day 21, no differences between treated or untreated mice were detected.

**Vaginal levels of NF-κB and AP-1 after microbicide treatment.** Inflammatory triggers may activate the NF-κB and AP-1 pathways, which play pivotal roles in immune regulation, apoptosis, and cell proliferation [24]. To explore the impact of microbicides on these pathways, nuclear levels were quantified by ELISA. NF-κB (p65) and AP-1 (cFos) were significantly increased in N-9–treated mice relative to HEC controls (P < .001 and P < .01, respectively), whereas PRO 2000 showed no significant increase relative to HEC (figure 4).

**Enhanced susceptibility to HSV infection in N-9–treated mice.** The biological significance of changes in the levels of individual cytokines or chemokines, cell populations, or transcriptional activators is not known. To address this gap, we
Figure 5. Increase in susceptibility to herpes simplex virus (HSV) infection caused by nonoxynol-9 (N-9) but not PRO 2000. Mice were challenged with 0.1 LD$_{90}$ HSV-2 (strain G) 12 h after receiving the seventh daily dose of N-9, PRO 2000, or hydroxyethylcellulose (HEC) and were observed daily for 15 days for signs and symptoms of disease. Results show survival pooled from 3 independent experiments (5 mice/group/experiment) (A). N-9 increased susceptibility to HSV compared with HEC and PRO 2000 ($P = .002$ and $P = .002$, respectively; log-rank test). The mean disease score is shown in panel B. Symptoms were scored on a 0–5 point scale: 0, no apparent infection; 1, slight redness of the vagina; 2, moderate redness and swelling of the vagina and surrounding tissue; 3, severe redness and swelling; 4, genital ulceration or hair loss of genital and surrounding tissue; and 5, evidence of hind limb paralysis. Mice reaching stage 4 or 5 disease were euthanized [23].

 Compared the susceptibility of mice after 7 daily applications of microbicides to a low inoculum of HSV-2 (strain G). The dose selected (0.1 LD$_{90}$) consistently causes disease in only 10%–30% of mice. Mice ($n = 15$ in total; $n = 5$/group; 3 independent experiments) received intravaginal N-9, PRO 2000, or HEC for 7 days. Twelve hours after the final drug administration, mice were challenged with 0.1 LD$_{90}$ of HSV-2 (strain G). N-9 treatment increased susceptibility to HSV compared with mice treated with PRO 2000 ($P = .002$) or HEC ($P = .03$) (figure 5). PRO 2000–treated mice showed no increase in susceptibility compared with HEC–treated mice. The differences did not reflect residual PRO 2000 in the genital tract, given that 80%–90% of mice in all 3 groups succumbed when challenged with the LD$_{90}$ dose. Moreover, no PRO 2000 was detected in vaginal washes obtained 12 h after the seventh dose by fluorescence assay (data not shown) [21].

**DISCUSSION**

A major gap in microbicide development is the absence of bioassays that are predictive of safety. This reflects an incomplete understanding of mucosal immunity in the female genital tract and what factors contribute to protection against or susceptibility to sexually transmitted infections. Despite its in vitro virucidal activity, N-9 increased the rate of HIV transmission with frequent application in a phase 3 trial [25]. Subsequent studies suggested that N-9 may trigger an inflammatory response, which presumably prevails over its virucidal activity [9]. Precisely how or whether the inflammatory response is responsible for the increased susceptibility to HIV infection is not known. In a study involving 10 healthy subjects, a single application of N-9 (Gynol-II) did not induce significant changes in levels of any of the cytokines measured in CVL samples compared with baseline values [26]. Significant increases in IL-1$\alpha$, IL-1$\beta$, and IL-8 levels were observed after 3 consecutive doses [26]; no placebo-gel group was included in these studies. Whether the magnitude of cytokine responses observed in these studies is functionally significant has not yet been determined.

The complex interactions between pro- and anti-inflammatory cytokines and other mediators of innate immunity suggest that reliance on measurements of a single or several cytokines may not prove to be predictive of microbicide safety. We sought to address this by developing a more comprehensive model focused on the functional significance of observed changes in immune mediators. We found that 3.5% N-9 triggered an increase, both at the protein and gene transcript level, in inflammatory cytokines and chemokines. This increase was associated with a modest influx of inflammatory cells and a significant increase in levels of the transcriptional activators NF-$\kappa$B and AP-1, relative to that observed with placebo gel. Twelve hours after the seventh dose, mice were challenged with a low inoculum of HSV-2 and displayed a significant increase in susceptibility to disease compared with mice that received HEC. Susceptibility to HSV infection may provide a surrogate marker of the functional integrity of mucosal immunity in the genital tract. Notably, an increase in susceptibility to HSV infection was also noted after a single application of N-9 in a recent study [16].

In contrast to results obtained with N-9, 2% PRO 2000 triggered only a modest and transient increase in levels of pro-inflammatory cytokines and chemokines, did not significantly increase tissue levels of NF-$\kappa$B or AP-1, and did not induce epithelial cell disruption. Most importantly, no increase in sus-
ceptibility to HSV infection was observed. If susceptibility to HSV infection proves to be predictive of mucosal immune function, then, even in the setting of intermittent application, 2% PRO 2000, unlike N-9, would not be anticipated to increase the risk of infection. Validation of this model will require correlation with results obtained in clinical trials.

Consistent with the murine model results, studies with human cell cultures have indicated that repeated exposure (2 h daily) to PRO 2000 at concentrations as high as 1 mg/mL were not cytotoxic, whereas N-9 was toxic at all concentrations tested [18]. Two percent PRO 2000 gel was well tolerated in phase 1 studies and was not associated with significant colposcopically visible changes. We extended these safety studies of PRO 2000 in pilot clinical studies and found no increase in proinflammatory cytokines in CVL samples after a single or 14 consecutive daily applications of 0.5% PRO 2000 gel [14, 21]. Additionally, there was no change in the intrinsic anti-HSV or antibacterial activity in CVL samples obtained throughout the 14-day study, which is consistent with no deleterious impact on innate immunity.

Although the increase in susceptibility to HSV infection in the mouse parallels the observed increase in HIV acquisition in the phase 3 N-9 trials, testing of additional microbicides is required before advancing this model as a biomarker of microbicide safety. At a molecular level, there are several pathways common to HIV and HSV that could contribute to an increased risk of both infections. Activation of NF-κB and/or AP-1 promotes transcription of the HIV-1 long terminal repeat in human CD4+ T cells. Interestingly, a virus-enhancing factor present in CVL samples obtained from a small number of women (3/38 HIV-seropositive women, 1/4 HIV-seronegative high-risk women, and 0/12 HIV-seronegative low-risk women) was shown to enhance HIV replication in vitro [27]. This HIV-inducing factor, distinct from cytokines, activates NF-κB and AP-1 and increases HIV-1 gene expression through the κB enhancer [28]. HSV triggers NF-κB activation [29–31], and activation of this pathway contributes to efficient virus replication [32]. In cells lacking an intact NF-κB pathway, HSV yields are reduced [33].

Another common mechanism could simply be disruption of the epithelium. Disruption could enhance infection by increasing the exposure to target cells. For HSV, epithelial disruption could expose dendritic cells, macrophages, or T cells, which are the primary target cells for HIV infection during sexual transmission. For HSV, epithelial disruption could increase access to nectin-1 coreceptors, which are more accessible after disruption of cell junctions [20, 34, 35].

Additionally, an increase in inflammatory mediators could directly enhance susceptibility to infection. Addition of individual cytokines to cell cultures activates HIV replication in vitro, although direct clinical data are lacking [36–39]. Chemokines can competitively inhibit HIV R5 virus infection through binding to the CCR5 coreceptor, but they also serve as potent T cell and monocyte attractants and induce signaling in T cells and macrophages that promotes HIV replication [37]. Less is known about the impact of cytokines or chemokines on HSV infection. It is presumed that the proinflammatory cytokine response to HSV facilitates viral clearance. However, murine studies have demonstrated enhanced pathogenicity in the presence of cytokines [40], suggesting that the impact may be complex. Although epithelial cells are the major target for HSV, the virus productively infects and modulates immune cells. The impact of cytokines or chemokines on HSV infection of epithelial or immune cells warrants further study.

Inflammatory responses may not be the only factor that contributes to susceptibility. Microbicides could increase the risk of infection by interfering with protective proteins, such as defensins [12, 13]. Whether the mouse will prove to be a model for protective proteins requires further study, because there are substantial species differences. A recent study found expression of α-defensins 1 and 2 as well as β-defensins 1, 2, and 4 in the uterus and vagina of mice by RT-PCR. α-defensin 5 was inconsistently detected in the uterus but was absent in the vagina [41].

In summary, the present study demonstrates that the cumulative mucosal effects of N-9, but not PRO 2000, increase susceptibility to HSV infection. The murine studies are consistent with clinical experiences and suggest that this model may prove to be predictive of microbicide safety, thus filling a major gap in the preclinical development of microbicides. Whether modest changes in epithelial integrity, cytokine levels, or increases in HSV susceptibility will prove to be “acceptable” will require testing of additional compounds and correlating results obtained in the murine model with those obtained in clinical trials. Some delivery systems, such as intravaginal rings, will not be readily adapted for evaluation in this model; however, the active drug itself could be tested. Although no single assay may prove to be predictive of microbicide safety, the results of these studies support inclusion of this murine safety model in preclinical assessment of microbicides to identify and prioritize the safest candidates.

Acknowledgments

We thank the Mount Sinai Department of Pathology; the Mount Sinai School of Medicine reverse-transcriptase polymerase chain reaction core facility; and Marcus Grisotto and the fluorescence-activated cell sorter core facility at the Mount Sinai School of Medicine.

References


33. Taddeo B, Zhang W, Lakeman F, Roizman B. Cells lacking NF-kappaB or in which NF-kappaB is not activated vary with respect to ability to sustain herpes simplex virus 1 replication and are not susceptible to apoptosis induced by a replication-incompetent mutant virus. J Virol 2004; 78:11613–21.


