Presently marketed vaginal barrier agents are cytotoxic and damage the vaginal epithelium and natural vaginal flora with frequent use. Novel noncytotoxic agents are needed to protect women from sexually transmitted diseases. One candidate compound is a high-molecular-mass form of soluble poly(sodium 4-styrene sulfonate) (T-PSS). The antimicrobial activity of T-PSS was evaluated in primary culture systems and in a genital herpes murine model. Results obtained indicate that T-PSS is highly effective against herpes simplex viruses, Neisseria gonorrhoeae, and Chlamydia trachomatis in vitro. A 5% T-PSS gel protected 15 of 16 mice from vaginal herpes, compared with 2 of 16 mice treated with a placebo gel. Moreover, T-PSS exhibited little or no cytotoxicity and has an excellent selectivity index. T-PSS is an excellent candidate topical antimicrobial that blocks adherence of herpes simplex virus at low concentrations, inactivates virus at higher concentrations, and exhibits a broad spectrum of antimicrobial activity.

Presently marketed vaginal contraceptives, the active ingredients of which include nonoxynol-9 (N-9) or other cytotoxic surfactants, may provide some prophylaxis against sexually transmitted diseases (STDs). However, their efficacy is limited, and they have not been successful in preventing human immunodeficiency virus infections [1–3]. Moreover, frequent use has been associated with vaginal irritation and the possible inactivation of the natural vaginal flora [4]. Novel agents need to be developed that specifically inactivate STD pathogens while having minimal or no effect on vaginal and cervical cells and the natural vaginal flora.

One candidate is polystyrene sulfonate (PSS) [5–7]. The Program for the Topical Prevention of Conception and Disease (Rush University and University of Illinois at Chicago) developed a method of synthesis for PSS that yields a soluble, high-molecular-mass (>700 kDa by high-pressure liquid chromatography) product (designated T-PSS) free of the chloro-hydrocarbon solvents used in conventional synthesis.

We hypothesized that T-PSS might prevent microbial adherence, particularly because we have found that sulfated polymers or polysaccharides competitively block attachment by some STD pathogens [8]. The antimicrobial activity of T-PSS was tested against herpes simplex virus (HSV) and Chlamydia trachomatis infection in primary cultures and against Neisseria gonorrhoeae growth in agar. Focusing on HSV, the mechanism of antiviral activity was explored and its efficacy evaluated in a murine model of genital herpes.

Materials and Methods

Preparation of compounds. T-PSS was synthesized by the Program for the Topical Prevention of Conception and Disease, under good manufacturing practice conditions, by free radical polymerization of sodium styrene sulfonate in water (C.J.C., R.A.A., D.P.W., and L.J.D.Z., unpublished data). A 5% wt/wt gel formulation was prepared by Advanced Care Products (North Brunswick, NJ). The gel uses hydroxyethyl cellulose as base material and contains 5% wt/wt T-PSS, in a proprietary mixture. A placebo gel with similar composition, without T-PSS and containing additional hydroxyethyl cellulose to control for viscosity (75–80 kcp), was used as control. The gels were prepared under good manufacturing prac-
tice conditions and were within established limits for purity, strength, and quality. Heparin, sodium tauroliothocholic acid-3-sulfate (NaTLC-3-SO₄), and N-9 were purchased from Sigma (St. Louis, MO). All compounds were dissolved in PBS.

Primary and permanent cell cultures. The human cervical epithelial cell lines HeLa 229 and CaSki were purchased from the American Type Culture Collection (Rockville, MD) and maintained as described elsewhere [8, 9]. Primary rabbit kidney cells were prepared as described elsewhere [10]. Primary human fallopian tube organ cultures (HFTOCs), epithelial cells derived from HFTOCs, and cervical cells were isolated from hysterectomy specimens, as described elsewhere [8, 9, 11].

HSV in vitro assays. HSV strains used were wild-type HSV-2 strains 333 and 186 and HSV-1 strain 17. Plaque reduction assays were conducted as described elsewhere [9]. Plaque assays were modified to explore the mechanism of anti-HSV activity [12]. Confluent monolayers of cells were precooled to 4°C in the presence of increasing concentrations of T-PSS at 4°C to assess anti-binding activity [12]. Alternatively, compound was added after binding, at the time cells were transferred to 37°C, to determine whether post-binding effects were affected. Modified infectious center assays were conducted to examine effects on infection mediated by intracellular virus and cell-cell spread. Briefly, cells were infected with virus at 37°C to allow entry. Cells were washed with a low-pH citrate buffer to inactivate residual extracellular virus 1–2 h after infection. Then, 4–5 h after infection, the infected cells were detached with trypsin–EDTA and plated onto monolayers of uninfected cells in the presence of medium containing test compound. Alternatively, to specifically examine effects on cell-cell spread, infected cells were plated onto monolayers of uninfected cells in the presence of compound and monoclonal antibody 1103 (anti–HSV-1 and –HSV-2 gD; Goodwin Institute, Plantation, FL) at a dilution of 1:500 to neutralize infection by virus released into the medium (extracellular virus). The direct inactivation of virus by drug was tested by mixing HSV, at a concentration of ~10⁵ pfu/mL, with compounds or PBS as control. After incubation for 3 h at 37°C, the mixtures were diluted to yield noninhibitory levels of compound (and to yield 100–500 pfu/flask on control plates) and were plated on CaSki cells to determine whether the compound had irreducible effects on virus infectivity. For all assays, effects of T-PSS and heparin were compared.

C. trachomatis. Infectivity assays were done with C. trachomatis, serotype E/UW-5/CX, on HeLa cells in the presence of T-PSS (or N-9 or NaTLC-3-SO₄ as controls) as described elsewhere [10]. For HFTOC, chlamydial elementary bodies were preincubated with compounds for 4 h at 4°C before infecting HFTOC by dropping the inoculum directly onto the tissue. After a 48-h incubation period, the tissue was removed from the inoculum, weighed, homogenized to a single suspension, and used to infect a monolayer of HeLa cells by centrifugation at 1100 rpm (228 g) for 5 min. After adsorption for 1 h, the inoculum was removed and cells were overlaid with fresh media. After 24 h, the infected HeLa cells were stained with a fluorescein isothiocyanate–conjugated monoclonal antibody to chlamydial lipopolysaccharide protein (Bio-Rad Laboratories, Redmond, WA) and enumerated by means of a FACSVantage flow cytometer (Becton Dickinson, San Jose, CA).

Data from 10,000 cells were collected in listmode for analysis with H-P-LYSYS software (Becton Dickinson).

N. gonorrhoeae. We assessed the effects of drugs directly on N. gonorrhoeae MS11 by incorporating various concentrations of T-PSS, N-9, or NaTLC-3-SO₄ into gonococcal agar plates (Difco, Detroit), as described elsewhere [9].

Cytotoxicity assays. The cytotoxic effect of T-PSS on primary human cervical cells was determined by quantitating cell viability through the uptake of neutral red dye [8, 9]. Viability of primary human epithelial cells derived from HFTOCs was determined with propidium iodide uptake by means of flow cytometric analysis [9, 11].

Animal studies. Studies were conducted by a modification of our mouse model of genital herpes [13]. Female Swiss Webster mice (18–21 g; Harlan Sprague-Dawley, Indianapolis) were pretreated with 3 mg of medroxyprogesterone acetate (Upjohn Pharmacal, Kalamazoo, MI) 7 days and 1 day before challenge with virus, then anesthetized with sodium pentobarbital, and the vaginal vault was preswabbed with a wet and then a dry calcium alginate–tipped swab (Fisher Scientific, Pittsburgh). Animals (16 per group) received 15 μL of 5% T-PSS gel, placebo gel, or PBS by intravaginal instillation followed 20 s later by 15 μL of inoculum containing 4.0 log₄₅ pfu of HSV-2 strain 186 (a dose that consistently produces >85% mortality). Vaginal swab samples were collected from all animals on day 2 after inoculation and assayed for virus by culture on primary rabbit kidney cells. Mice were evaluated daily through day 21 after inoculation for evidence of symptomatic infection, including hair loss and erythema around the perineum, hind-limb paralysis, and mortality. Animals that remained asymptomatic were defined as uninfected if no virus was isolated from the swab cultures obtained on day 2 after infection.

Statistical analysis. Data are mean ± SD. For the animal studies, incidence data were compared by Fisher’s exact test (2-tailed).

Results

Spectrum of antimicrobial activity. Primary human cervical cells and HFTOCs were used with HSV and C. trachomatis, respectively. These cells or tissues are probably important in sexual transmission of infectious microbes. T-PSS completely inhibited HSV-1 and HSV-2 infection at concentrations of ~1 μg/mL (table 1). T-PSS also inhibited C. trachomatis infection of HFTOCs and HeLa cells. The inhibitory effects were greater than those observed with NaTLC-3-SO₄ or N-9. For example, at a concentration of 1 mg/mL, T-PSS and NaTLC-3-SO₄ inhibited 81% and 65% of chlamydial infection, respectively; inhibition by N-9 was observed only at concentrations that were toxic to cells (data not shown) [9]. T-PSS was highly effective in vitro against N. gonorrhoeae and completely inhibited growth at 1 μg/mL; similar inhibitory effects were observed with NaTLC-3-SO₄ at a concentration of 1 mg/mL. In contrast, even at cytotoxic concentrations, N-9 only modestly inhibited gonococci (data not shown) [9].

Mechanism of anti-HSV activity. Experiments were conducted to examine the mechanism(s) of T-PSS antiviral activity compared with heparin, a known inhibitor of HSV binding to
Table 1. Antimicrobial and cytotoxic effects of high-molecular-mass soluble poly(sodium 4-styrene sulfonate) (T-PSS) in prevention of sexually transmitted infections, as measured in various culture media.

<table>
<thead>
<tr>
<th>Measure</th>
<th>HSV-1 (PHCC)</th>
<th>HSV-2 (PHCC)</th>
<th>Chlamydia trachomatis (HFTOCs)</th>
<th>Neisseria gonorrhoeae (agar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED₅₀, µg/mL</td>
<td>0.06</td>
<td>0.04</td>
<td>20</td>
<td>0.1</td>
</tr>
<tr>
<td>CD₅₀, mg/mL</td>
<td>~5</td>
<td>~5</td>
<td>&gt;1</td>
<td>NA</td>
</tr>
<tr>
<td>SI, CD₅₀ : ED₅₀</td>
<td>8 × 10⁴</td>
<td>1.25 × 10⁴</td>
<td>&gt;50</td>
<td>NA</td>
</tr>
</tbody>
</table>

**NOTE.** HFTOCs, human fallopian tube organ cultures; HSV, herpes simplex virus; PHCC, primary human cervical cells; NA, not applicable; SI, selectivity index.

A Dose of T-PSS that inhibited 50% of HSV-1 or HSV-2 infection of PHCCs, or 50% of C. trachomatis infection of HFTOCs, or 50% of N. gonorrhoeae viability. These values were determined from dose-response curves that were generated from 2–3 independent experiments conducted in duplicate or triplicate. Each point in the dose-response curves represented the pfu formed (HSV), the inclusions formed (C. trachomatis), or the number of viable gonococci in the presence of the stated compound, as a percentage of the pfu, the organisms, or the gonococci in the absence of the compound.

B Dose of T-PSS that killed 50% of primary cervical cells, as measured by neutral red dye uptake or primary human epithelial cells derived from HFTOCs as measured by propidium iodide uptake after exposure of cells to drug for 24 h.

cell surface heparan sulfate [14]. T-PSS was as effective as heparin in inhibiting the adsorption of virus to cells (figure 1, left). The effect of T-PSS on virus binding paralleled its effect on plaque formation. This suggests that inhibition of virus adsorption is the predominant mechanism of antiviral activity. T-PSS or heparin was also added to the cultures after the initial binding period at 4°C to determine whether there were post-binding effects on viral infection (figure 1, left). Results suggest that T-PSS inhibits postbinding steps at concentrations >10 µg/mL.

The direct effects of T-PSS or heparin on HSV were evaluated by preincubating virus with drug (or PBS) for 3 h at 37°C. The virus-drug mixture was then diluted to yield 10²–10³ pfu/mL and noninhibitory concentrations of drug. Preincubation with T-PSS reduced viral plaque formation, whereas heparin enhanced infection (figure 1, right).

Traditional plaque assays test the infectivity of extracellular virus. However, the source of sexual acquisition may also be intracellular [15]. Modified infectious center assays were conducted to determine whether T-PSS could inhibit intracellular infection or cell-cell spread. T-PSS inhibited infectious center formation more than did heparin (figure 1, center).

**Cytotoxicity of T-PSS.** T-PSS had minimal effect on cell viability at concentrations as high as 2.5 mg/mL (table 1). After a 48-h exposure to 1 mg/mL T-PSS, 99% of primary human epithelial cells derived from HFTOCs were viable, compared with none of the cells that had been exposed to 0.01% N-9 (data not shown). Notably, T-PSS reduced cellular DNA synthesis; this increased with duration of exposure of cells to compound. Primary cervical cellular DNA synthesis was reduced...
by 50% after a 21-h exposure to 0.25 mg/mL T-PSS or 0.4 mg/mL heparin (data not shown).

Efficacy of T-PSS in the mouse genital herpes model. All 16 PBS-treated mice became infected and developed symptomatic disease. Similarly, all mice that received placebo gel became infected; 14 of 16 developed symptoms. These data were consistent with no protective effect of the placebo gel. In contrast, T-PSS gel provided protection against infection. Only 3 of 16 mice became infected; only 1 developed symptoms of disease \((P < .001)\).

Discussion

These studies demonstrated high efficacy for T-PSS against HSV-1, HSV-2, N. gonorrhoeae, and C. trachomatis at concentrations that exhibited little cytotoxicity. On the basis of these data, T-PSS exhibits a 10,000-fold higher selectivity index than does N-9, the active ingredient in most contraceptive preparations. T-PSS was also highly effective in preventing genital herpes in the mouse. Notably, T-PSS was more effective against C. trachomatis than were other candidate compounds, including NaTLC-3-SO₄, N-9, or sulfated carbohydrates \([8, 9]\).

The mechanisms of sexual transmission for most pathogens remain poorly defined. For example, the importance of intracellular virus in the transmission of HSV remains unresolved \([15]\). HSV may spread from infected cell to uninfected neighboring cells by direct cell-cell spread. Our data suggest that T-PSS inhibits HSV infectivity by both extra- and intracellular pathways.

T-PSS appears to inhibit infection by extracellular virus by blocking virus binding (figure 1, left) \([11]\). Infection was reduced when T-PSS was preincubated with virus and the mixture was diluted to noninhibitory drug concentrations. In contrast, preincubation of virus with heparin enhanced viral infection. The reason heparin enhances infection under these conditions is unclear. Possibly, T-PSS, but not heparin, directly inactivates HSV. Alternatively, T-PSS might bind irreversibly to HSV glycoproteins and inhibit viral attachment to cells.

T-PSS also inhibits infection mediated by intracellular virus, as suggested by the modified infectious center assay data (figure 1, center). Together, these results suggest that the mechanism(s) of antimicrobial activity may involve more than competition with cell surface receptors for microbial attachment, although this most likely substantially contributes to the observed biologic effects. More importantly, the observations that T-PSS can inhibit virus binding, infection by intracellular HSV, and cell-cell spread and may directly inactivate viral particles suggest that it may be a very effective topical antimicrobial.

These results support the concept that a topical formulation containing T-PSS may provide protection against multiple STD pathogens. Importantly, T-PSS is easy to synthesize, is highly soluble, and can be easily formulated. Thus, T-PSS deserves further development and clinical evaluation.

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References