Seminal Plasma Reduces the Effectiveness of Topical Polyanionic Microbicides

Sarju Patel,1,3 Ehsan Hazrati,1 Natalia Cheshenko,1 Benjamin Galen,1 Heyi Yang,2 Esmeralda Guzman,1 Rong Wang,2 Betsy C. Herold,1 and Marla J. Keller3

Departments of 1Pediatrics, 2Genetics and Genomic Sciences, and 3Medicine, Mount Sinai School of Medicine, New York, New York

The objective of this study was to test the activity of microbicides against herpes simplex virus type 2 (HSV-2) introduced in seminal plasma. We found that seminal plasma interfered with the activity of PRO 2000 and of cellulose sulfate, increasing by 100-fold the concentration of drug required to inhibit 90% of viral plaque formation. Seminal plasma competitively inhibited binding of the microbicides to the HSV-2 envelope. Most of the interference was found in a high molecular-weight fraction; tandem mass spectrometry identified the proteins as fibronectin-1 and lactoferrin. In a murine model, the interference translated in vivo into a loss in protection. We found that 2% PRO 2000 gel protected 100% of mice challenged intravaginally with HSV-2 introduced in PBS, whereas only 55% of mice were protected if virus was introduced in seminal plasma (, log rank test). If these findings are reflective of what occurs in humans, modifications to microbicides to ensure that they retain activity in the presence of seminal plasma are indicated.

Herpes simplex virus type 2 (HSV-2) is the leading cause of genital ulcer disease [1], and its impact as a public health threat is amplified because of its epidemiologic synergy with HIV [2]. The vulnerability of women to HIV and HSV infection underscores the need for preventative strategies that target both viruses. A vaccine fully protective against HIV and/or HSV is unlikely to be available in the near future [3, 4]. Oral prophylaxis may be indicated for specific populations, but has limitations, including the potential for selection of resistance. Thus, development of vaginal microbicides is a priority.

Most preclinical studies focus on the antiviral activity of microbicides in cell, tissue and animal models, with virus being introduced in buffer or media. However, in vivo, infection occurs in the presence of cervicovaginal secretions, with virus being introduced in semen. Therefore, it is important to determine microbicide activity under conditions that simulate what occurs in vivo. We have shown that several sulfated and/or sulfonated microbicides retain activity over a broad pH range and in the presence of cervicovaginal secretions [5, 6]. The objective of this study was to examine the anti-HSV activity of microbicides in the presence of seminal plasma. We tested PRO 2000 and cellulose sulfate, as both of these candidate microbicides have been advanced to Phase IIb/III clinical trials.

MATERIALS AND METHODS

Seminal and cervicovaginal fluid samples. Following institutional review board approval, informed consent was obtained to collect cervicovaginal and semen samples from healthy volunteers with a low number of identifiable risk factors for sexually transmitted infec-
tions. Semen was obtained by masturbation after 48 h of sexual abstinence, allowed to liquefy at room temperature for 30–60 min, and then centrifuged at 3000 g for 15 min to separate spermatozoa from seminal plasma. Cervicovaginal lavage (CVL) fluid samples were collected by washing with 10 mL of 0.9% saline [7]. CVL samples were also collected from women who participated in an unrelated study to assess the safety of PRO 2000 gel [8]. Participants were randomized to receive 0.5% PRO 2000 gel or placebo gel. CVL samples were obtained from a subset of subjects 1 h after application of the first dose. Penicillin, streptomycin, and amphotericin B were added to CVL and seminal plasma supernatants, which were then stored as aliquots at −80°C [7].

Reagents. The Program for the Topical Prevention of Conception and Disease (Rush University) provided unformulated cellulose sulfate. Unformulated and formulated PRO 2000 were provided by Indevus Pharmaceuticals. Fluorescent-labeled PRO 2000 (PRO 2000-F) was provided as a gift by S. Regen (Lehigh University). Hydroxethylcellulose placebo gel was obtained from the Contraceptive Research and Development Program (CONRAD; Eastern Virginia Medical School). To match the seminal plasma pH and protein concentration, HEPES (pH, 7.8) containing 25 mg/mL of bovine serum albumin (Fisher Scientific) and penicillin, streptomycin, and amphotericin B served as a control.

Cells and virus. CaSki cells (human cervical epithelial cells; American Type Culture Collection) and the wild-type strain HSV-2(G) were used for all studies [9]. Viral envelopes were labeled for confocal studies by infecting cells for 48 h and then incubating them with the lipophilic tracer DiD (Molecular Probes, Invitrogen) at a concentration of 1 μmol/L for 10 min before purification on sucrose gradients [10].

Plaque assays. Cells were pretreated for 1 h at 37°C with microbicides (0 to 500 μg/mL) diluted in PBS or a CVL sample and then challenged with an equivalent volume of HSV (∼200 pfu/well) that was immediately diluted to inoculation, in PBS (pH 7.4), HEPES, or pooled seminal plasma (3–5 donors per pool) diluted at a ratio of 1:5 in HEPES. After incubation for 1 h, cells were washed and overlaid with medium, and viral titers were determined by immunoassay 48 h after infection. To determine the overall anti-HSV activity of the microbicides, cells in 96-well plates were pretreated with 40 μL of microbicide diluted in PBS or CVL fluid then challenged with 10-fold dilutions of HSV-2 (0.01–1000 pfu/cell) diluted in seminal plasma or buffer (40 μL/well). Only wells containing 10–50 plaques were used to calculate the viral yield [10]. Additional experiments were conducted with CVL samples obtained 1 h after vaginal application of PRO 2000 gel or placebo gel [8].

Binding studies. Binding studies were conducted with cells fixed in 1% paraformaldehyde for 1 h at 37°C or with live cells at 4°C, as described elsewhere [10, 18]. Cells were pretreated with 100 μg/mL of microbicide diluted in PBS and inoculated with HSV-2(G) introduced in PBS, HEPES, or seminal plasma; fixed cells were allowed to incubate for 90 min at 37°C, and live cells for 5 h at 4°C. After incubation, cells were washed thrice with PBS to remove unbound virus, and cell-bound virus was detected by preparation of Western blot analyses of cell lysates probed with anti-glycoprotein D monoclonal antibody 1103 (Goodwin Institute).

Confocal microscopy. Cells were exposed to DiD-labeled virus diluted in PBS or seminal plasma (10 pfu/cell) in the absence or presence of 100 μg/mL PRO-2000-F for 5 h at 4°C and then washed thrice. To label cell membranes, cells were stained for 30 min with sulfoalkylbiotin reagent at a dilution of 1:1000 (EZ-Link; Pierce) before binding, fixed with 4% paraformaldehyde after binding, and then reacted with Alexa Fluor-350 conjugated streptavidin at a dilution of 1:1000 (Molecular Probes). Images were examined using a Zeiss LSM 510 Meta confocal microscope. Quantification of cell-associated DiD and PRO 2000-F was performed with National Institutes of Health Image densitometric software (100 cells counted).

Preincubation of virus or cells with microbicides. To examine whether seminal plasma interacts irreversibly with viral particles, 10^4 pfu/mL of HSV-2(G) was incubated for 1 h at 37°C with pooled seminal plasma or PBS and then the mixture was serially diluted in HEPES and inoculated onto CaSki cells in the presence of PRO 2000 (10 or 100 μg/mL). Alternatively, to determine whether seminal plasma interacts irreversibly with CaSki cells, cells were preincubated for 1 h at 37°C with pooled seminal plasma or buffer, and then washed thrice prior to inoculating with HSV-2(G) in the presence of PRO 2000. Cell viability was assayed 24 and 48 h after a 1-h exposure to seminal plasma, nonoxynol-9, or buffer by means of a cell proliferation assay (CellTiter96; Promega).

Fractionation and protein identification. Pooled seminal plasma was applied to the upper chamber of a Centricon-100 centrifugal filter (Millipore) and centrifuged at 5000 g for 45 minutes at room temperature, and the fraction with molecular weights of >100 kD was collected. The process was repeated and the fractions adjusted to their original volumes by dilution in PBS. The fraction containing molecules of <100 kD was transferred to a Centricon-50 centrifugal filter, and the procedure was repeated with centrifugation at 10,000 g. Fractions containing molecules of <50, 50–100, and >100 kD were aliquoted and stored at −80°C and tested in plaque assays.

The active fraction (ie, molecules of >100 kD) was further separated by size-exclusion chromatography (Sephacryl S-300 column; GE Healthcare Life Sciences). The column was equilibrated with distilled water and then PBS (pH, 7.2) at a flow rate of 0.5 mL/min. Samples (0.5–1 mL) of seminal fractions
Figure 1. Comparison of the activity of PRO 2000 and cellulose sulfate against herpes simplex virus 2 (HSV-2) infection when virus is introduced in PBS, HEPES buffer, or seminal plasma. CaSki cells were pretreated for 1 h with the indicated concentration of microbicide and were challenged with 200–300 pfu of HSV-2 (A) or with serial dilutions of virus (B). Plaque-forming units were counted 48 h after challenge, and mean values for 3 wells were calculated; similar results were obtained in 3 independent experiments. An asterisk (*) indicates that no plaques were visualized.

were loaded, and the elution flow rate was adjusted to 0.25 mL/min. Protein elution was monitored by UV absorbance at 280 nm, and elution peak samples were collected and concentrated using Amicon-10 (Millipore) centrifugal filters, adjusted to the original volume by dilution in PBS, and tested in plaque assays.

The active fraction samples were further purified using pre-packed anion-exchange gel matrices (HiTrap Q FF and HiTrap ANX FF; GE Healthcare Life Sciences). The columns were washed with 5 column volumes each of distilled water, binding buffer (for HiTrap Q FF, 0.20 mmol/L Tris, pH 8.0; for HiTrap ANX FF, 0.20 mmol/L ethanolamine buffer, pH 9.5), elution buffer (binding buffer plus 1.0 M NaCl) and subsequently with 10 volumes of the binding buffer before applying fractions. The active fraction peak samples obtained from the size-exclusion column were diluted 1:5 in buffer (the peak 1 sample in the Tris buffer and the peak 2 sample in the ethanolamine buffer) and applied to the column. Proteins were eluted over a 1.0 M NaCl gradient and individual protein peak samples were collected and evaluated in plaque assays.

Proteins in the active fraction samples were reduced, alkylated, and sequentially digested with 10 mmol/L dithiothreitol (Sigma), 50 mmol/L iodoacetamide (Sigma), and trypsin (Promega). The resulting peptides were analyzed by high-performance liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) using an LCQ ion trap mass spectrometer (ThermoFinnigan) with an online MicroPro HPLC system (Eldex). Peptides were separated on a reversed-phase C-18 column (0.2 × 150 mm, 5 μm, 200 Å) (Michrom BioResources) by linear gradient elution of 30%–80% B with solvent A, which contained 95% water, 5% methanol, 0.5% acetic acid, and 0.01% trifluoroacetic acid; and with solvent B, which contained 95% methanol, 5% water, 0.5% acetic acid, and 0.01% trifluoroacetic acid. Separated peptides were directly eluted into the
mass spectrometer by electrospray ionization. Mass spectra were acquired in a data-dependent mode; for each full-scan mass spectrum obtained (mass-to-charge ratio [m/z], 300–2000), 3 MS/MS spectra were obtained. The resulting MS and MS/MS spectra were searched against the National Center for Biotechnology Information nonredundant human protein database (http://www.ncbi.nlm.nih.gov) using Sonar software (Genomics Solutions).

**Immunoaffinity columns.** Immunoaffinity columns were prepared by coupling fibronectin antibodies (F09-16; Sigma, and MS-838-P1; Lab Vision) or a lactoferrin antibody (L 3262; Sigma) to a 1-mL N-hydroxysuccinimide (NHS)–activated HiTrap column. Seminal plasma (pooled from 3 subjects and diluted 1:10 in PBS) was applied to each of the columns and allowed to cycle 3 times, and the unbound fractions were collected, concentrated in PD-10 Centricon tubes (Millipore), desalted, and diluted to yield a final protein concentration of 1.5 mg/mL, after which each fraction was evaluated for interfering activity in a plaque assay.

**Mouse model of genital herpes.** With the approval of the Institutional Animal Care and Use Committee, female BALB/c mice were pretreated with 2 mg of medroxyprogesterone acetate subcutaneously and, 5 days later, were inoculated intravaginally with 40 μL of 2% PRO 2000 or hydroxyethylcellulose gel. Fifteen minutes later, mice were challenged with 20 μL of HSV-2(G) (5 log10 pfu per mouse) that had been diluted in pooled seminal plasma or buffer. The mice were evaluated for 14 days for signs of disease; 10 mice per group per experiment were evaluated in 2 independent studies [11].

**Statistical analysis.** Plaque assay results were compared by means of the Student t test, and Kaplan-Meier survival curves were assessed by log-rank test using Prism software (version 4; GraphPad).

**RESULTS**

**Seminal plasma interferes with anti-HSV activity of microbicides.** Cells were pretreated with PRO 2000 or cellulose sulfate and then challenged with virus diluted in PBS, HEPES, or seminal plasma. In pilot studies, we found that the addition of antibiotics to the PBS or HEPES buffer had no impact on HSV plaque formation (not shown). At a 1:5 dilution, the pooled seminal plasma had little effect on infection, as similar numbers of plaques were observed in the wells containing no microbicide (figure 1A). PRO 2000 and cellulose sulfate inhibited HSV if virus was introduced in PBS; the calculated concentrations that inhibited 90% of viral plaques (IC_{90}) were ~4 and 5 μg/mL, respectively. Introduction of virus in HEPES buffer resulted in an increase in the calculated IC_{90} to ~40 and 50 μg/mL, respectively. However, little or no anti-HSV activity was observed if the virus was introduced in seminal plasma, even at concentrations as high as 100 μg/mL. Similar results were obtained with additional laboratory and clinical isolates of both HSV serotypes (not shown).

These findings were extended to further quantify how much anti-HSV activity was lost if virus was introduced in seminal plasma. Cells were pretreated with the microbicides and then challenged with serial dilutions of HSV-2(G) diluted in PBS or pooled seminal plasma, and the viral titer was calculated. If virus was introduced in PBS, both microbicides inhibited HSV infection at least 1000-fold at 100 μg/mL. However, if virus was introduced in seminal plasma, the antiviral activity was observed if the virus was introduced in seminal plasma, even at concentrations as high as 100 μg/mL. Similar results were obtained with additional laboratory and clinical isolates of both HSV serotypes (not shown).

To determine whether cervical secretions might overcome this interference, we took advantage of CVL samples that had been obtained from subjects who were participating in an unrelated study of PRO 2000 [8]. The CVL samples obtained from 2 subjects who had received PRO 2000 gel inhibited HSV-2 infection 5000-fold to 20,000-fold for cells challenged with virus introduced in PBS. There was a modest reduction in antiviral activity if virus was introduced in HEPES, but almost all of the activity was lost if virus was introduced in seminal plasma (figure 2). The PRO 2000 concentrations in the CVL samples from subjects 1 and 2 were 97 and 166 μg/mL, respectively. These findings demonstrate that seminal plasma significantly interferes with the anti-HSV activity of these microbicides, even in the presence of cervical secretions.

**Mechanism of interference.** PRO 2000 and cellulose sulfate competitively inhibit the binding of HSV-2 to heparan sulfate receptors, primarily by interacting with viral glycoproteins [5].
To examine whether seminal plasma interferes with this, binding studies were conducted either with fixed cells at 37°C or live cells at 4°C, a temperature permissive only for binding. Comparable results were obtained with both drugs under either experimental condition; a representative blot for cellulose sulfate with fixed cells is shown (figure 3A). Cellulose sulfate inhibited viral binding if virus was introduced in PBS or HEPES. However, the antibinding activity was lost if virus was introduced in seminal plasma.

Confocal microscopy binding studies revealed that seminal plasma significantly interfered with the ability of PRO 2000-F to block viral binding (P < .0001) (figure 3B and 3C). In addition, there was a significant increase in the amount of cell-associated drug if virus was introduced in seminal plasma, compared with introduction in PBS (P < .0001). This possibly reflects competition between seminal plasma and PRO 2000 for viral envelope binding sites.

Seminal plasma might interfere with microbicide function.
Figure 4. Results of tests to determine whether seminal plasma interacts with CaSki cells or herpes simplex virus 2 (HSV-2) viral particles to mediate interference with the activity of PRO 2000. A, Results for virus particles pretreated with seminal plasma or PBS and then diluted prior to infection of CaSki cells in the presence of the microbicide. B, Results for CaSki cells treated with undiluted seminal plasma before being infected with HSV-2 in the presence of the microbicide. (For both A and B, plaque-forming units (pfu) were counted 48 h after infection; mean values were calculated for 2 wells and are representative of results from 3 independent experiments.) C, Viability of CaSki cells 24 h and 48 h after exposure to seminal plasma, nonoxynol-9, or HEPES. Mean percentages were calculated with data from controls as the denominator; data are from a representative experiment, and similar results were obtained in 2 independent experiments. Asterisks indicate no plaques visualized.

by interacting with the drugs, virus, and/or the target cell. Mixing PRO 2000 with seminal plasma failed to alter the intrinsic fluorescence of PRO 2000 (not shown), and seminal plasma did not interfere with interactions between PRO 2000 and epithelial cells, as determined by microscopy (figure 3B). In tests to determine whether seminal plasma interacts directly and irreversibly with the viral particle, we found that interference with the activity of PRO 2000 persisted if virus was pretreated with seminal plasma and then diluted prior to infection (figure 4A). Conversely, PRO 2000 retained its activity at a concentration of 100 μg/mL if the cells were first treated with undiluted seminal plasma for 1 h and then washed extensively prior to being infected in the presence of drug. Pretreating the cells with undiluted seminal plasma reduced the susceptibility to HSV-2 infection (figure 4B). No loss in cell viability was detected following 1 h of exposure to the undiluted seminal plasma (figure 4C). Thus, seminal plasma interacts with both the virus and the cell, but the interactions with the virus contribute more to the interference.

**Seminal plasma components that contribute to microbicide interference.** The inhibitory activity was lost if the seminal plasma was subjected to proteolysis with proteinase K, suggesting that proteins contribute to the interference activity (not shown). Initial fractionation indicated that almost all of the interfering activity mapped to the >100 kD fraction (figure 5A). When the >100 kD fraction was further separated by size ex-
Figure 6. Mass spectrometry identification of lactoferrin and fibronectin peptides and their effect on the observed interference of seminal plasma with microbicidal activity. A, Fragment ion mass spectrum of the peptide YLGPQYVAGITNLK, a tryptic peptide of lactoferrin; B, list of tryptic lactoferrin peptides identified; C, Fragment ion mass spectrum of SSPVVIDAIDAPSNLR, a tryptic peptide of fibronectin; D, list of tryptic fibronectin peptides identified. E, Comparison of the impact of unfractionated seminal plasma and seminal plasma partially depleted of each protein on antiviral activity of PRO 2000 (25 μg/mL). PFU were counted 48 h after infection; mean values were calculated from 2 independent experiments. m/z, mass-to-charge ratio.

In vivo murine study. As expected, >90% of the mice that were pretreated with hydroxyethylcellulose succumbed to infection independent of whether virus was introduced in PBS...
Seminal plasma significantly interfered with the in vitro activity of the naphthalene sulfonate polymer PRO 2000 and of cellulose sulfate, and this translated into a reduction in protection in a murine model. The experimental design of these studies represented an effort to simulate how drug and virus might interact in vivo. When human cervical cells were exposed to drug at concentrations found in CVL samples 1 h after gel application and challenged with virus diluted in seminal plasma, microbicide anti-HSV activity was significantly diminished (figure 3). No interference was observed in the presence of seminal plasma contributed to the absence of efficacy in the recent prematurely halted clinical trial of cellulose sulfate. Although the mechanism(s) have not yet been elucidated, the observation that fibronectin and lactoferrin bind HIV gp120 suggests that a mechanism similar to that observed for the interference with HSV may be involved.

A recent study found that the anti-HIV activity of several polyionmic microbicides also was diminished in the presence of seminal plasma in vitro [19], and ongoing studies from our lab indicate that seminal plasma reduces the anti-HIV activity of PRO 2000 and cellulose sulfate. Although the mechanism(s) have not yet been elucidated, the observation that fibronectin and lactoferrin bind HIV gp120 suggests that a mechanism similar to that observed for the interference with HSV may be involved.

Whether a reduction in anti-HIV activity of cellulose sulfate in the presence of seminal plasma contributed to the absence of efficacy in the recent prematurely halted clinical trial of cellulose sulfate and whether these in vitro findings will impact the effectiveness of PRO 2000 in ongoing clinical studies is not yet known. If partial protection with PRO 2000 is observed, a possible strategy to enhance the antiviral activity of PRO 2000 is to combine the microbicide with others that are not dele-
seriously impacted by seminal plasma. If validated by clinical studies, these findings support the testing of antiviral activity in the presence of cervical secretions and seminal plasma in preclinical development, to facilitate the identification of optimal microbicides, alone or in combination, prior to advancing products to clinical trials.

References