Two Novel Vaginal Microbicides (Polystyrene Sulfonate and Cellulose Sulfate) Inhibit *Gardnerella vaginalis* and Anaerobes Commonly Associated with Bacterial Vaginosis

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This is the first report demonstrating the in vitro inhibitory activity of two novel microbicides (cellulose sulfate and polystyrene sulfonate) against bacterial vaginosis (BV)-associated bacteria. Vaginal application of these microbicides not only may reduce the risk of acquisition of human immunodeficiency virus and other sexually transmitted infection-causing organisms but may also decrease the incidence of BV.

AIDS and other sexually transmitted infections (STIs) continue to be major health threats. An attractive approach for women to prevent the acquisition of these diseases through vaginal intercourse is the use of antimicrobial products (microbicides) (12, 24). Nonoxynol-9, a highly cytotoxic surfactant and detergent commonly used as an active ingredient in marketed spermicidal and contraceptive formulations, possesses broad-spectrum in vitro activity against the human immunodeficiency virus (HIV) and many other STI-causing organisms. Unfortunately, nonoxynol-9-containing products also inactivate lactobacilli and can increase the occurrence of bacterial vaginosis (BV) (16, 17). In addition, vaginal ulcerations can result from frequent use of this compound (25). These adverse effects are given as reasons for the unacceptability of nonoxynol-9-containing spermicidal products for vaginal use to prevent HIV transmission (20), and these products may even enhance transmission (21). Alternate vaginal topical agents need to be identified.

Two new, noncytotoxic microbicides that have reached clinical trial include polystyrene sulfonate (T-PSS) and cellulose sulfate (Ushercell). Ushercell is a long-chain sulfated polysaccharide (~1,900 kDa) and T-PSS is a long-chain sulfonated polymer (~751 kDa). Both have broad-spectrum antimicrobial activity. They are known to inhibit HIV, human papillomavirus, herpes simplex virus type 1 (HSV-1), HSV-2, *Chlamydia trachomatis*, and *Neisseria gonorrhoeae* in vitro (1, 2, 7, 9, 26). These compounds do not inhibit *Lactobacillus gasseri* in vitro at concentrations of 5 to 50 mg/ml and have been proven to be safe in a variety of animal studies (1, 2, 26; L. J. D. Zaneveld, D. P. Waller, R. A. Anderson, C. Chany, and S. Garg, Program Abstr. Microbicides 2000 Conf., p. 30, 2000). As a result, the

U.S. Food and Drug Administration approved an Investigational New Drug application for both products.

Since vaginal microbicides may be used frequently, it is important that these products minimally alter the endogenous vaginal microflora. A concern is that these polymers may enhance the growth of *Gardnerella vaginalis* and BV-associated anaerobes, possibly resulting in an increased incidence of BV. BV is defined as a clinical syndrome in which the normal, predominantly lactobacilli-containing vaginal flora are replaced by high concentrations of *G. vaginalis*, anaerobic bacteria, and genital mycoplasmas (11, 19). *G. vaginalis* appears to play a key role in creating an environment conducive to the development of BV (S. Faro, Editorial, Infect. Dis. Obstet. Gynecol. 8:75). BV is one of the most common vaginal disorders among reproductive-age women. It is associated with gynecological and obstetric complications, as well as with an increased risk of acquisition and transmission of HIV (6, 8, 13, 18, 22).

The purpose of this study was to evaluate how T-PSS and Ushercell affect the growth of *G. vaginalis* and anaerobes commonly associated with BV.

Procurement of compounds. High-molecular-weight sodium cellulose sulfate (Ushercell) was synthesized at Dextran Products, Ltd. (Scarborough, Ontario, Canada) according to the method described by Usher et al. (23). Polystyrene sulfonate (T-PSS) was synthesized by the Program for the Topical Prevention of Conception and Disease (TOPCAD; Chicago, Ill.) by free-radical polymerization of sodium styrene sulfonate in water (C. J. Chany, R. A. Anderson, D. P. Waller, and L. J. D. Zaneveld, unpublished data). Both were synthesized under good manufacturing practice conditions. Ushercell and T-PSS have peak molecular masses of 2,300 and 864 kDa, respectively, and average molecular masses of 1,900 and 751 kDa, respectively.

Bacterial strains. A total of 33 clinical isolates and two American Type Culture Collection (ATCC) strains were used for this study, including six genera of bacteria commonly asso-

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ciated with BV: *Gardnerella*, *Bacteroides*, *Peptostreptococcus*, *Prevotella*, *Fusobacterium*, and *Eubacterium*. The isolates were obtained from the Alden Research Laboratory collection, UCLA Medical Center, Santa Monica, Calif., and from the Obstetrics and Gynecology Research Laboratory collection, Rush Medical Center, Chicago, Ill. All the isolates came from patients with BV and/or pelvic infections, such as endometritis. The isolates were stored at -70°C in skim milk (Difco Laboratories, Detroit, Mich.) and subcultured two or three times in brucella broth or in human blood bilayer Tween (HBT) agar before testing.

***G. vaginalis* agar diffusion bioassay.** Since *G. vaginalis* is consistently associated with BV, the effect of T-PSS and Usher-cell on *G. vaginalis* growth was studied first. An agar diffusion bioassay was performed with four clinical isolates as indicator strains. HBT agar (BD-Microbiology Systems, Cockeysville, Md.) was chosen as a selective medium for *G. vaginalis*. The polymers have a considerably larger molecular size than most antibiotics; therefore, their diffusion through media was expected to be slower. In an effort to overcome this difficulty, the compounds were initially tested in wells punched in the agar plates as described below. A suspension containing approximately 10^7 CFU of *G. vaginalis*/ml was prepared and used to inoculate two HBT agar plates. After drying for 30 min, four wells were punched in each HBT top layer agar with a number 8 cork borer. Different concentrations of each compound in phosphate-buffered saline (PBS) were prepared (10, 5, 2.5, 1, 0.5, 0.25 and 0.125 mg/ml) and aliquoted (200 μl) into the wells of two sets of HBT agar plates. The last well was filled with 200 μl of PBS as the negative control. After a 2-h period of prediffusion, the plates were incubated overnight under a 5% CO_2 atmosphere at 37°C . Comparative studies were performed with ampicillin (8 $\mu\text{g}/\text{ml}$) as a positive control in a separate plate. The sizes of the zones of inhibition surrounding the well (distance from the edge of the well to the bacteria growth) were measured in millimeters with a vernier caliper.

Evaluation of antibacterial activity by broth dilution method. The macrodilution broth method was performed according to a method described by the National Committee for Clinical Laboratory Standards (NCCLS) (15). T-PSS and Usher-cell were serially diluted in brucella broth (Difco Laboratories) supplemented with sheep blood, vitamin K, and hemin, as described in NCCLS M11-A5 (15). The medium did not contain NaHCO_3 and was reduced in an anaerobic chamber in an atmosphere containing 5% CO_2 for approximately 2 h prior to inoculation. The highest concentration tested was 10 mg/ml (10, 5, 2.5, 1.25, 0.625, and 0.313 mg/ml). Aliquots (1 ml) of the diluted compounds were added to 12- by 75-mm plastic tubes. The inoculum was prepared by suspending each bacterial strain to a turbidity equal to the 0.5 McFarland standard in unsupplemented brucella broth and diluted 1:50 in supplemented brucella broth. Then, 1-ml aliquots of each test strain were dispensed into the tubes that contained the diluted compounds. The final concentration was 1×10^6 to 5×10^6 CFU/ml. The mixtures were incubated at 37°C under anaerobic conditions. After a 48-h incubation, the tubes were examined for growth. Comparative studies were performed with metronidazole as the control drug. Tubes without either microorganism or drug were also tested simultaneously as negative and positive controls, respectively. The MIC was defined

TABLE 1. Zones of *G. vaginalis* growth inhibition by polystyrene sulfonate (T-PSS) and by cellulose sulfate (Usher-cell) in agar diffusion assay

Compound	Concn	Inhibition zones (mm) ^a			
		Gv284	Gv422	Gv712	Gv743
T-PSS	10 mg/ml	4	5	6	6
	5 mg/ml	4	5	5	5
	2.5 mg/ml	3	3	4	3
	1 mg/ml	2	2	1	2
	0.5 mg/ml	2	2	1	1
	0.25 mg/ml	0	1	0	0
	0.125 mg/ml	0	0	0	0
Usher-cell	10 mg/ml	2	5	3	1
	5 mg/ml	2	4	2	1
	2.5 mg/ml	1	3	2	0
	1 mg/ml	1	2	1	0
	0.5 mg/ml	1	1	1	0
	0.25 mg/ml	0	0	0	0
	0.125 mg/ml	0	0	0	0
Ampicillin	8 $\mu\text{g}/\text{ml}$	7	8	8	7
PBS		0	0	0	0

^a Each measurement is the average inhibition distance from the edge of the well to the edge of growth.

as the lowest concentration of each compound that completely inhibited growth of the tested bacteria.

Results and discussion. Both T-PSS and Usher-cell inhibited the four *G. vaginalis* clinical isolates in the agar diffusion bioassay. *G. vaginalis* was inhibited by T-PSS in a concentration-dependent manner (Table 1). The growth-inhibition effect against the four indicator strains began at 0.25 to 0.5 mg of T-PSS/ml, depending on the strain. At the highest concentration tested (10 mg/ml), the inhibitory effect was similar to that of ampicillin (8 $\mu\text{g}/\text{ml}$).

Usher-cell also had a dose-dependent growth-inhibition effect on *G. vaginalis* clinical isolates. Inhibition started at 0.5 mg/ml for three isolates. One of the isolates (Gv743) demonstrated decreased susceptibility to the inhibitory activity of Usher-cell, with growth inhibition starting at 5 mg/ml (Table 1).

Table 2 depicts the MIC endpoints obtained with the broth dilution method. Both T-PSS and Usher-cell inhibited the growth of most test isolates. In general, T-PSS was effective at lower concentrations than Usher-cell. The compounds inhibited both strains of *G. vaginalis* and both strains of *Eubacterium lentum*. All eight strains of *Peptostreptococcus* were inhibited by Usher-cell, and six were inhibited by T-PSS. The two strains of *Prevotella melaninogenica* were not inhibited by Usher-cell, and only one strain was inhibited by T-PSS. However, both compounds inhibited all strains of the other *Prevotella* species (*P. intermedia*, *P. bivia*, and *P. disiens*). One of the three tested strains of *Bacteroides thetaiotaomicron* was inhibited. None of the *Bacteroides fragilis* strains was inhibited by T-PSS, and only one strain was inhibited by Usher-cell. Under the same conditions, metronidazole was much more effective against all of these microbes than either T-PSS or Usher-cell, at least on a weight/volume basis.

T-PSS and Usher-cell are under clinical evaluation as active

TABLE 2. Inhibition of BV-associated anaerobes by polystyrene sulfonate (T-PSS) and cellulose sulfate (Ushercell) in broth dilution assay

Isolate ^a	Bacteria species	MIC endpoints ^b		
		T-PSS (mg/ml)	Ushercell (mg/ml)	Metronidazole (μg/ml)
10481	<i>Fusobacterium nucleatum</i>	1.25	5	1
11518	<i>Fusobacterium nucleatum</i>	1.25	10	0.25
11423	<i>Fusobacterium gonidiaformans</i>	NE ^c	5	0.125
11653	<i>Fusobacterium gonidiaformans</i>	NE	5	0.25
9052	<i>Prevotella melaninogenica</i>	1.25	NE	0.25
5657	<i>Prevotella melaninogenica</i>	NE	NE	0.125
11142	<i>Prevotella intermedia</i>	1.25	10	0.5
11168	<i>Prevotella intermedia</i>	1.25	10	0.25
11697	<i>Prevotella bivia</i>	2.5	5	2
11683	<i>Prevotella bivia</i>	1.25	5	0.5
653	<i>Prevotella bivia</i>	2.5	10	2
11579	<i>Prevotella disiens</i>	1.25	10	0.25
11698	<i>Prevotella disiens</i>	0.6	10	0.125
12066	<i>Gardnerella vaginalis</i>	1.25	5	1
12262	<i>Gardnerella vaginalis</i>	1.25	5	0.5
11658	<i>Peptostreptococcus magnus</i>	10	5	≤0.06
11598	<i>Peptostreptococcus magnus</i>	NE	5	0.25
11287	<i>Peptostreptococcus tetradius</i>	1.25	5	≤0.06
11253	<i>Peptostreptococcus tetradius</i>	NE	5	0.5
443	<i>Peptostreptococcus anaerobicus</i>	5	10	≤0.06
11587	<i>Peptostreptococcus asaccharolyticus</i>	2.5	10	≤0.06
11607	<i>Peptostreptococcus asaccharolyticus</i>	5	10	0.125
4C	<i>Peptostreptococcus asaccharolyticus</i>	10	5	0.125
9420	<i>Eubacterium lentum</i>	5	5	0.5
11700	<i>Eubacterium lentum</i>	1.25	10	1
ATCC	<i>Bacteroides thetaiotaomicron</i>	5	10	2
11604	<i>Bacteroides thetaiotaomicron</i>	NE	NE	0.25
11651	<i>Bacteroides thetaiotaomicron</i>	NE	NE	0.5
ATCC	<i>Bacteroides fragilis</i>	NE	10	0.5
11647	<i>Bacteroides fragilis</i>	NE	NE	0.25
11652	<i>Bacteroides fragilis</i>	NE	NE	0.5

^a The numbers represent clinical isolates. *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 are the NCCLS-recommended reference strains for anaerobic antimicrobial susceptibility testing.

^b Lowest concentration that completely inhibited the bacteria growth.

^c NE = no effect by 10 mg/ml (the highest concentration tested).

ingredients for vaginal microbicide products (e.g., see reference 14). These compounds are not cytotoxic to spermatozoa or the host cells used in the herpes and HIV assays and do not inactivate *Lactobacillus gasseri* (1, 2, 26; Zaneveld et al., Program Abstr. Microbicides 2000 Conf.). The present study demonstrates that T-PSS and Ushercell also inhibit the growth of many bacteria commonly associated with BV, eliminating the concern that they may enhance the incidence of this disease. Both compounds are effective against most or all tested isolates of *G. vaginalis*, *Peptostreptococcus*, *Prevotella*, *Eubacterium*, and *Fusobacterium* at concentrations of ≤10 mg/ml. Less or no inhibition was found with respect to *Bacteroides* isolates, particularly to *B. fragilis* strains. Although previous studies have shown that sulfated or sulfonated polymers inhibit the in vitro growth of STI-causing bacteria and the infectivity of enveloped viruses, including HIV (1, 2, 3, 4, 7, 9, 10, 26), this is the first report on the inhibition of BV-associated anaerobic bacteria by such compounds.

The recommended treatment for BV is metronidazole, orally or intravaginally (5). However, this drug is not always highly effective, particularly when cure rates after 1 month or more are considered (11). Compared to the MIC levels of metronidazole, T-PSS and Ushercell were much less effective on a weight/volume basis against the anaerobic microbes tested in the present study. However, the high molecular weights and

charge densities of T-PSS and Ushercell make vaginal absorption extremely unlikely, in contrast to metronidazole. In addition, Ushercell and, to a lesser extent, T-PSS have bioadhesive properties, delaying leakage from the vagina (2, 26). Such properties may make these compounds safer. In addition, they may remain for longer periods of time in the vagina. It would be worthwhile to test them as an alternative treatment for BV.

Little is known about the possible mechanism of action whereby T-PSS and Ushercell can prevent growth of microbes. Previous studies have shown that the mechanism of antimicrobial activity towards several STI-causing pathogens by these compounds may involve receptor antagonism or mimeticism during cell-cell fusion (1, 2, 9). This study does not address the mechanism by which T-PSS and Ushercell exert their antimicrobial activity against *G. vaginalis* and BV-associated anaerobic bacteria.

The present data on the in vitro inhibitory activity of T-PSS and Ushercell against *G. vaginalis* and other BV-associated anaerobic bacteria provide additional information regarding their safety as vaginal microbicides. The compounds have a direct inhibitory effect on HIV and other STI-causing pathogens. We hypothesize that a secondary mechanism of anti-STI action may be through a reduction in the occurrence of BV, a condition that can increase acquisition and transmission of

HIV and other STIs. Clinical studies are required to prove this hypothesis.

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