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Evaluation of Poly(Styrene-4-Sulfonate) as a Preventive Agent for Conception and Sexually Transmitted Diseases

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ABSTRACT: A commercial preparation of a sodium polystyrene sulfonate (designated as N-PSS; its molecular weight is 500 000 daltons) was tested as an inhibitor of sperm function and as a preventive agent for conception and the transmission of sexually transmitted diseases. The polymer is an irreversible inhibitor of hyaluronidase and acrosin; its IC_{50} values are 5.7 μg/mL and 0.5 μg/mL, for hyaluronidase and acrosin, respectively. N-PSS is also a stimulus of human sperm acrosomal loss. It produces maximal acrosomal loss at 2.5 μg/mL. Contraception in rabbits is nearly complete when rabbit spermatozoa are pretreated with 0.5 mg/mL of N-PSS before artificial insemination; however, N-PSS does not immobilize spermatozoa at concentrations as high as 50 mg/mL. N-PSS has broad spectrum antiviral and antibacterial activities. Infection by human immunodeficiency virus and herpes simplex virus are inhibited by N-PSS; 3-log reductions are produced by 7 μg/mL and 3 μg/mL, respectively. N-PSS is active against *Chlamydia trachomatis* and *Neisseria gonorrhoeae.* At 1 mg/mL, N-PSS inhibits chlamydial infectivity by more than 90%. N-PSS produces a 3-log reduction in gonococcal growth at 15 μg/mL. In contrast, N-PSS (5 mg/mL) does not affect the growth of *Lactobacillus* (normal component of the vaginal flora). N-PSS can be classified as a noncytotoxic contraceptive antimicrobial agent. These properties justify bringing a polystyrene sulfonate into clinical trials for its evaluation as a preventive agent for conception and several sexually transmitted diseases.

Key words: Hyaluronidase inhibitor, acrosin inhibitor, acrosomal loss, contraceptive, STD preventive, preclinical studies.

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The world population of approximately 6 billion people will likely increase by 50% by 2050 (United Nations, 1998). Overpopulation, particularly in developing countries, is complicated by the pandemic of human immunodeficiency virus (HIV) infections (Fenton, 1996) and the prevalence of other sexually transmitted diseases (STDs). Nonoxynol-9 (N-9) and other spermicides with detergent and cytotoxic properties have been proposed as preventives against STD organisms (Jennings and Clegg, 1993; Voelker, 1995); however, findings with N-9 at doses that do not irritate or damage the female reproductive tract are inconsistent (Roddy et al, 1998a,b; Stafford et al, 1998). Increased protection and alternative approaches against unplanned pregnancies and STDs are clearly needed.

In 1993, we launched the Program for the Topical Prevention of Conception and Disease (TOPCAD). The program is multidisciplinary, consisting of collaborators from several universities and not-for-profit institutions. Its purpose is to develop safe, noncytotoxic, broad spectrum active ingredients that can protect women during sexual activity.

Our strategy for discovery of active ingredients is based on the premise that common mechanisms exist for fertilization and microbial infection. Activities of hyaluronidase and proteinases are among the factors involved in tissue invasion by several STD-causing microorganisms, and in mammalian fertilization. Release of these lytic enzymes into the extracellular milieu is a prerequisite or is supportive of their actions.

Fertilization is inhibited by agents or factors that inhibit acrosomal hyaluronidase (EC 3.2.1.35; Pincus et al, 1948;

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Parkes, 1953; Parkes et al, 1954; Joyce and Zaneveld, 1985) and acrosin (EC 3.2.21.10, a trypsin-like enzyme; Zaneveld et al, 1970; Joyce et al, 1979; Beyler and Zaneveld, 1982; Kaminski et al, 1985). Agents that alter the acrosome (eg, acrosome reaction) also inhibit fertilization (Rogers et al, 1977; Rogers, 1982; Endo et al, 1987; Nuzzo and Zaneveld, 1988).

Hyaluronidase is involved in *Treponema* (syphilis) invasion (Fitzgerald and Gannon, 1983; Fitzgerald and Repesh, 1987). Increased vaginal hyaluronidase is associated with trichomoniasis, candidiasis, and other forms of vaginitis (Rosso, 1975); and with vaginosis caused by *Gardnerella vaginalis, Mobiluncus* spp, and *Mycoplasma hominis* (McGregor et al, 1994).

Bacterial immunoglobulin A (IgA) proteinase specifically hydrolyzes secretory IgA. It is present in *Neisseria gonorrhoeae* (Kornfeld and Plaut, 1981; O'Reilly and Bhatti, 1986; Mulks and Knapp, 1987; Simpson et al, 1988), and in *Ureaplasma* and nongenital pathogens (Kornfeld and Plaut, 1981; Kapatais-Zoubmos et al, 1985; Proctor and Manning, 1990). *Neisseria gonorrhoeae* contains 2 types of IgA proteinase. The form associated with disseminated gonococcal disease is susceptible to inhibition by thiol- and serine-specific agents (Mulks and Knapp, 1987; Simpson et al, 1988).

Vaginal proteinase activity increases in women with gonorrhea (Blake et al, 1979). Similarly, extracellular proteinase levels correlate with the pathogenesis of *Candida albicans* (Shimizu et al, 1987). Virulent strains of group B streptococci produce six-fold more proteinase that nonvirulent strains (Straus et al, 1980).

A cellular serine (trypsin-like) proteinase is involved in cell penetration by HIV. During viral fusion, an epitope of gp120 (a viral glycoprotein) interacts with a trypsin-like enzyme and binds to the target cell CD4 receptor (Koito et al, 1989). Furthermore, a trypsin-like enzyme is required for the intracellular processing of the precursor glycoprotein, gp160, to gp120 after the cell is infected (Koito et al, 1989; Kido et al, 1993). Trypsin inhibitors are anti-HIV agents (Hallenberger et al, 1992; Bourinbaiar and Nagorny, 1994). An aspartyl proteinase and cysteine proteinase are also involved in post-translational processing of HIV and herpes simplex virus (HSV) proteins, respectively (Zezulak and Spear, 1984). Although hyaluronidase and proteinases are not the only factors for microbial infectivity, there is much evidence that hyaluronidase and proteinase have a role in infection.

The safety of contraceptive anti-STD agents is important because of their elective and likely frequent use. The active ingredient should show low cytotoxicity and be specific for 1 or more pathogenic microorganisms in the vagina. Beneficial microflora (eg, lactobacilli) should be minimally affected.

We identified a commercial polystyrene sulfonate (PSS) for further development. PSS exists in different molecular weight forms and at different levels of sulfonation. The United States Pharmacopeia (1995) describes 1 form of PSS as a water-insoluble, cross-linked polymer. The present high molecular weight, water-soluble material is designated N-PSS to differentiate it from other molecular forms.

N-PSS was chosen, in part, because of its contraceptive, nonspermicidal properties (Homm et al, 1985; Foldesy et al, 1986; Hahn and McGuire, 1986). The purpose of this study was to confirm and extend the data regarding effects of the polymer on sperm function and to evaluate its antimicrobial activity.

Our data confirm that N-PSS is a highly effective contraceptive. They further show that 1) N-PSS has broad spectrum activity against STD-causing microorganisms, 2) it does not affect lactobacilli, and 3) it exerts its activities by one or more noncytotoxic mechanisms of action. These findings provide justification for synthesis of PSS under conditions of good manufacturing practices and its testing in clinical trials.

Materials and Methods

Overview

Testing was designed to identify the contraceptive potential of N-PSS and to evaluate its ability to act against 1 or more STD-causing microbes. Tests fell into 2 broad categories: sperm function and microbial infectivity, or growth. All tests were conducted in vitro with the exception of contraceptive evaluation in the rabbit.

Sperm function tests were chosen on the basis of our current understanding of sperm processes or properties, which are prerequisite to successful oocyte penetration. These include adequate enzymic activities of hyaluronidase and acrosin and the ability to release these and other critical acrosomal factors at the appropriate time and place in relation to the oocyte. Activities of both enzymes were estimated by spectrophotometric determination of their reaction products. The natural substrate (hyaluronic acid) was used for hyaluronidase assays. The esterase activity of acrosin was measured with a synthetic substrate. Acrosomal loss was determined by direct visualization of the stained acrosomal region of the sperm head.

Antimicrobial assays were further divided into viral and bacterial. HSV and HIV were selected as representative sexually transmitted viruses. *N. gonorrhoeae* and *Chlamydia trachomatis* were selected as representative sexually transmitted bacteria. Except for *N. gonorrhoeae*, tests were designed to measure microbial infectivity. After timed exposure of N-PSS to the microbes and inoculation of target cells, N-PSS was removed by dilution before the microbe was allowed to replicate within the target cells. Therefore, inhibition in N-PSS-treated samples was due to its effect on viral/bacterial entry into the target cells.

One test each of sperm function (sperm immobilization) and microbial growth (*Lactobacillus*) were chosen for safety assess-

ment. The effect of an agent on the percentage of mobile sperm in a sample can be used as an indirect measure of the cytotoxic effect of that agent on spermatozoa. Active ingredients (eg, N-9) in commercial vaginal contraceptives are spermicidal (ie, they kill spermatozoa). The spermicidal effect of N-9 and other detergents is caused by a nonspecific cytotoxic disruption of plasma membranes of spermatozoa; other membranes are also disrupted. This nonspecific property also gives N-9 its unwanted side effects, such as vaginal lesions. An agent may immobilize spermatozoa without killing them; however, it is not possible to kill spermatozoa without causing immobilization. In this context, failure of the test agent to reduce the fraction of mobile sperm in a sample (while at the same time showing contraceptive activity) is a desirable outcome.

A healthy vagina contains a normal complement of microbial flora, the most prevalent of which is *Lactobacillus* (Mardh, 1991). These beneficial bacteria help protect the vagina from pathogenic microbes. Agents with activity against STD-causing microbes that do not adversely affect *Lactobacillus* would likely cause less risk for secondary infections. Failure of an active antimicrobial agent to inhibit growth of *Lactobacillus* is a favorable outcome. *Lactobacillus* growth was estimated spectrophotometrically on the basis of the light-scattering property of particulate suspensions.

Materials

Sodium poly(4-styrenesulfonate), lot 78-7063 (N-PSS; code EE-7731) was kindly provided by National Starch and Chemical Company (Bridgewater, NJ). This product is characterized as a stable polymer with a molecular weight of 500 000 daltons. In its dry form, it is free-flowing, tan in color, and contains 12.56% sulfur by weight. N-PSS is highly soluble in aqueous solutions (>200 mg/mL). A 1% aqueous solution of N-PSS has a pH of 5.53.

Sheep testicular hyaluronidase (Type III), hyaluronic acid from bovine vitreous humor, N-acetylglucosamine, p-dimethylaminobenzaldehyde, Nα-benzoyl L-arginine ethyl ester, Bismarck Brown and Rose Bengal stains, pregnant mares' serum, and human chorionic gonadotropin (hCG) were products of Sigma Chemical Company (St Louis, Mo). *Lactobacillus* MRS broth and Gas Pak anaerobic pouches were purchased from Fisher Scientific (Itasca, Ill). The calcium ionophore, A23187 (free acid), was obtained from Calbiochem (San Diego, Calif). N-9 was obtained from Ortho Pharmaceutical Corporation (North Brunswick, NJ), and a suspension of penicillin (10 000 U/mL) and streptomycin (10 mg/mL) was from Gibco BRL (Grand Island, NY). *L. gasseri* (vaginal isolate) was obtained from the American Type Culture Collection (ATTC; Rockville, Md).

Sperm Function Testing

Inhibition of Hyaluronidase Activity—Hyaluronidases from testes and spermatozoa are immunologically and enzymically identical. Furthermore, the enzymic properties of spermatozoal hyaluronidase from different species are indistinguishable (Zaneveld et al, 1973), and the inhibitor sensitivities of testicular and spermatozoal hyaluronidase are very similar (Joyce et al, 1986). Therefore, results obtained with bovine testicular hyaluronidase are applicable to the spermatozoal enzyme.

Hyaluronidase (EC 3.2.1.35) activity was quantified with hyaluronic acid as a substrate. This was measured by determining the concentration of N-acetylglucosamine-reactive material that results from enzyme action.

Reaction mixtures contained the following, in a total volume of 0.25 mL: sodium acetate (0.1 M) containing 0.15 M NaCl, pH 5.5 (acetate buffer), hyaluronidase (7.2 U) from a stock solution dissolved in the acetate buffer, and hyaluronic acid (0.3 mg/mL). The enzyme was preincubated with test agent for 10 minutes before starting the reaction by adding hyaluronic acid. Enzyme reactions were determined by the method of Aronson and Davidson (1967). Incubations were carried out for 30 minutes at ambient temperature. The reaction product was determined colorimetrically as previously described (Reissig et al, 1955). The absorbency of the resultant adduct was immediately determined at 545 nm.

Differences were determined between absorbencies for reactions that were run in the presence and absence of hyaluronidase (blank, in which hyaluronidase was added after ending the reaction). A dose-response curve was generated at several concentrations (5–7) of N-PSS.

IC_{50} values (ie, concentrations of the inhibitor that produce 50% inhibition under the conditions of the assay) were determined with curve-fitting software (TableCurve 2D, version 3.02; Jandel Scientific, now SPSS, Inc, Chicago, Ill) by analyzing enzyme activity as a function of inhibitor concentration, as described previously (Anderson et al, 1994). Reversibility of hyaluronidase inhibition was determined by the method of Ackermann and Potter (1949), in which the level of inhibited enzyme activity was determined in the presence of different amounts of the enzyme.

Inhibition of Human Acrosin Activity—Human acrosin (EC 3.4.21.10) activity was measured spectrophotometrically (253 nm); the hydrolysis of the ethyl ester of Nα-benzoyl-L-arginine (BAEE) was quantified. Enzyme isolation, partial purification, and measurement were carried out by methods described in detail by Anderson et al (1981).

Enzyme reaction mixtures contained the following: sodium phosphate (50 mM) pH 7.5 (Anderson et al, 1985), enzyme-containing protein (3–5 mIU in which 1 mIU hydrolyzes 1 nmol BAEE per min), BAEE (0.05 mM), and N-PSS (from zero to 0.5 mg/mL). The total incubation volume was 1 mL. The enzyme was preincubated with N-PSS for 5 minutes before starting the reaction by adding BAEE. Reaction blanks, in which either substrate or enzyme had been eliminated, were run in parallel to the reactions. The hydrolysis of 1 μmol of BAEE (in a total volume of 1 mL) corresponds to a change in absorbency of 1.15 (Anderson et al, 1981).

Enzymic activity in the presence of each agent was compared with that for the control reaction (no test agent added). A dose-response curve was generated at several concentrations (5–7) of N-PSS that resulted in enzyme inhibition that ranged from 20% to 85%. IC_{50} values and reversibility of acrosin inhibition was determined as described for hyaluronidase.

Stimulation of Human Acrosomal Loss—In this context, acrosomal loss refers to the disruption of the sperm acrosome. This could be in response to a treatment or to a chemical entity. No inference is made whether or not this response is identical to the

physiological acrosome reaction during which the acrosome is also lost (Kopf and Wilde, 1990; Zaneveld et al, 1993).

Within 90 minutes of collection, semen was layered over buffered 11% Ficoll, and spermatozoa were sedimented by centrifugation. Spermatozoa were resuspended (5×10^6 cells/mL) in Biggers, Whitten Whittingham (BWW) medium (Biggers et al, 1971), less bovine serum albumin. The sperm suspension was divided into 0.5-mL portions. A small portion (approximately 10 μL) was reserved for sperm mobility assessment by light microscopy (see *Human sperm immobilization*, which follows). After 5 minutes of equilibration at 37°C, either N-PSS at different concentrations, or 1 nM calcium ionophore, A23187 (Anderson et al, 1992), was added. The ionophore (10 mM) was dissolved in dimethyl sulfoxide. This was diluted to 0.1 μM in BWW medium before it was added to sperm suspensions. Fifteen minutes after these additions, 10 μL of each suspension was removed to assess viability as measured by sperm movement. The remaining spermatozoa were fixed with buffered glutaraldehyde (Anderson et al, 1992) and stained for acrosome visualization with Bismarck Brown and Rose Bengal (De Jonge et al, 1989b).

Human Sperm Immobilization—The method was modified from that described by Sander and Cramer (1941). Sperm immobilizing activity of N-PSS was compared with that of a control preparation of N-9.

For screening the sperm immobilizing activity of N-PSS, a solution of 50 mg/mL was prepared in 0.9% NaCl. This solution was adjusted to pH 7.0 with HCl and was further diluted in 0.9% NaCl to concentrations of 25, 10, 5, and 2.5 mg/mL. N-9 was dissolved in 0.9% NaCl at concentrations from 50 to 150 μg/mL.

The agents were tested by mixing 250 μL of their working stock solutions with 50 μL of freshly ejaculated semen. Just before and 30 seconds after mixing at ambient temperature, the percentage of mobile spermatozoa was determined by microscopic observation (400×) of the suspensions on a standard microscope slide. All data were collected by a single observer. A spermatozoon that showed movement, whether or not it exhibited forward progression, was considered mobile. Semen samples that showed less than 50% immobilization in response to 100 μg/mL N-9 were not included in data analysis. A dose-response curve was constructed from these data.

Contraception in the Rabbit—The effect of N-PSS on the ability of pretreated spermatozoa to fertilize rabbit oocytes in vivo was evaluated essentially as described by Joyce et al (1979). Female rabbits (age 8–12 months, 4.4 kg) were injected IM with 200 IU of pregnant mares' serum. After 96 hours, 200 IU of hCG was administered via the ear vein. Semen was collected from New Zealand white rabbits (age 8–12 months, 4 kg) with the aid of an artificial vagina and a ''teaser'' doe. Spermatozoa were washed in modified Tyrode's albumin lactate pyruvate (TALP) medium (Kovacs and Foote, 1992). The medium was modified to exclude albumin. The suspension (adjusted to a sperm concentration of 25×10^6/mL) was divided into 2 equal portions, 1 of which contained only modified TALP, and served as control. N-PSS was added to the second portion 15 minutes before its use in artificial insemination.

Immediately after treatment with hCG, the female rabbits were artificially inseminated with 0.5 mL of the treated or the control sperm suspension. Approximately 28–34 hours after being inseminated, rabbits were killed with Sleepaway (Fort Dodge Laboratories, Fort Dodge, Iowa), the uteri were sectioned about 2 cm distal to the oviductal/uterine junction, and the oviducts and ovaries were excised. Ovaries were dissected from the oviducts, and the oviducts were flushed from the uterine end with 0.9% saline. The perfusate was collected into a culture dish. Oocytes recovered in the perfusate were microscopically examined for cleavage (fertilization). Data were reported as the percentage of recovered oocytes fertilized for each rabbit. Data were subjected to arcsine transformation before further analysis; the number of rabbits, rather than the number of oocytes recovered, was used as the sample size.

Antimicrobial Testing

Inhibition of HIV Infectivity—HIV-1 infectivity in the presence of N-PSS was evaluated with a viral binding inhibition assay. Serial dilutions of test compound (from 0.1 μg/mL to 1 mg/mL) were added to MT2 cells (Resnick et al, 1990). These cells were seeded in 96-well cell culture plates at 10 000 cells per well in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, 100 μg/mL streptomycin, 20 μg/mL gentimicin, and 25 mM Hepes. HIV-1 (strain III_B; NIAID, Bethesda, Md) was propagated in a T4-lymphoblastoid cell line, harvested, and diluted, so that an inoculum (50 μL) produced 70–100 syncytia per well in untreated MT2 cell cultures. The virus was added to the preseeded wells containing the test compound, or only the control medium.

Each serial dilution of compound with the virus was added to each of 3 wells containing the MT2 cells. A fourth well contained only N-PSS and MT2 cells, and served as a screen for possible cytotoxicity. Virus control samples (no compound added) were also included, in triplicate. The virus control wells received an equal volume of RPMI medium, which was used to dissolve the test compound. The virus/cell cocultures were incubated at 37°C (5% CO_2) for 48–72 hours. At this time, cultures were microscopically scored for syncytia formation.

Data are expressed as percent of control value (number of syncytia induced in wells with no compound added). N-PSS concentration was plotted against the percent of control viral infectivity to calculate the concentration of N-PSS that causes 50% inhibition of viral replication (IC_{50}). Analyses were performed with curve-fitting software (TableCurve, Jandel Scientific).

Inhibition of C. trachomatis *Infectivity*—Inhibition of *Chlamydia* infectivity was measured as described by our laboratories (Cooper et al, 1990; Herold et al, 1997). Before experimentation, cryopreserved *C. trachomatis* (serotype E/UW-5/CX) were quickly thawed (37°C) and suspended by mild sonication. Serial 1:10 dilutions of the bacterial suspension were made, ranging from 10^{-1} to 10^{-7}.

HeLa monolayers (on coverslips) were inoculated with 100 μL of the different dilutions of the *Chlamydia* suspension (elementary bodies), in the presence or absence of N-PSS. The compound was tested at 0.1, 1, 10, 100, and 500 μg/mL. After 1 hour, the monolayers were washed to remove N-PSS and *Chlamydia* that remained in solution. They were incubated for 48 hours.

Medium was removed, and the HeLa monolayers were fixed

in methanol, washed, and treated with Kallsted Chlamydia Culture Confirmation fluorescein-conjugated monoclonal antibody. Reaction of the labeled antibody with the cells was carried out for 30 minutes at room temperature (no light) in a humidified chamber. The cells were washed with water; a drop of mounting medium was placed on a glass slide, and the coverslip was applied.

Inclusions due to chlamydial infection were visualized with a fluorescent microscope as green fluorescence. Data were reported as the number of inclusion forming units (IFU) per mL of undiluted chlamydial titer, and were adjusted to control values of 2.6×10^7 IFU/mL for control cultures (no test agent) in each experiment. After logarithmic transformation of all data, dose-response curves were created with TableCurve curve-fitting software.

Evaluation of HSV infectivity and the growth of *N. gonorrhoeae* and *L. gasseri* in the presence of N-PSS was carried out as described by Anderson et al (1998). Methods for these tests are summarized here.

Inhibition of HSV Infectivity—A sensitive plaque reduction assay was used to detect the effects of N-PSS on infection of primate cells by HSV type 1 (HSV-1). These cells (vero) were derived from African green monkey kidney cells and obtained from ATCC. HSV-1, strain KOS [HSV-1(KOS)] was used. Two-mL samples of a virus suspension that contained approximately 1400 plaque-forming units (PFU) per mL were prepared in phosphate-buffered saline (PBS). They were mixed with equal volumes of serial dilutions of N-PSS. This yielded 4 mL of mixture that contained the virus at half the concentration of the original suspension. The concentration of N-PSS ranged from 0 to 500 μg/mL. One-mL samples of each mixture were immediately plated in triplicate on 25 cm^2 monolayers of vero cells. Inoculated cells were incubated with rocking for 2 hours. The inoculum was removed and replaced with medium 199 that contained 1% fetal bovine serum and 0.1% pooled human gamma-globulin (Armour, Kankakee, Ill). Gamma globulin is a source of anti-HSV antibodies that inhibits the formation of secondary plaques but allows the formation of primary plaques. After 2–3 days of incubation, the cells were stained with Giemsa for visualization and counting of plaques. A dose-response was evaluated with TableCurve software. PFU per mL were plotted as a function of N-PSS concentration.

N-PSS was also tested against clinical isolates of HSV-2. The method of analysis was similar to that described for HSV-1 except that CaSki cells, a human cervical epithelial cell line obtained from ATTC, were used as the target cells. Stock virus (10^9 PFU/mL) from each isolate was serially diluted to a titer of 10^5 PFU/mL; this was tested against N-PSS at 10-fold serial dilutions ranging from 0.01 to 100 μg/mL. Clinical isolates were designated as BBKC, MMA (Terhune et al, 1998), DT2 (Oram et al, 2000), and H1. The H1 isolate was obtained from a genital lesion from a man with HIV with persistent, extensive mucosal disease (unpublished).

Inhibition of N. gonorrhoeae—Strain MS11 was isolated from an uncomplicated case of gonorrhea. The identity was verified by gram reaction, oxidase reactivity, and sugar fermentation. The inoculum was prepared, and overnight cultures were grown on gonococci (GC) agar. Bacteria were resuspended to a density equal to a 0.5 MacFarland standard (approximately 10^8 colony forming units [CFU] per mL). This suspension was diluted 1:100 with GC broth to a concentration of 10^6 CFU/mL. This concentration of gonococci without compounds served as a control. Different concentrations of N-PSS (0.1 μg to 1 mg/mL) were added directly to the GC agar, and the agar was poured into Petri dishes. The agar plates were inoculated with different concentrations of gonococci. Five serial 1:10 dilutions were made in GC broth and 20-μL aliquots of the dilutions were inoculated onto compound-containing agar plates. The plates were incubated overnight, and the colonies were enumerated following overnight incubation in 5% CO_2 at 37°C.

Data for each concentration of compound were expressed as the number of CFU per mL of the original bacterial suspension. A dose-response was estimated with TableCurve software. Bacterial titer (CFU/mL) was plotted as a function of N-PSS concentration.

Inhibition of Lactobacillus.—*L. gasseri* was cultured under anaerobic conditions at 37°C as suggested by ATCC. Bacteria were grown in *Lactobacillus* MRS broth contained in 33-mm culture dishes. The dishes were placed into anaerobic Gas Pak pouches. An appropriate amount of the bacterial suspension was added to 10 mL of nitrogen-purged medium, contained in a sterile Erlenmeyer flask (25 mL) so that the absorbency of the suspension at 550 nm was approximately 0.025. The flasks were topped with sterilized rubber serum stoppers. Experimental flasks contained N-PSS at a concentration of 5 mg/mL. A control flask that contained no N-PSS was also included.

Culture growth was determined turbidometrically. Measurements were taken beginning at 120 minutes after the start of incubation and at 20-minute intervals for a total length of 260 minutes. Samples (1 mL) were removed from the incubation flasks and the absorbency of each suspension at 550 nm was determined as an estimate of cell density. Data were fit to the equation:

$$\text{Ln(absorbency)} = a + b(\text{time})$$

where a is the absorbency at zero time, b is the slope of the curve, and time is measured in minutes. The doubling time (T_D) was calculated from the equation:

$$T_D = (\text{Ln } 2)/b.$$

Values are given as the doubling time of bacterial growth with 90% confidence limits. Inhibition of bacterial growth by N-PSS was directly calculated from the reciprocal values of the doubling time (ie, 75% inhibition of the reciprocal of the calculated doubling time is equal to 75% inhibition of bacterial growth).

Statistical Analysis

Hyaluronidase and acrosin activities are expressed as averages, with the standard errors of the mean. Data were best fit to curves generated with TableCurve software, from which were calculated IC_{50} values of enzyme inhibition and coefficients of determination of the fitted curves.

Percentage data collected for the acrosomal loss, rabbit contraception, and sperm immobilization tests were subjected to arcsine transformation (Sokal and Rohlf, 1981) before further analysis, and data were back-transformed for presentation. They are

presented as either average percent of maximal acrosomal loss, percent of fertilization, or percent of mobile sperm, with 90% confidence limits. Differences in acrosomal loss and rabbit conception in the presence and absence of N-PSS were compared with the Student's *t* test on the transformed data.

Data on the antimicrobial activity of N-PSS against HSV, *N. gonorrhoeae*, and *C. trachomatis* were subjected to logarithmic transformation (Sokal and Rohlf, 1981) before further analysis. Values are presented as the back-transformations of average titers with 90% confidence limits. Differences in *C. trachomatis* titers among treatment groups were evaluated with the Newmann-Keuls multiple range test (Woolf, 1968). IC_{50} values and concentrations of N-PSS yielding 3-log reductions in infectivity (99.9% inhibition of microbial titer) were calculated from curves that were fit to the data with TableCurve software. *Lactobacillus* growth was expressed as a change in absorbency at 550 nm over time. This was measured in the presence and absence of N-PSS. Slopes of growth curves generated from the data were compared, from which doubling times and their 90% confidence limits were calculated. If the 90% confidence limits of the slopes overlapped, the slopes were considered to be not different.

All differences were considered significant at the 0.05 level of confidence. Values were considered to be not different at the 0.1 level of confidence.

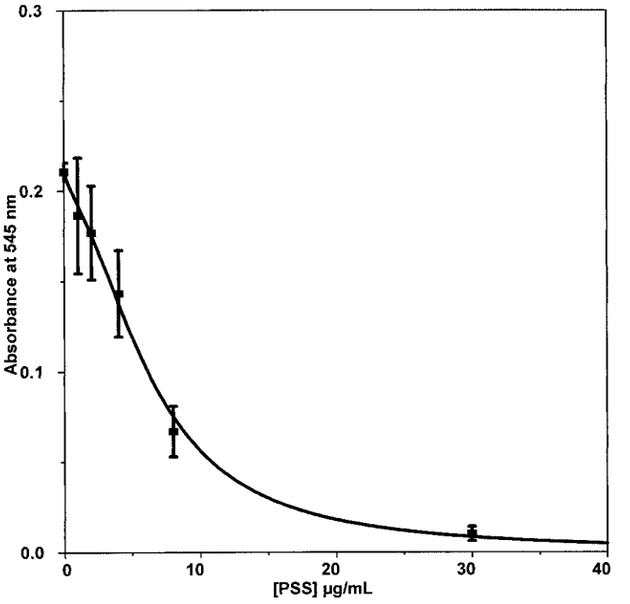

Figure 1. Hyaluronidase activity in the presence of N-PSS was measured spectrophotometrically with hyaluronic acid as substrate. Data are presented as average product formation, expressed as the absorbency at 545 nm. Error bars represent standard errors of the mean of quadruplicate samples at each concentration of N-PSS, measured over 2 independent experiments. The curve that runs through the data is described by the equation: $Y^{-1} = A + BX^2 - C^{-x}$, where A = 5.2631, B = 0.12558, and C = 0.50768. Its coefficient of determination (r^2) is 0.995. This curve was used to calculate the IC_{50} (5.7 µg/mL) and 3-log reduction (195 µg/mL) values for hyaluronidase inhibition by N-PSS.

Results

Sperm Function Inhibition

N-PSS is an effective inhibitor of the sperm-related enzymes, hyaluronidase and acrosin. The IC_{50} for hyaluronidase inhibition is 5.7 µg/mL, with nearly complete inhibition (3-log reduction) at 195 µg/mL (Figure 1). Apparent reversibility of enzymic inhibition can be evaluated by an Ackermann-Potter plot of hyaluronidase inhibition vs the amount of the added enzyme. This plot suggests irreversible hyaluronidase inhibition by N-PSS (Figure 2). The Y-intercept for the control activity is 0.0024 (95% confidence limits = −0.021 to 0.0258), whereas the intercept for the reaction in the presence of 5 µg/mL N-PSS is −0.0803 (95% confidence limits = −0.1281 to −0.0325). Acrosin inhibition shows a sharper dose-response than hyaluronidase inhibition at low concentrations of N-PSS. The IC_{50} for acrosin inhibition is approximately 1 order of magnitude lower than for hyaluronidase inhibition (0.5 µg/mL). However, acrosin inhibition levels off at higher N-PSS concentrations, with only approximately 80% inhibition seen at 500 µg/mL (Figure 3). As with hyaluronidase inhibition, acrosin inhibition by N-PSS seems irreversible (graph not shown). The Y-intercept for the control reaction is −0.0052 (95% confidence limits = −0.0228 to 0.0124) and that for the N-PSS-inhibited (0.4 µg/mL) reaction is −0.0952 (95% confidence limits = −0.1519 to −0.0385).

N-PSS is a potent stimulus of human sperm acrosomal loss. When compared with the response to a maximally stimulating concentration of the calcium ionophore, A23187 (Anderson et al, 1992), one-half maximal acrosomal loss occurs in the presence of 0.17 µg/mL N-PSS; near maximal acrosomal loss is produced by 2 µg/mL N-PSS (Figure 4). The fraction of mobile spermatozoa was unaffected by N-PSS or A23187 in this assay. Averages for samples that contained no additions (control), ionophore, and N-PSS (2 µg/mL) were 65%, 65%, and 62%, respectively.

In contrast, N-PSS does not immobilize human spermatozoa. Therefore, it produces no overt cytotoxic effect on these cells. N-PSS immobilizes sperm by less than 20% at a concentration of 50 mg/mL (Figure 5). Sperm mobility does not differ among experimental groups at N-PSS concentrations from zero to 50 mg/mL ($F_6^5 = 3.026$, $P > .1$). These data are in contrast to the sperm immobilization by N-9. All samples tested were inhibited by at least 80% by 100 µg/mL of N-9. The IC_{50} for N-9 is 88 µg/mL, with a 3-log reduction in mobility seen at 238 µg/mL (data not shown).

Consistent with its action on sperm function, N-PSS inhibits conception in the rabbit by 99% ($P < .01$, *t* test) when mixed with spermatozoa (final concentration = 0.5 mg/mL) before artificial insemination (Table 1). Out of 112 oocytes recovered from rabbits inseminated with

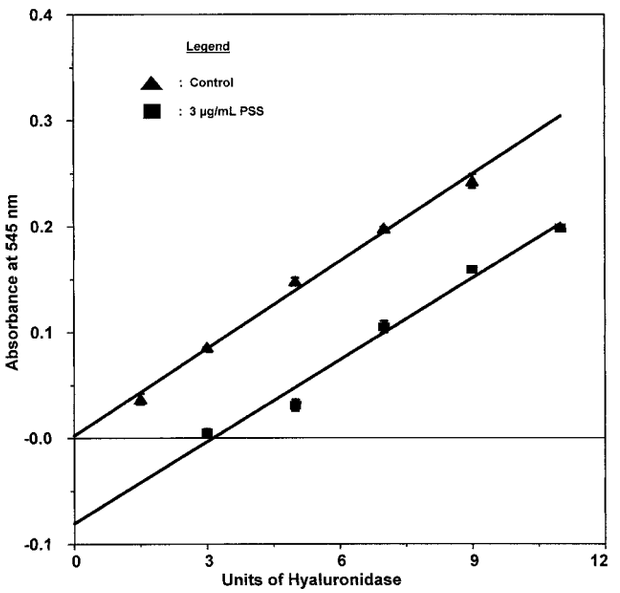

Figure 2. Reversibility of hyaluronidase inhibition by N-PSS was determined by the method of Ackermann and Potter (1949). Values are expressed as average product formation, as measured by the absorbency at 545 nm. Error bars are the standard errors of the mean of duplicate determinations at each concentration of enzyme added to the reaction mixture. Error bars not visible indicate a standard error less than the physical dimension of the symbol. Linear curves were fit to the data obtained in the presence (■) and absence (▲) of 3 μg/mL N-PSS and are described by the equations: $Y = -0.0803 + 0.0257X$ and $Y = 0.0024 + 0.02743X$, respectively. Coefficients of determination for the control and inhibited reactions were 0.994 and 0.982. Curves for reversible inhibition should pass through the origin. The Y-intercept for irreversible inhibitors should be less than zero. In this instance, the X-intercept reflects the amount of enzyme that has been removed from the reaction by the inhibitor.

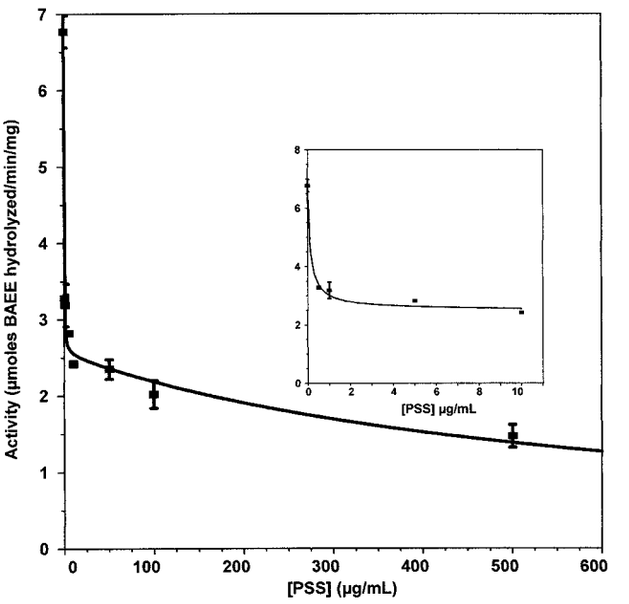

Figure 3. Human acrosin inhibition by PSS was measured spectrophotometrically with benzoyl-L-arginine ethyl ester as substrate. Values are expressed as average enzyme activity (expressed as nmol substrate hydrolyzed per min per mg enzyme protein) of 4 to 5 separate determinations. Error bars represent standard errors of the mean. The curve that passes through the data is described by the equation: $Y = A/(1 + ABX) + C/(1 + CDX)$, where $A = 4.2202$, $B = 1.8598$, $C = 2.5471$, and $D = 6.62 \times 10^{-4}$. The coefficient of determination (r^2) for this curve is 0.999. This curve was used to calculate the IC_{50} (0.5 μg/mL) for acrosin inhibition by N-PSS. Inset: Detailed view of acrosin activity in the presence of N-PSS at concentrations up to and including 10 μg/mL.

spermatozoa treated with N-PSS before insemination, only 1 oocyte was fertilized.

Antimicrobial Activity

HIV-1 and HSV-1, both of which are enveloped viruses, are inhibited by N-PSS (Figures 6 and 7). The IC_{50} for HIV inhibition by N-PSS is 7 μg/mL; N-PSS produces a 3-log reduction in viral titer at 15 μg/mL. N-PSS was more active against HSV-1. Its IC_{50} is less than 0.1 μg/mL, and the calculated 3-log reduction concentration is approximately 3 μg/mL. Similar results were obtained with several clinical isolates of HSV-2 (Table 2). For the isolates, IC_{50} values were approximately 0.1 μg/mL, with 3-log reduction concentrations less than 10 μg/mL in all instances. The polymer produces no overt toxic effects on either of the host cells (MT2 cells, vero cells, and CaSki cells, respectively) used in the antiviral assays. The highest concentrations tested were 100 μg/mL for HIV, and 500 μg/mL for HSV.

Antibacterial activities of N-PSS include inhibition of *C. trachomatis* and *N. gonorrhoeae. Chlamydia* infectivity is inhibited by approximately 75% in the presence of 100 μg/mL. Inhibition increases to more than 90% when the concentration of N-PSS is increased to 1 mg/mL (Figure 8). This concentration of N-PSS does not affect the HeLa host cells of this assay. N-PSS is more active as an inhibitor of *N. gonorrhoeae* growth, with an IC_{50} of 6 μg/mL. N-PSS produces a 3-log reduction in growth at 15 μg/mL; complete inhibition occurs at 100 μg/mL (Figure 9).

However, N-PSS does not affect the growth of *Lactobacillus*, a normal component of the vaginal flora (Figure 10). The slight (10%) difference in the calculated doubling time of *Lactobacillus* growth in the presence of N-PSS (5 mg/mL) as compared to the doubling time of the control culture (111 minutes) is not significant ($P = .1$). This lack of effect contrasts with the inhibitory effect of N-9. The IC_{50} of N-9 against *L. gasseri* is 0.4 mg/mL (data not shown).

Discussion

The present report shows that N-PSS has both contraceptive and antimicrobial properties. This high molecular weight, commercially available preparation is highly effective as a contraceptive agent in the rabbit. Furthermore, it is effective in inhibiting the infectivity or growth (or both) of several STD-causing microbes, including HIV, HSV, *Chlamydia*, and gonococci, without having an effect on beneficial vaginal microbes such as lactobacilli.

Previous data (Homm et al, 1985) had already shown PSS to be an effective contraceptive. Subsequent studies

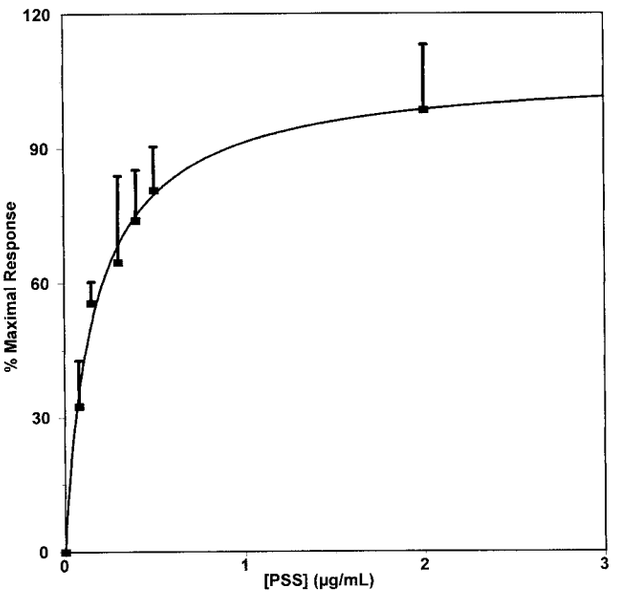

Figure 4. Induction of acrosomal loss in human spermatozoa was evaluated by brightfield microscopy after acrosomal staining. Data were subjected to arcsine transformation prior to analysis. Values are expressed as the average percentage of maximal loss produced by the addition of the calcium ionophore, A23187. The proportion of total spermatozoa that lacked acrosomes after addition of ionophore was 30% (90% confidence limits = 27.8% to 31.6%; n = 4). Acrosomal loss among the control samples was 8.7% (8.2%–9.2%). Error bars represent the standard errors of 4 replicate determinations at each concentration of N-PSS. The curve that passes through the data is described by the equation: $Y = A + B/(1 + (X/C)^D)$, where A = 0.19679, B = 107.56, C = 0.16902, and D = 0.95994. Its coefficient of determination (r^2) = 0.993.

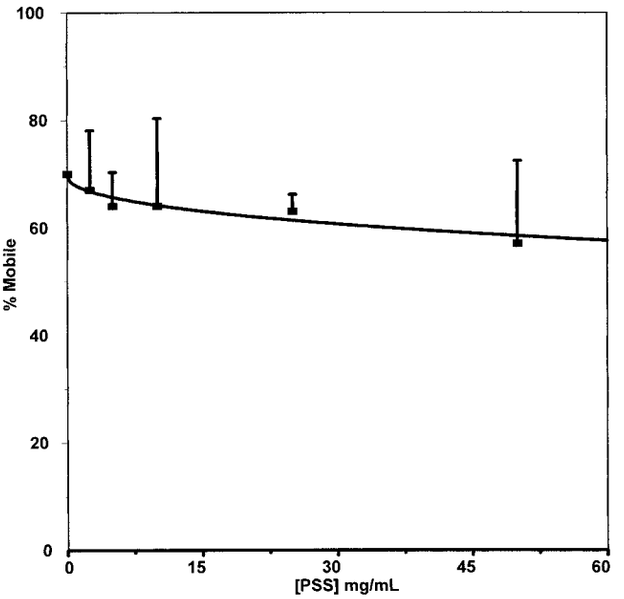

Figure 5. Sperm immobilization was measured in the presence of several concentrations of N-PSS by a modification of the method of Sander and Cramer (1941). Data were subjected to arcsine transformation prior to analysis. The value at each concentration of N-PSS is the average and upper 90% confidence limits of duplicate determinations performed with each of 2 semen samples. Analysis of variance shows no difference in mobility among different concentrations of N-PSS up to 50 mg/mL ($F_6^5 = 3.026$; $P > .1$). The curve that passes through the data is described by the equation: $Y^{-1} = A + BX^{0.5}$, where A = 0.014356 and B = 3.8853×10^{-4}.

by the same investigators showed that PSS may be acting, at least in part, by its ability to inhibit acrosin (Foldesy et al, 1986; Hahn and McGuire, 1986). These observations were confirmed and extended in the present report. Our data regarding the IC_{50} for acrosin inhibition (Figure 3) and the decreased dose-response as N-PSS concentrations increased agree well with these previous studies (Foldesy et al, 1986). Irreversible acrosin inhibition (*Results*) by this polymer has not been previously reported.

Quantitative data were not already available on the effect of PSS on hyaluronidase; however, early work on hyaluronidase inhibitors suggested that N-PSS may be active on the basis of its polyanionic character (Spensley and Rogers, 1954; Bernfeld et al, 1961). As with acrosin inhibitors, hyaluronidase inhibitors are contraceptive (Pincus et al, 1948; Sieve, 1952; Parkes, 1953; Doring, 1954; Joyce and Zaneveld, 1985; Anderson et al, 1998). N-PSS is a highly effective irreversible hyaluronidase inhibitor (Figures 1 and 2).

The failure of N-PSS to immobilize sperm (Figure 5) agrees well with earlier reports with similar material (Homm et al, 1985). This finding shows that N-PSS does not exert an overt cytotoxic effect on spermatozoa. It is acting distinctly from currently marketed topical contraceptive formulations that use N-9 and other spermicidal surfactants as the active ingredient.

Unexpectedly, however, N-PSS acts as a highly effective stimulus of human acrosomal loss (Figure 4). Agents that inhibit the stimulus-induced acrosome reaction are contraceptive (Rogers et al, 1977; Beyler and Zaneveld, 1982; Dravland et al, 1984; Kaminski et al, 1985; Joyce et al, 1987; De Jonge et al, 1989a). The timing and response of an acrosome to appropriate stimuli in proximity to an oocyte are critical in the ability of a spermatozoon

Table 1. *Inhibition of in vivo fertilization in the rabbit after pretreatment of spermatozoa with Polystyrene sulfonate**

Treatment	Control	0.5 mg/mL N-PSS
Rabbits treated, n	4	3
Oocytes recovered	115	112
Fertilization, %	93	1†
(90% confidence limits)	(83.6–98.8)	(3.2–12.9)

* Fertilization rates were determined in rabbits after artificial insemination with spermatozoa to which was added either phosphate-buffered saline (control) or 0.5 mg/mL N-PSS (experimental). Additional details are provided in ''Materials and Methods.'' Values are expressed as the percentage of recovered oocytes that had been fertilized as indicated by the first cell division. Confidence limits are based on the number of rabbits used in each group. Only 1 oocyte in the treated group showed signs of fertilization.

† Value differs from control ($P < .001$, *t* test).

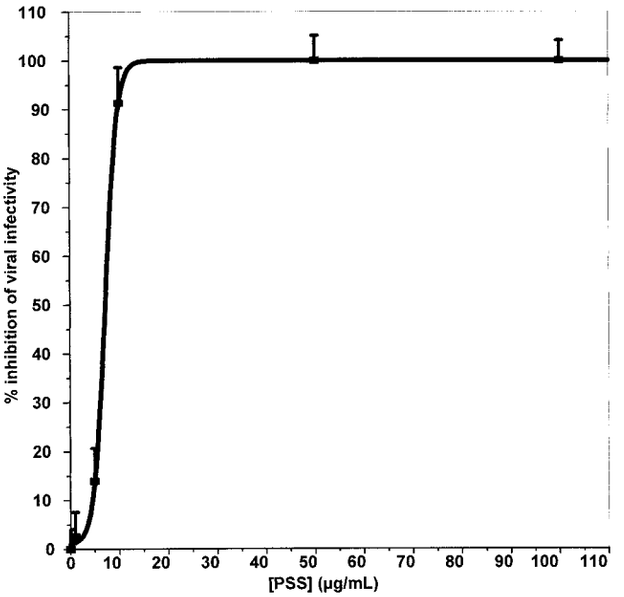

Figure 6. Inhibition of HIV infectivity by N-PSS was measured by quantifying HIV-induced syncytia in MT2 cells. Data were subjected to arcsine transformation prior to analysis. Values are expressed as the average percentage of control syncytia produced at each concentration of N-PSS from triplicate determinations. Error bars are the upper 90% confidence limits. The curve that passes through the data is described by the equation: $Y = A + B/(1 + e^{-(X-C)/D})$, where A = 0.89353, B = 99.118, C = 7.2299, and D = 1.1858. The coefficient of determination (r^2) for this curve is 0.999. This curve was used to calculate the IC_{50} (7 µg/mL) and 3-log reduction (15 µg/mL) values for HIV inhibition by N-PSS.

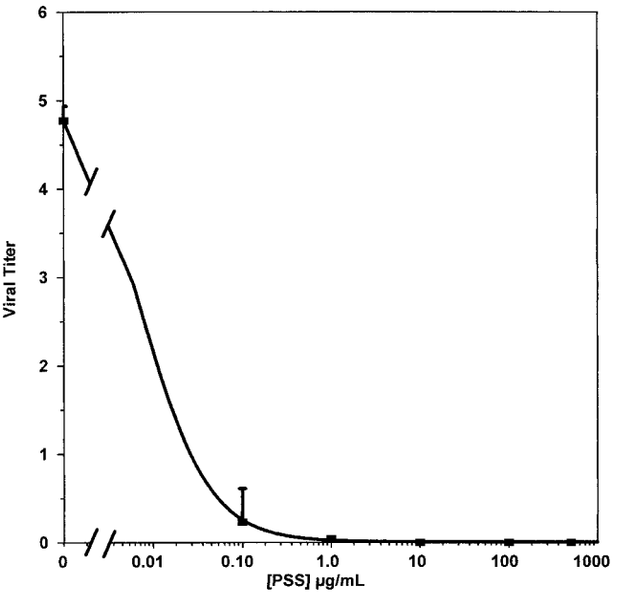

Figure 7. Inhibition of HSV infectivity by N-PSS was measured with a plaque reduction assay. Data were subjected to logarithmic transformation prior to analysis. Values represent the average of triplicate determinations (PFU/mL $\times 10^{-8}$) surviving incubation in control medium or with the concentrations of N-PSS indicated. Error bars are the upper 90% confidence limits. The curve that passes through the data is described by the equation: $Y = A + B/(1 + (X/C)^D)$, where A = 5.74×10^{-3}, B = 5.256, C = 7.31×10^{-3}, and D = 1.141. The coefficient of determination (r^2) = 0.999. This curve was used to calculate the IC_{50} and 3-log reduction values for HSV inhibition by N-PSS.

to fertilize an oocyte. Furthermore, spermatozoa that have lost their acrosomes are shorter lived and therefore less likely to fertilize than intact spermatozoa (Jones, 1990; Tarlatzia et al, 1993). An agent that induces premature acrosomal loss would therefore have contraceptive activity similar to that found in agents that inhibit the stimulus-induced acrosome reaction.

Available evidence suggests that proteinase and hyaluronidase activities are involved in the infectivity by 1 or more STD-causing microbes (Rosso, 1975; Romano and Moiseiev, 1982; Fitzgerald and Repesh, 1987; Shimizu et al, 1987; Arroyo and Alderete, 1989; Steiner and Cruce, 1992; Kato et al, 1994; Bourinbaiar and Lee-Huang, 1995). If so, inhibitors of these enzymes (eg, trypsin and hyaluronidase) should have a negative influence on the microbial infectivity. The present work supports this contention. Independent studies with polymers of different molecular weights and from different sources have shown that PSS can have anti-HIV (Mohan et al, 1992; Tan et al, 1993) and anti-HSV (Zeitlin et al, 1997) properties. The present study confirms this work and extends it by showing that a single preparation of this polymer has a broad spectrum of antimicrobial activity (Figures 6–9).

Among several sulfonated polymers, PSS was particularly active as an anti-HIV agent (Mohan et al, 1992; Tan et al, 1993). The molecular weights of the polymers used in those studies (8 to 70kd) were much less than that of the polymer used in the present study. Furthermore, their IC_{50} values for preventing viral infectivity differed from each other by more than an order of magnitude.

Table 2. *N-PSS inhibits clinical isolated of HSV-2**†

Isolate	IC50 (µg/mL)	3-Log reduction (µg/mL)	Percentage of inhibition at 0.1 µg/mL (90% Confidence Limits)
MMA	0.18	7.0	26 (25.2–26.8)
BBKC	0.09	1.4	55 (55.3–55.5)
DT2	0.08	4.5	55 (53.9–55.8)
H1	0.14	1.4	40 (34.6–46.6)

* N-PSS was tested against infectivity of several clinical isolates of HSV-2, as described in ''Materials and Methods.'' Inhibition constants (IC_{50} and concentration required for 3-log reduction of infectivity) were calculated from regression analysis of each dose-response curve. Dose-responses of N-PSS inhibition of each isolate were fit to the following equations. For isolate MMA, $\text{Titer} = a + b/(1 + ([\text{PSS}]/c)^d$, where $a = -0.012089$, $b = 4.9502$, $c = 0.17045$, and $d = 1.4961$ ($r^2 = 0.999$). For isolate BBKC, $\text{Titer}^{-1} = a + b[\text{PSS}]^{2.5} + c[\text{PSS}]^{0.5}$, where $a = 0.2$, $b = 85.238$, and $c = -0.06678$ ($r^2 = 0.999$). For isolate DT2, $\text{Titer}^{-1} = a + be^{[\text{PSS}]}$, where $a = -2.1128$ and $b = 2.3133$ ($r^2 = 0.998$). For isolate H1, $\text{Titer} = ae^{(-[\text{PSS}]/b)}$, where $a = 4.8889$ and $b = 0.16953$ ($r^2 = 0.992$). These equations were used to estimate the inhibition (with 90% confidence limits) produced by 0.1 µg/mL N-PSS.

† HSV-2 = herpes simplex type 2; PSS, polystyrene sulfonate.

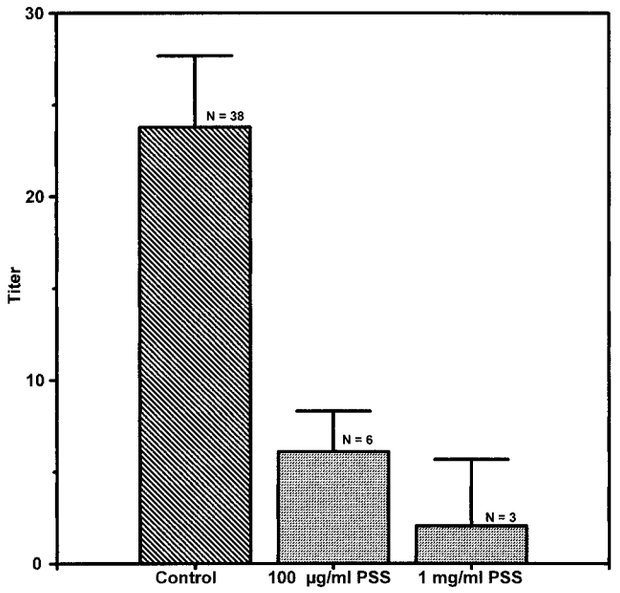

Figure 8. Chlamydial infectivity in the presence of N-PSS was measured by immunofluorescent microscopy. Data were subjected to logarithmic transformation prior to analysis. Values represent average of bacterial titer (IFU/mL $\times$ 10^{-6}) at each concentration of N-PSS. Numbers over each bar are the number of determinations of titer. Error bars are the upper 90% confidence limits. Differences among all means are significant ($P < .005$, Newman-Keuls multiple range test).

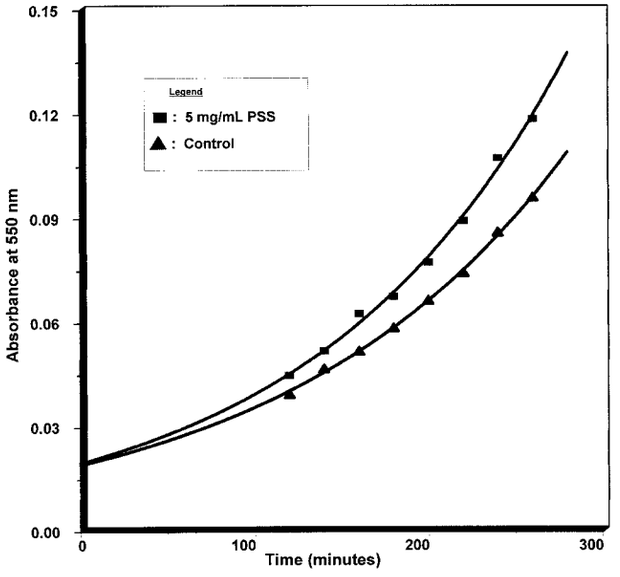

Figure 10. *L. gasseri* growth in suspension culture was measured spectrophotometrically. Values are expressed as average absorbency at 550 nm. Error bars are standard errors of the mean of duplicate determinations at each time point. The curves passing through the control (▲) and 5 mg/mL N-PSS-treated (■) cultures are described by the equation: Ln Y = A + BX, where A = -3.9483 and -3.4023, and B = 6.194×10^{-3} and 5.495×10^{-3} for the control and N-PSS data, respectively. Coefficients of determination equal 0.998 and 0.994 for the control and N-PSS curves. Doubling times were calculated from the equation: TD = Ln 2/B, where B is the slope of the curve. These values for the control (112 minutes) and N-PSS-treated (126 minutes) cultures were not different ($P > .1$, *t* test)

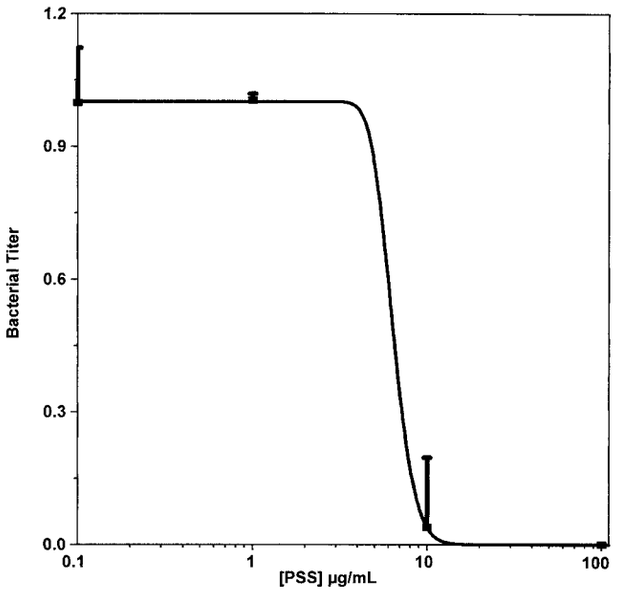

Figure 9. *N. gonorrhoeae* was grown on agar plates in the presence or absence of N-PSS. Data were subjected to logarithmic transformation prior to analysis. Values represent averages of 3 to 4 determinations of bacterial titer (CFU/mL $\times$ 10^{-8}). Error bars represent upper 90% confidence limits. The curve that passes through the data is described by the equation: Y = A + B*exp($-e^{-(X-D\ln(\ln 2)-C)/d}$), where A = 1.0014, B = -1.0014, C = 6.3213, and D = 1.287. The coefficient of determination (r^2) = 0.999. This curve was used to calculate the IC_{50} (6 µg/mL) and 3-log reduction (15 µg/mL) values for gonococcal inhibition by N-PSS.

Values reported by Mohan et al (1992) are very similar to our values (Figure 6). HIV inhibition by another preparation of PSS has recently been extended to other forms of the virus. A broad spectrum of activity was seen against laboratory strains (including monocytotropic ADA and BaL) and clinical isolates, with IC_{50} values ranging from 0.3 to 7.9 µg/mL (unpublished).

Our work shows a lower IC_{50} for HSV inhibition by N-PSS than other work published while our study was ongoing (Zeitlin et al, 1997). However, at higher concentrations, the difference in sensitivity is not apparent. Zeitlin et al reported an IC_{100}, which is the same as our calculated concentration that produces a 3-log reduction in infectivity (3 µg/mL; Figure 7).

N-PSS produced similar inhibition of clinical isolates of HSV-2 (Table 2). These data may be somewhat more clinically relevant because HSV-2 is primarily responsible for cases of genital herpes infections (Nahmias et al, 1980). Efficacy was essentially the same when N-PSS was tested against several isolates, suggesting that its activity is directed toward a basic mechanism of infectivity of this virus. Activity among several types of HSV and HIV (see earlier discussion) supports the use of laboratory

strains for the screening of N-PSS and related products as antiviral agents.

To our knowledge, no other work has reported the inhibition of either *Chlamydia* infectivity or gonococcal growth by PSS. Our finding of inhibited chlamydial infectivity is consistent with chlamydial inhibition by sulfated polyanions, such as pentosan polysulfate, dextran sulfate, and glycosaminoglycans (Zaretzky et al, 1995).

The present study did not address the mechanism by which N-PSS exerts its broad spectrum of antimicrobial and contraceptive activities; however, available evidence suggests that this polymer may be acting as a receptor antagonist or mimetic during cell-cell fusion.

Different forms of PSS, with molecular weights different from that used in the present study, inhibit HIV infectivity at several levels of the infectious process (Mohan et al, 1992). The main effect, however, is believed to be inhibition of binding of viral gp120 to CD4+ target cells.

HSV infectivity depends on the interaction of viral ligands with glycosaminoglycan (heparan sulfate) receptors on the target cells (WuDunn and Spear, 1989; Shieh et al, 1992). Its inhibition by polyanions, such as heparin (Spear, 1993), suggests that N-PSS may bind to glycosaminoglycan receptors. The present data agree with our recent work reported for HSV-1 and HSV-2 inhibition by another preparation of PSS. This was further extended to the inhibition of in vivo infectivity by HSV in the mouse (Herold et al, 2000).

The multiple actions of N-PSS on sperm function suggest that more than a single mechanism may be (although not necessarily) involved in its contraceptive activity. PSS binds to the receptor for the sea urchin sperm ligand, bindin. Bindin specifically binds to sulfated fucan polysaccharides on the egg surface and mediates the attachment of a sperm to an egg during fertilization (DeAngelis and Glabe, 1990). Interaction of mammalian sperm ligands for glycoprotein zona receptors in the rabbit can be blocked by sulfated polymers. Mammalian sperm proacrosin (a zymogen precursor to acrosin) may be analogous to sea urchin bindin for its property as an egg receptor ligand (Jones, 1990). This may be of interest in view of the ability of N-PSS to inhibit acrosin (Figure 3). The stimulus-induced acrosome reaction is impaired by acrosin inhibitors (Meizel and Lui, 1976; De Jonge et al, 1989a). On the other hand, whereas N-PSS inhibits acrosin, it is also highly effective in inducing acrosomal loss (Figure 4). This finding suggests that the role of proacrosin in sperm-egg fusion may be more than that of an acrosin precursor (Jones, 1990).

Issues of safety regarding further development of PSS should not be problematic. This is based on the information presented in the present report and on the characteristics of this polymer. For example, N-PSS exerts its antimicrobial activity with no evidence of cytotoxicity to cells used in the assays for HIV, HSV, and *Chlamydia.* The polymer shows no cytotoxicity toward spermatozoa (Figure 5) at concentrations up to 100 times that required to produce nearly complete contraception in the rabbit (Table 1). Furthermore, N-PSS exerts no adverse effect on normal beneficial vaginal flora such as *Lactobacillus* (Figure 10). *Lactobacillus* growth is unaffected by N-PSS at a concentration nearly 3 orders of magnitude higher than that required for inhibition of HIV (Figure 6), HSV (Figure 7), and gonococci (Figure 9). N-PSS is classified as nontoxic in the material safety data sheet provided by National Starch and Chemical Company.

Prevention of STDs (as with any disease), rather than cure, is the strategy of choice. This is particularly true for HIV and HSV infections, cures for which are currently unavailable. Toward this end, N-PSS is particularly suitable as a preventive agent because its contraceptive and antimicrobial activities do not appear to be mediated by one or more cytotoxic mechanisms of action. The inhibition of gonococcal growth could suggest a cytotoxic effect against this microbe; however, additional studies are required to identify the exact mechanism by which N-PSS inhibits the growth of *N. gonorrhoeae.*

The efficacy of PSS as a contraceptive antimicrobial agent is likely dependent upon the method of administration. Vaginal delivery of PSS is a logical choice. First, the active ingredient is focused on the portal of entry for both spermatozoa and pathogenic microbes. Localized effects make it possible for active ingredients to retain their activity at lower concentrations, thus minimizing undesirable and systemic side effects. Through vaginal application, effective prophylaxis can be accomplished by agents that otherwise would be ineffective because of their poor systemic absorption. This is particularly true for N-PSS, which is characterized by a high charge and molecular weight. Furthermore, vaginal application of a preventive agent gives the female partner direct control over her reproductive health.

N-PSS (or PSS) has been chosen as a candidate for an efficacious contraceptive antimicrobial agent for several reasons. First, it has satisfied several criteria that we established for an active ingredient. Second, PSS is highly soluble in aqueous solutions, which makes it compatible with water-based formulations, increases the range of achievable dosing, and increases its bioavailability within the vaginal compartment. Third, PSS is a high molecular weight polymer with a high charge density. These properties reduce the probability that PSS may produce systemic side effects because its presence would be restricted to the site of application (the vagina). Limited distribution to other parts of the body would further reduce the dose of PSS required for its desired effect. Fourth, the nature of the polymer, including its anionic group (sulfonate, in contrast to sulfate), reduces its metabolic breakdown (at

least by mammalian cells). This further increases the time over which target cells are exposed to effective concentrations of the agent, thus further decreasing the required dose.

The preclinical experiments presented in this report show that PSS is an attractive candidate for use as a safe, effective vaginal contraceptive, with a broad spectrum of activities against STD microorganisms. These findings support our choice of PSS for further evaluation and development; however, the data should be viewed with cautious optimism because all of the activities of N-PSS have been evaluated in vitro or in nonprimate models. This is a common constraint among new drug substances throughout preclinical studies. The clinical efficacy of PSS is currently unknown, and will be critically evaluated in Phase 2 trials. If PSS is clinically active, this material will offer substantial benefits, and should find global acceptance in the goal to improve women's reproductive health.

Acknowledgments

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References

Ackermann WW, Potter VR. Enzyme inhibition in relation to chemotherapy. *Proc Soc Exp Biol Med.* 1949;72:1–9.

Anderson RA, Beyler SA, Mack SR, Zaneveld LJD. Characterization of a high-molecular-weight form of human acrosin. Comparison with human pancreatic trypsin. *Biochem J.* 1981;199:307–316.

Anderson RA, Feathergill K, Kirkpatrick R, Zaneveld LJD, Coleman KT, Spear PG, Cooper MD, Waller DP, Thoene JG. Characterization of cysteamine as a potential contraceptive anti-HIV agent. *J Androl.* 1998;19:37–49.

Anderson RA, Feathergill KA, De Jonge CJ, Mack SR, Zaneveld LJD. Facilitative effect of pulsed addition of dibutyryl cAMP on the acrosome reaction of noncapacitated human spermatozoa. *J Androl.* 1992;13:398–408.

Anderson RA, Feathergill KA, Drisdel RC, Rawlins RG, Mack SR, Zaneveld LJD. Atrial natriuretic peptide (ANP) as a stimulus of the human acrosome reaction and a component of ovarian follicular fluid: correlation of follicular ANP content with in vitro fertilization outcome. *J Androl.* 1994;15:61–70.

Anderson RA, Oswald C, Leto S, Zaneveld LJD. Evidence for multiple catalytic sites of human acrosin from kinetic evaluation of fructose-induced acrosin inhibition. *Arch Biochem Biophys.* 1985;241:509–520.

Aronson NN, Davidson EA. Lysosomal hyaluronidase from rat liver. I. Preparation. *J Biol Chem.* 1967;242:437–440.

Arroyo R, Alderete JF. Trichomonas vaginalis surface proteinase activity is necessary for parasite adherence to epithelial cells. *Infect Immunol.* 1989;57:2991–2997.

Bernfeld P, Tuttle LP, Hubbard RW. Factors influencing the activity of testicular hyaluronidase. *Arch Biochem Biophys.* 1961;92:232–240.

Beyler S, Zaneveld L. Inhibition of in-vitro fertilization of mouse gametes by proteinase inhibitors. *J Reprod Fertil.* 1982;66:425–431.

Biggers J, Whitten W, Whittingham D, Freeman W. The culture of mouse embryos in vitro. In: Daniel J, ed. *Methods In Mammalian Embryology.* San Francisco: WH Freeman, 1971:86–116.

Blake M, Holmes KK, Swanson J. Studies on gonococcus infection. XVII. IgA1-cleaving protease in vaginal washings from women with gonorrhea. *J Infect Dis.* 1979;139:89–92.

Bourinbaiar AS, Lee-Huang S. Acrosin inhibitor, 4′-acetamidophenyl 4-guanidinobenzoate, an experimental vaginal contraceptive with anti-HIV activity. *Contraception.* 1995;51:319–322.

Bourinbaiar AS, Nagorny R. Effect of serine protease inhibitor, N-α-tosyl-L-lysyl-chloromethyl ketone (TLCK), on cell-mediated and cell-free HIV-1 spread. *Cell Immunol.* 1994;155:230–236.

Cooper MD, Rapp J, Jeffery-Wiseman C, Barnes RC, Stephens DS. Chlamydia trachomatis infection of human fallopian tube organ cultures. *J Gen Microbiol.* 1990;136:1109–1115.

De Jonge CJ, Mack SR, Zaneveld LJD. Inhibition of the human sperm acrosome reaction by proteinase inhibitors. *Gamete Res.* 1989a;23:387–397.

De Jonge CJ, Mack SR, Zaneveld LJD. Synchronous assay for human sperm capacitation and the acrosome reaction. *J Androl.* 1989b;10:232–239.

DeAngelis PL, Glabe CG. Specific recognition of sulfate esters by bindin, a sperm adhesion protein from sea urchins. *Biochim Biophys Acta.* 1990;1037:100–105.

Doring GK. Practical experience with a new vaginal contraceptive. *Int J Sexol.* 1954;8:93–94.

Dravland JE, Llanos MN, Munn RJ, Meizel S. Evidence for involvement of a sperm trypsinlike enzyme in the membrane events of the hamster sperm acrosome reaction. *J Exp Zool.* 1984;232:117–128.

Endo Y, Schultz RM, Kopf GS. Effects of phorbol esters and a diacylglycerol on mouse eggs: inhibition of fertilization and modification of the zona pellucida. *Dev Biol.* 1987;119:199–209.

Fenton KA. The XI International AIDS Conference in Vancouver: perspectives from epidemiology and public health. *Genitourinary Med.* 1996;72:370–373.

Fitzgerald TJ, Gannon EM. Further evidence for hyaluronidase activity of Treponema pallidum. *Can J Microbiol.* 1983;29:1507–1513.

Fitzgerald TJ, Repesh LA. The hyaluronidase associated with Treponema pallidum facilitates treponemal dissemination. *Infect Immun.* 1987;55:1023–1028.

Foldesy RG, Homm RE, Levinson SL, Hahn DW. Multiple actions of a novel vaginal contraceptive compound, ORF 13904. *Fertil Steril.* 1986;45:550–555.

Hahn DW, McGuire JL. Prototype of a new long-acting spermistatic agent. In: Zatuchni GI, Goldsmith A, Spieler JM, Sciarra JJ, eds. *Male Contraception: Advances and Prospects.* Philadelphia: Harper and Row; 1986:218–226.

Hallenberger S, Bosch V, Angliker H, Shaw E, Klenk H-D, Garten W. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. *Nature.* 1992;360:358–361.

Herold BC, Bourne N, Marcellino D, Kirkpatrick R, Strauss DM, Zaneveld LJD, Waller DP, Anderson RA, Chany II CJ, Cooper MD, Stanberry L. Poly(sodium 4-styrenesulfonate): an effective candidate topical anti-microbial for the prevention of sexually transmitted diseases. *J Infect Dis.* 2000;181:770–773.

Herold BC, Siston A, Bremer J, Kirkpatrick R, Wilbanks G, Fugede P, Peto C, Cooper M. Sulfated carbohydrate compounds prevent microbial adherence by sexually transmitted disease pathogens. *Antimicrob Agents Chemother.* 1997;41:2776–2780.

Homm RE, Foldesy RG, Hahn DW. ORF 13904, a new long-acting vaginal contraceptive. *Contraception.* 1985;32:267–274.

Jennings R, Clegg A. The inhibitory effect of spermicidal agents on replication of HSV-2 and HIV-1 in-vitro. *J Antimicrob Chemother.* 1993;32:71–82.

Jones R. Identification and functions of mammalian sperm-egg recognition molecules during fertilization. *J Reprod Fertil.* 1990;42(suppl): 89–105.

Joyce C, Freund M, Peterson RN. Contraceptive effects of intravaginal application of acrosin and hyaluronidase inhibitors in rabbit. *Contraception.* 1979;19:95–106.

Joyce CL, Mack SR, Anderson RA, Zaneveld LJD. Effect of hyaluronidase, β-glucuronidase and beta-N-acetyl-glucosaminidase inhibitors on sperm penetration of the mouse oocyte. *Biol Reprod.* 1986;35:336–346.

Joyce CL, Nuzzo NA, Wilson L, Zaneveld LJD. Evidence for a role of cyclooxygenase (prostaglandin synthetase) and prostaglandins in the sperm acrosome reaction and fertilization. *J Androl.* 1987;8:74–82.

Joyce CL, Zaneveld LJD. Vaginal contraceptive activity of hyaluronidase and cyclooxygenase (prostaglandin synthetase) inhibitors in the rabbit. *Fertil Steril.* 1985;44:426–428.

Kaminski JM, Nuzzo NA, Bauer L, Waller DP, Zaneveld LJD. Vaginal contraceptive activity of aryl 4-guanidinobenzoates (acrosin inhibitors) in rabbits. *Contraception.* 1985;32:183–189.

Kapatais-Zoubmos K, Chandler DKF, Barile MF. Survey of immunoglobulin A protease activity among selected species of ureaplasma and mycoplasma: specificity of host immunoglobulin A. *Infect Immunol.* 1985;47:704–709.

Kato M, Irisawa T, Muramatu M. Antibacterial effects of esters of guanidino- and amidino-acids trypsin inhibitors. *J Enzyme Inhib.* 1994;8: 25–37.

Kido H, Kamoshita K, Fukutomi A, Katunuma N. Processing protease for gp160 human immunodeficiency virus type I envelope glycoprotein precursor in human $T4^+$ lymphocytes. *J Biol Chem.* 1993;268: 13406–13413.

Koito A, Hattori T, Murakami T, Matsushita S, Maeda Y, Yamamoto T, Takatsuki K. A neutralizing epitope of human immunodeficiency virus type 1 has homologous amino acid sequences with the active site of inter-α-trypsin inhibitor. *Int Immunol.* 1989;1:613–618.

Kopf GS, Wilde MW. Signal transduction processes leading to acrosomal exocytosis in mammalian spermatozoa. *Trends Endocrinol Metab.* 1990;1:362–368.

Kornfeld SJ, Plaut AG. Secretory immunity and the bacterial IgA proteases. *Rev Infect Dis.* 1981;3:521–534.

Kovacs A, Foote RH. Viability and acrosome staining of bull, boar and rabbit spermatozoa. *Biotech Histochem.* 1992;67:119–124.

Mardh P-A. The vaginal ecosystem. *Am J Obstet Gynecol.* 1991;165: 1163–1168.

McGregor JA, French JI, Jones W, Milligan K, McKinney PJ. Bacterial vaginosis is associated with prematurity and vaginal fluid mucinase and sialidase: results of a controlled trial of topical clindamycin cream. *Am J Obstet Gynecol.* 1994;170:1059–1060.

Meizel S, Lui CW. Evidence for the role of a trypsin-like enzyme in the hamster sperm acrosome reaction. *J Exp Zool.* 1976;195:137–144.

Mohan P, Schols D, Baba M, De Clercq E. Sulfonic acid polymers as a new class of human immunodeficiency virus inhibitors. *Antiviral Res.* 1992;18:139–150.

Mulks MH, Knapp JS. Immunoglobulin A1 protease types of Neisseria gonorrhoeae and their relationship to auxotype and serovar. *Infect Immunol.* 1987;55:931–936.

Nahmias AJ, Dannenbarger J, Wickliffe C, Muther J, eds. *Clinical Aspects of Infection with Herpes Simplex Viruses 1 and 2.* Amsterdam: Elsevier; 1980.

Nuzzo NA, Zaneveld LJD. Wheat germ agglutinin treatment of follicle cell-free mouse oocytes inhibits fertilization. *Gamete Res.* 1988;21: 213–221.

Oram RJ, Marcellino D, Strauss D, Gustafson E, Talarico CL, Root AK, Sharma PL, Thompson K, Fingeroth JD, Crumpacker C, Herold BC. Characterization of an acylovir-resistant herpes simplex virus type 2 strain isolated from a premature neonate. *J Infect Dis.* 2000;181:1458–1461.

O'Reilly TM, Bhatti AR. Proteases of the pathogenic Neisseriae: possible role in infection. *Microbios.* 1986;45:113–129.

Parkes AS. Prevention of fertilization by a hyaluronidase inhibitor. *Lancet.* 1953;2:1285–1287.

Parkes AS, Rogers HJ, Spensley PC. Biological and biochemical aspects of the prevention of fertilization by enzyme inhibitors. *Proc Soc Study Fertil.* 1954;6:65–80.

Pincus G, Pirie NW, Chang MC. The effects of hyaluronidase inhibitors on fertilization in the rabbit. *Arch Biochem.* 1948;19:388–396.

Proctor M, Manning PJ. Production of immunoglobulin A protease by streptococcus pneumoniae from animals. *Infect Immunol.* 1990;58: 2733–2737.

Reissig JL, Strominger JL, Leloir LF. A modified colorimetric method for the estimation of N-acetylamino sugars. *J Biol Chem.* 1955;217: 959–966.

Resnick L, Busso ME, Duncan RC. Anti-HIV screening technology. In: *Heterosexual Transmission of AIDS.* New York, NY: Alan R Liss Inc; 1990:311–325.

Roddy RE, Cordero M, Ryan KA, Figueroa J. A randomized controlled trial comparing nonoxynol-9 lubricated condoms with silicone lubricated condoms for prophylaxis. *Sex Transm Infect.* 1998a;74:116–119.

Roddy RE, Zekeng L, Ryan KA, Tamoufe U, Weir SS, Wong EL. A controlled trial of nonoxynol 9 film to reduce male-to-female transmission of sexually transmitted diseases. *New Engl J Med.* 1998b; 339:504–510.

Rogers BJ. Capacitation, acrosome reaction, and fertilization. In: Zaneveld L, Chatterton R, eds. *Biochemistry of Mammalian Reproduction.* New York: John Wiley and Sons; 1982:203–230.

Rogers BJ, Ueno M, Yanagimachi R. Inhibition of hamster sperm acrosome reaction and fertilization by oligomycin, antimycin A, and rotenone. *J Exp Zool.* 1977;199:129–136.

Romano A, Moiseiev J. Bacterial enzymes in viral infections: a new concept. The possible role of hyaluronidase in herpes simplex virus keratitis. *Metab Pediatr Systemic Opthalmol.* 1982;6:361–365.

Rosso G. Vaginal hyaluronidase activity in some pathological conditions. III. Cervico-vaginitis. *Minerva Ginecol.* 1975;27:537–544.

Sander FV, Cramer SD. A practical method for testing the spermicidal action of chemical contraceptives. *Hum Fertil.* 1941;6:134–137, 153.

Shieh M-T, WuDunn D, Montgomery RI, Esko JD, Spear PG. Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J Cell Biol.* 1992;116:1273–1281.

Shimizu K, Kondoh Y, Tanaka K. Proteinase production and pathogenicity of Candida albicans. I. Invasion into chorioallantoic membrane by Candida albicans strains of different proteinase activity. *Microbiol Immunol.* 1987;31:407–414.

Sieve BF. A new antifertility factor (a preliminary report). *Science.* 1952; 116:373–385.

Simpson DA, Hausinger RP, Mulks MH. Purification, characterization and comparison of the immunoglobulin A1 proteases of Neisseria gonorrhoeae. *J Bacteriol.* 1988;170:1866–1873.

Sokal R, Rohlf FJ. *Biometry*. San Francisco, Calif: WH Freeman; 1981: 400–453.

Spear PG. Membrane fusion induced by Herpes simplex virus. In: Bentz J, ed. *Viral Fusion Mechanisms.* Boca Raton, Fla: CRC Press; 1993: 201–233.

Spensley PC, Rogers HJ. Enzyme inhibition. *Nature.* 1954;173:1190.

Stafford MK, Ward H, Flanagan A, Rosenstein IJ, Taylor-Robinson D, Smith JR, Weber J, Kitchen VS. Safety study of nonoxynol-9 as a vaginal microbicide: evidence of adverse effects. *J Acquir Immune Defic Syndr.* 1998;17:327–331.

Steiner B, Cruce D. A zymographic assay for detection of hyaluronidase

activity on polyacrylamide gels and its application to enzymatic activity found in bacteria. *Anal Biochem.* 1992;200:405–410.

Straus DC, Mattingly SJ, Milligan TW, Doran TI, Nealon TJ. Protease production by clinical isolates of type III group B streptococci. *J Clin Microbiol.* 1980;12:421–425.

Tan GT, Wickramasinghe A, Verma S, Hughes SH, Pezzuto JM, Baba M, Mohan P. Sulfonic acid polymers are potent inhibitors of HIV-1 induced cytopathogenicity and the reverse transcriptases of both HIV-1 and HIV-2. *Biochim Biophys Acta.* 1993;1181:183–188.

Tarlatzia BC, Danglis J, Kolibianakis EM, Papadimas J, Bontis J, Lagos S, Mantalenakis S. Effect of follicular fluid on the kinetics of the human sperm acrosome reaction in vitro. *Arch Androl.* 1993;31:167–175.

Terhune SS, Coleman KT, Sekulovich R, Burke RL, Spear PG. Limited variability of glycoprotein gene sequences and neutralizing targets in herpes simplex virus type 2 isolates and stability on passage in cell culture. *J Infect Dis.* 1998;178:8–15.

United States Pharmacopeial Convention, Inc. *The United States Pharmacopeia/The National Formulary.* Rockville, Md: USP; 1995:1430–1431.

United Nations. World population nearing 6 billion projected close to 9 billion by 2050. In: *1998 Revision of the Official United Nations Population Estimates and Projections.* United Nations: Population Division of the Department of Economic and Social Affairs; 1998.

Voelker R. Scientists zero in on new HIV microbicides. *JAMA.* 1995;273:979–980.

Woolf CM. *Principles of Biometry*. Princeton, NJ: Van Nostrand Co.; 1968:83–115.

WuDunn D, Spear PG. Initial interaction of Herpes simplex virus with cells is binding to heparan sulfate. *J Virol.* 1989;63:52–58.

Zaneveld LJD, Anderson RA, Mack SR, De Jonge CJ. Mechanism and control of the human sperm acrosome reaction. *Hum Reprod.* 1993;8:2006–2008.

Zaneveld LJD, Polakoski KL, Robertson RT, Williams WL. Trypsin inhibitors and fertilization. In: *Proceedings of the International Research Conference on Proteinase Inhibitors, Munich.* Berlin: Walter de Gruyter; 1970:236–244.

Zaneveld LJD, Polakoski KL, Schumacher GFB. Properties of acrosomal hyaluronidase from bull spermatozoa. *J Biol Chem.* 1973;248:564–570.

Zaretzky FR, Pearce-Pratt R, Phillips DM. Sulfated polyanions block Chlamydia trachomatis infection of cervix-derived human epithelia. *Infect Immunol.* 1995;63:3520–3526.

Zeitlin L, Whaley KJ, Hegarty TA, Moench TR, Cone RA. Tests of vaginal microbicides in the mouse genital herpes model. *Contraception.* 1997;56:329–335.

Zezulak KM, Spear PG. Limited proteolysis of herpes simplex virus glycoproteins that occurs during their extraction from vero cells. *J Virol.* 1984;50:258–262.