Journal of Andrology, Vol. 23, No. 6, November/December 2002

Lignosulfonic Acid Blocks In Vitro Fertilization of Macaque Oocytes When Sperm Are Treated Either Before or After Capacitation

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Received for publication January 18, 2002; accepted for publication June 17, 2002.

Abstract

Lignin-derived macromolecules (LDMs) are biologically active compounds that affect a variety of cell-to-cell interactions including the inhibition of fertilization and embryo development in a number of nonmammalian species. The effect of ligno-sulfonic acid (LSA), a highly sulfonated LDM, on cynomolgus macaque sperm-oocyte interaction was evaluated with a zona pellucida binding assay and by in vitro fertilization (IVF). Sperm were treated with LSA (1.5 mg/mL) either before washing or after capacitation. Capacitation included centrifugation through 80% Percoll followed by 2 consecutive washes with medium, overnight incubation, and activation with dibutyryl cyclic adenosine monophosphate and caffeine. The zona binding assay was performed using immature oocytes that had adhered to the center of glass "binding chambers." The number of capacitated sperm that attached to the zona over a 3-minute period was recorded. Sperm attachment was significantly inhibited by LSA as compared to controls whether treatment occurred after capacitation (92.5%; $P < .001$) or before washing (82.5%; $P < .001$). When sperm were treated similarly with fucoidin, a sulfated polysaccharide known to inhibit sperm-oocyte interaction, sperm-zona binding was significantly inhibited by postcapacitation treatment but not by prewash treatment. Treatment of sperm with LSA consistently blocked fertilization over 4 IVF cycles both before washing and after capacitation. Fertilization rate for controls was 65% ± 17%. No LSA-treated sperm were observed on the surface of lightly rinsed oocytes after 4 hours of coincubation. Localization of biotinylated LSA showed labeling over the entire sperm surface with the greatest intensity observed over the head and midpiece. LSA treatment had no

effect on the percentage of motile sperm or quality of sperm motility. Due to the antifertility properties of this nontoxic molecule, LSA appears to have potential as a vaginal contraceptive.

Key words: Contraception, fucoidin, microbicide, polysulfonation, zona pellucida

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The development of vaginal contraceptives is an important goal of applied research in reproductive biology. The action of most vaginal contraceptives involves nonspecific cytotoxic effects on sperm rather than the targeting of specific sperm functions. However, spermicides currently in use (eg, Nonoxynol-9) are also cytotoxic to vaginal epithelia, resulting in a greater risk of sexually transmitted infections (Maguire et al, 2001). In light of this problem, there is great need for development of sperm-specific contraceptives that are not cytotoxic. The most well understood functions of sperm are those involved in fertilization, but it is challenging to develop strategies for interference with these functions at locations such as the vagina that are distant from the site of fertilization. Freshly ejaculated sperm undergo numerous changes required for fertilization during capacitation and transport in the female reproductive tract, and thus may not be vulnerable to vaginal contraceptives that are not directly spermicidal. In this manuscript we report the discovery of a naturally occurring, noncytotoxic compound that blocks primate fertilization without affecting sperm viability, even when sperm are exposed to the compound before capacitation.

Lignosulfonic acid (LSA) is a member of a family of related lignin-derived macromolecules (LDMs) that are byproducts that form as a result of the conversion of wood pulp into paper. LSA is derived from the sulfite pulping process whereby wood chips are extracted with acidic aqueous sulfur dioxide, resulting in the depolymerization and dissolution of lignin, to produce a cellulose fiber (reviewed by McCubbins, 1983). The aqueous effluent of this process is believed to consist largely of the polar breakdown products of lignin such as LSA (Kringstad and Lindstrom, 1984). LSA is a highly sulfonated macromolecule ranging in molecular weight from 5 kd to several hundred kd, and is composed of substituted guaiacylpropane monomers (Loomis and Beyer, 1953; Pearl, 1967; Vocac and Alphin, 1968).

LDMs have been shown to inhibit fertilization in a number of nonmammalian species without being cytotoxic to gametes (Higashi et al, 1992; Cherr et al, 1993; Pillai et al, 1997). LSA is virtually nontoxic when administered orally (LD_{50} > 40 g/kg) and has been used for many years as an animal feed additive because of its antipepsin activity and the protection it provides against the development of gastric ulcers (Vocac and Alphin, 1968, 1969; Luscombe and Nicholls, 1973). LSA has also been shown to have interesting biological activities such as macrophage activation (Suzuki et al, 1989a) and inhibition of the human immunodeficiency virus (HIV) in vitro (Suzuki et al, 1989b, c).

In this communication, we report that when ejaculated, noncapacitated macaque sperm are treated with LSA they remain motile but are rendered infertile. Furthermore, this antifertility effect is maintained even after hours of capacitation and activation. This property of LSA was compared with that of fucoidin, another sulfated molecule that has been reported to inhibit sperm-oocyte interaction. However, unlike LSA, fucoidin was effective when it was present during coincubation with gametes, but not when sperm were treated before capacitation. LSA appears to have unique potential as a vaginal contraceptive because of the persistence of its antifertility effect and its lack of cytotoxicity.

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Chemicals

High-performance liquid chromatography—grade water was obtained from Fisher Scientific (Santa Clara, Calif). Dulbecco phosphate-buffered saline (DPBS) and modified Biggers, Whitten, and Whittingham medium (Hepes-buffered BWW) were prepared by Irvine Scientific (Irvine, Calif). CMRL 1066 medium was prepared by Gibco (Rockville, Md), heparin was obtained through Elkins-Sinn Inc (Cherry Hill, NJ), fetal bovine serum (FBS) was provided by HyClone (Logan, Utah), and buffalo rat liver (BRL) cells were from American Type Culture Collection (Manassas, Va). Fluorescent probes (fluorescein isothiocyanate-*Pisum sativum* [FITC-PSA] and fluorescein-conjugated goat antibiotin antibody) were obtained from Vector Laboratories (Burlingame, Calif). Sulfosuccinimidyl-6-(biotinamido) hexanoate (EZ-Link sulfo-NHS-LC-biotin) was obtained from Pierce (Rockford, Ill). All recombinant hormones were generously supplied by Ares Serono (Randolph, Mass). All other chemicals and salts for media preparation were purchased from Sigma Chemical Company (St Louis, Mo). LSA sodium salt was purchased from Aldrich Chemical Company and was additionally purified to remove contaminants according to the procedures described by Higashi et al (1992). Briefly, this procedure involved sequential solvent extraction (methylene chloride and acetonitrile) followed by dialysis (3.5 kd cutoff) and lyophilization.

Sperm Collection, LSA Treatment, Washing, and Capacitation

Four adult male cynomolgus macaques were caged individually at the California Regional Primate Research Center (CRPRC) in compliance with the Federal Animal Welfare Act and the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*. The animals were maintained on a 12L: 12D cycle at 25 ° -27 ° C and were given a diet of Purina monkey chow and water ad libitum. Semen samples were collected by electroejaculation (Sarason et al, 1991) into 15-mL centrifuge tubes containing 5 mL of Hepes-buffered BWW maintained at room temperature. After 15-30 minutes, the coagulum was removed, the semen samples were further diluted with an additional 5 mL of Hepes-buffered BWW containing 3 mg/mL bovine serum albumin (BSA), and then concentrated into a 1 mL pellet by centrifugation at 300 x *g* for 10 minutes. LSA dissolved in Hepes-buffered BWW salts was added to half the pellet to give a final concentration of 1.5 mg LSA/mL of sperm (this constituted prewash treatment with LSA). An equal volume of Hepes-buffered BWW salts was added to the other half of the pellet (control). Both aliquots were incubated at room temperature for 40 minutes and then washed and capacitated as described previously (Tollner et al, 2000). Briefly, sperm were centrifuged through 3.5 mL of 80% Percoll for 25 minutes at 400 x *g*. The supernatant containing Percoll and any remaining sperm was removed and the sperm pellet was resuspended in bicarbonate-buffered BWW (Overstreet et al, 1980) containing 30 mg/mL of BSA. Sperm were washed 2 more times by centrifugation at 300 x *g* for 10 minutes and were diluted in this medium. Sperm were finally resuspended at a concentration of 10-20 x 10^6/mL in the bicarbonate-buffered BWW and were capacitated by a series of incubations beginning with a 24-hour incubation at room temperature in 4.5% CO_2. Following this room temperature incubation, sperm suspensions were incubated at 37 ° C in 4.5% CO_2 for 2 more hours, at which point the sperm concentration was adjusted to 4 x 10^6/mL for zona pellucida binding experiments or 500 000 motile sperm/mL for in vitro fertilization (IVF). The sperm suspensions were activated by incubation for an additional hour at 37 ° C in 4.5% CO_2 in media containing 1 mM caffeine and 1 mM dibutyryl cyclic adenosine monophosphate. Forty-five minutes following activation, an aliquot of control sperm was treated with 1.5 mg/mL LSA for 15 minutes (postcapacitation treatment) before introducing oocytes into the sperm suspensions for IVF or addition of sperm to the zona pellucida binding chamber (see below). Five minutes before either IVF

or zona pellucida binding assays, 200 sperm from each of the control, prewash, and postcapacitation treatments were scored for percentage motility at 200x magnification with phase-contrast optics using a BH-2 series Olympus microscope. Progression was assessed with a 0-4 rating system, as follows: 0 = no progressive sperm; 1 = 1%-25% of motile sperm with forward (space gaining) progressive motility; 2 = 26%-50% of motile sperm with forward progressive motility; 3 = 51%-75% of motile sperm with forward progressive motility; and 4 = 76%-100% of motile sperm with forward progressive motility.

For 3 different males, sperm from each of the control, prewash, and postcapacitation treatments were also assessed for acrosomal reactions following the completion of activation. An aliquot from each treatment was washed 3 times at 300 x *g* for 5 minutes with DPBS to remove excess BSA and permeablized with 95% cold EtOH for at least 15 minutes. Acrosome reactions were detected according to the methods described by Cross et al (1986). Briefly, sperm were dried onto glass slides and stained with 100 µg/mL FITC-PSA in DPBS. Excess FITC-PSA was removed by rinsing slides with distilled H_2O. Antifading solution (DPBS containing 32% glycerol, 0.5% paraformaldehyde, and 0.01% sodium azide) was deposited over regions of dried sperm and overlaid with a glass coverslip. Sperm were observed with a Lietz Laborlux S microscope equipped with 200 W mercury fluorescence vertical illuminator and a 1-Lambda Ploemopac incident light fluorescence illuminator employing an I3 filter cube with a BP 450-490 excitation filter, an RKP 0510 dichromatic mirror, and an LP 515 suppression filter. Two hundred to 300 sperm from each treatment were scored for presence of the acrosome.

In a separate set of experiments, sperm were treated with 1.5 mg/mL of fucoidin either before Percoll washing or after activation (as described above for LSA) and were then assessed for sperm—zona pellucida binding as described below.

Sperm Binding to Zonae Pellucidae

Ovaries were obtained at necropsy from adult female cynomolgus macaques at CRPRC. Zona pellucida—intact immature oocytes were collected from the ovaries and were frozen at -80 °C in 2 M dimethyl sulfoxide (DMSO) in DPBS according to previously published protocols (Vandevoort et al, 1992). the oocytes were thawed at 22 °C and rinsed through 3 dishes, each containing 0.5 mL of Hepes-buffered BWW medium, to remove DMSO prior to experiments. Oocytes were then deposited onto glass slides (1 oocyte per slide) and allowed to air-dry for more than 10 minutes. Within 5 minutes before sperm-zona coincubation, 2 µL of Hepes-buffered BWW containing activators were added to each air-dried oocyte. Four posts of silicon grease containing 50-75 µm beads were then deposited at 4 corners around the oocyte. A 22 x 22 mm glass coverslip was carefully pressed onto the posts until the grease was completely flattened. The slide was warmed on a microscope stage warmer set at 37.5 °C for 5 minutes before the addition of sperm. A 40-µL aliquot of activated sperm preparation (either control, prewash, or postcapacitation treatments) was added to the edge of the coverslip over the rehydrated oocyte and was drawn by capillary action to fill the entire 22 x 22 mm space. Binding of sperm to the zona surface was observed with a Lietz Laborlux microscope with phase contrast optics at 400x. A timer was started at the moment the first motile sperm attached to the zona. Due to the depth of the preparation, sperm were restricted to bind to the outer edges of the zona. The total number of bound sperm was counted after 3 minutes starting at the 12 o'clock position of the oocyte and working clockwise to the starting point. The count required on average about 15 seconds to complete. Two oocytes (replicates) were used for each treatment for each male.

In Vitro Fertilization

Four female cynomolgus macaques maintained at CRPRC as described above were superovulated using injections of recombinant human gonadotropins as described previously (Zelinski-Wooten et al, 1997).

Beginning on the morning of day 1 to 4 of menses, females received follicle-stimulating hormone (FSH) injections twice daily through treatment days 1-6 and FSH/luteinizing hormone through treatment days 7-9. Females also received concurrent treatment with Antide (1.0 mg/kg s.c., once daily), a gonadotropin-releasing hormone antagonist. Animals were evaluated by ultrasound for follicular development on treatment day 7. Females with good follicular development received recombinant human chorionic gonadotropin (hCG) the next morning and 27 hours after the administration of hCG, follicles were aspirated at laparoscopy.

On treatment day 11, oocytes were collected by laparoscopic retrieval. Briefly, a Verres needle was introduced via a small midventral incision into the abdominal cavity of anesthetized females. Carbon dioxide was used to insufflate the abdomen and a 5-mm trocar was then introduced into the same incision followed by the laparoscope. Grasping forceps were introduced through a port placed into the right caudal abdomen. A 3-inch, 22-gauge needle attached to mild vacuum pressure was introduced into the abdomen, and each visible follicle was then aspirated into a 15 mL sterile tissue culture tube containing Tyrode albumin lactate pyruvate medium (Wolf et al, 1996). The instruments were then withdrawn and each incision was closed using standard procedures. The pooled aspirates from left and right ovaries were placed in TH3 medium and immediately transported to the laboratory for recovery of oocytes.

Cumulus-oocyte complexes or denuded oocytes were placed in TH3 medium (Nusser et al, 2001) containing 50 units/mL heparin at 37 ° C and graded according to maturation status using a dissecting microscope. Mature oocytes (at metaphase II stage with first polar body) with or without cumulus layers were washed (3 times) in warm and equilibrated CMRL 1066 medium containing 10% FBS, 10 mM L-glutamine, 5 mM sodium pyruvate, 1 mM sodium lactate, 100 units/mL penicillin, and 100 µg/mL streptomycin (Nusser et al, 2001) and kept in this medium in a CO_2 incubator at 37 ° C prior to fertilization.

Oocytes were incubated in the insemination drops covered with oil for 4 hours, and then washed 3 times in CMRL 1066 medium, and cultured for 4-7 days at 37 ° C in 5% CO_2 on BRL cells in CMRL medium supplemented with 10% FBS (Nusser et al, 2001). Three to 5 oocytes were used for each treatment in each IVF cycle (4 different females were used, each female represented 1 IVF cycle). Oocytes that contained 2 pronuclei and 2 polar bodies (12-16 hours after insemination) and then cleaved into 2 cells (24-30 hours after insemination) were considered fertilized and maintained in culture. Embryos were transferred to fresh plates of BRL cells every other day.

During the fourth IVF cycle, 2 to 3 oocytes were removed from each insemination drop after the 4-hour coincubation period for observation of sperm on the zona pellucida. Oocytes were lightly rinsed once and placed in a fresh drop of CMRL 1066 maintained under oil in a clear culture dish. Oocytes were photographed with an AxioCam digital camera (Carl Zeiss Vision GmbH, Germany) installed on an inverted Olympus Ix70 microscope with Hoffman modulation contrast optics.

Labeling of Sperm With LSA

In order to verify the presence of LSA on the sperm surface throughout the washing and capacitation procedure, sperm were treated with biotinylated LSA (LSA-b). LSA, purified and lyophilized as described above, was dissolved in 0.05 M borate buffer and made amine-rich through reaction with 1 M ethylenediamine. The reaction was stopped with glucose after 1 hour and aminated LSA (NH-LSA) was dialyzed extensively with a 3500 molecular weight cutoff (3.5K MWCO; Slide-A-Lyzer; Pierce) against phosphate buffer at pH 7.2 (Hermanson, 1996). Five milligrams of NH-LSA was than reacted with 2.78 mg of sulfo-NHS-LC-biotin (NHS-biotin) in 1 mL of Hepes-buffered distilled H_2O for 1 hour at room

temperature at pH 7.2. The reaction mixture was dialyzed (3.5K MWCO) first against 5 mM Hepes solution with distilled H_2O pH 8.0 for 24 hours and then into BWW salts (pH 7.4) for an additional 24 hours. The resulting solution was stored at 5 °C and used within the next 2-3 days. To control for the possible presence of free, nonreacted NHS-biotin in solution with LSA-b, both untreated LSA incubated with NHS-biotin and NHS-biotin alone were dialyzed as above.

Sperm were treated with LSA-b as described previously with prewash treatment of unlabeled LSA. Briefly, following the initial washing of sperm from semen, LSA-b was added to half of the sperm pellet to give a final concentration of 1.5 mg LSA-b/mL. An equal volume of BWW salts (pH 7.4) was added to the other half of the pellet (control). Sperm were incubated for another 40 minutes and then washed through Percoll and BWW, incubated overnight, and activated as described previously. An aliquot of sperm from both LSA-b treated and control groups were removed for labeling with fluorescein conjugated anti-biotin goat antibody (anti-biotin) 4 times during the process; 40 minutes after LSA-b addition, after Percoll washing, after overnight incubation, and 60 minutes after activation. Forty-five minutes following activation, an aliquot of control sperm was treated with LSA-b for 15 minutes (postcapacitation treatment). Each aliquot was washed once by centrifugation (300 x *g* for 5 minutes) and resuspended into 1 mL of DPBS without BSA. Aliquots were treated with antibiotin at a final concentration of 20 µg/mL for 30 minutes and then washed twice (each at 300 x *g* for 5 minutes) with DPBS. The resulting pellet was fixed with 0.8% paraformaldehyde for 15 minutes. Fixed sperm were resuspended in DPBS and centrifuged again at 300 x *g* for 10 minutes and observed for fluorescence as described previously for FITC-PSA.

Imaging of fluorescent sperm for the production of micrographs was performed as described previously (Yudin et al, 1998). Briefly, sperm were viewed with an Olympus upright BH-2 microscope (Scientific Instruments, Sunnyvale, Calif) using a 60x oil immersion objective. The microscope was equipped with a Bio-Rad MRC-600 Laser scanning confocal system including a 15 mW krypton-argon mixed gas multiline laser (Bio-Rad, Hercules, Calif). Sperm were optically sectioned (0.25 µm) and the full Z-series of images was collected and projected in order to confirm surface labeling patterns. Images were digitally converted with Adobe Photoshop (Adobe Systems, San Jose, Calif) and printed using dye sublimation.

Results

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Sperm Binding to Zona Pellucida

LSA had a highly significant effect on the ability of capacitated sperm to bind to the zona pellucida of immature oocytes (Figure 1). Postcapacitation addition of LSA to sperm inhibited binding on average by 92.5% ($P < .001$; ANOVA at alpha = .01 with pairwise comparisons using the Tukey range test; Statistical Analysis Systems software; SAS institute, Cary, NC) compared with that of controls. Furthermore, LSA still inhibited sperm-zona binding by 82.5% ($P < .001$) when added to sperm before washing through Percoll and overnight incubation. The inhibitory effect of LSA on sperm-zona binding following pre- and postcapacitation treatments did not differ significantly, although postcapacitation treatment appeared to result in slightly greater inhibition (Figure 1). Neither the percentage of motile sperm nor sperm progression were affected by LSA treatment (Table 1). A high percentage of LSA-treated and control sperm (>75%) developed hyperactivated motility patterns consistent with capacitation. LSA-treated sperm appeared to strike the zona with equal frequency as control sperm, but they were more likely to either immediately bounce off or pull away after several seconds before making a more "secure" attachment.

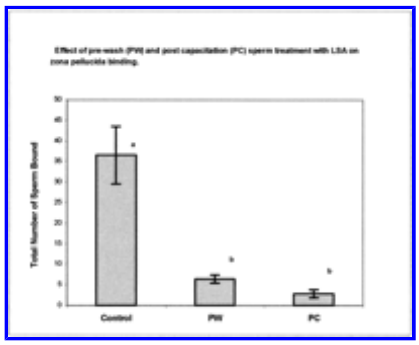

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Figure 1. Zona pellucida binding assay with control sperm or sperm treated with 1.5 mg/mL LSA either before centrifugation through Percoll (before capacitation; Pre-C) or after activation (postcapacitation; Post-C). Columns represent averages of sperm bound per zona with sperm from 4 different males and a total of 8 zona per treatment. Error bars represent SEM. Different letters above columns indicate significant differences between treatment means ($P < .001$).

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Table 1. *Percentage of motile sperm and sperm progression affected by LSA treatment*[*]

LSA treatment of sperm either before washing or after capacitation had no effect on the acrosome reaction. The percentage of prewash, postcapacitation, and control sperm exhibiting acrosomal loss (lack of fluorescence over the anterior head of the sperm) was 21% ± 4%, 15% ± 5%, and 25% ± 6%, respectively (mean ± SD; n = 3). Although acrosome reaction rates were lower on average for LSA-treated sperm, differences were not significant.

Zona pellucida binding experiments were repeated using fucoidin, a polysulfated compound previously shown to inhibit sperm—zona pellucida binding in several mammal species (Huang and Yanagimachi, 1984; Peterson et al, 1985; Oehninger et al, 1990, 1992). As with LSA, the effect of fucoidin was compared following treatment before and after capacitation. Fucoidin significantly inhibited binding by 91.5% ($P < .001$; ANOVA at alpha = .01 with Tukey range testing) compared with that of controls when the compound was added after capacitation (Figure 2). A 36% inhibition of binding resulted from addition of fucoidin before capacitation, but this change was not significantly different from controls (Figure 2). No motility changes were observed in sperm following addition of fucoidin either before or after capacitation (data not shown).

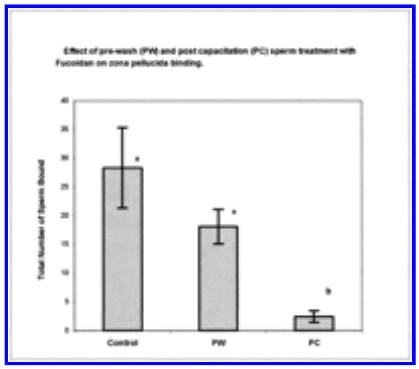

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Figure 2. Zona pellucida binding assay with control sperm or sperm treated with 1.5 mg/mL fucoidin either before centrifugation through Percoll (before capacitation; Pre-C) or after activation (postcapacitation; Post-C). Columns represent averages of sperm bound per zona with sperm from 4 different males and a total of 8 zonae per treatment. Error bars represent SEM. Different letters above columns indicate significant differences between treatment means ($P < .001$).

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In Vitro Fertilization

In all 4 IVF cycles, both pre- and postcapacitation treatment of sperm with LSA blocked fertilization (Table 2; a total of 19 oocytes were used for each of the LSA treatments). Only those oocytes that were inseminated with control sperm formed pronuclei and underwent cleavage (Table 2; 11 of 18 oocytes were fertilized). As in the sperm-zona binding studies, there was no difference between treatments in their effect on sperm motility (Table 1). There was a drop in percent sperm motility following dilution and activation in 2 of the replicate experiments that may account for the lower fertilization rates in the control groups in those IVF cycles (Table 1). A difference in sperm binding to the zona pellucida was apparent between oocytes incubated with control sperm and those incubated with LSA-treated sperm (Figure 3).

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Table 2. *In vitro fertilization rates*[*]

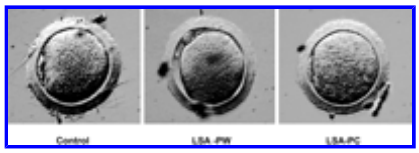

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Figure 3. Following 4 hours of coincubation of gametes for IVF, oocytes inseminated with control sperm **(A)**, sperm treated with LSA precapacitation **(B)**, and sperm treated with LSA after capacitation **(C)** were lightly rinsed once and photographed with an AxioCam digital camera (Carl Zeiss Vision GmbH, Germany) installed on an inverted Olympus Ix70 microscope with Hoffman modulation contrast optics.

Localization of LSA on Sperm Surface

Sperm labeled with LSA-b before washing were observed for surface labeling before and after Percoll separation, following overnight incubation, and after activation. At all stages, sperm retained a surface label that did not appear to change in distribution or intensity. Images of representative LSA-b labeled sperm captured shortly after Percoll separation and after capacitation, approximately 24 hours later, are shown in Figure 4. LSA-b appeared to bind to the entire sperm, but label intensity was greatest over the head and midpiece (Figure 4, B2 and C2). Before fixation, this pattern was observed in 100% of living sperm with little variation. A small percentage of dead or nonmotile sperm fluoresced intensely along their entire length, presumably from a nonspecific association of antibiotin. This was occasionally seen in the NHS-biotin controls as well. Two separate controls for label specificity were run in parallel. Sperm incubated with untreated LSA (no added amine groups) that was reacted with NHS-biotin exhibited the same labeling patterns, but at 25%-50% of the intensity. This could result from limited reaction of NHS-biotin with untreated LSA or from insufficient removal of NHS-biotin following dialysis. As a control for the dialysis, NHS-biotin alone was dialyzed. The retained solution was used as the labeling control. In this case, light labeling of the midpiece was observed in all stages (Figure 4, A2) but this was also observed with antibiotin treatment of previously untreated sperm. Labeling patterns differed slightly with

postcapacitation addition of LSA-b. LSA did not appear to bind to either the principal piece of the flagellum or the posterior head of capacitated sperm in most sperm (Figure 4, D2). Addition of LSA-b after capacitation results in more intense fluorescence over the head in 80%-90% of sperm. To verify that LSA-b maintains antifertility effects, sperm treated with LSA-b before capacitation were assessed for ability to bind to the zona pellucida. LSA-b significantly inhibited binding compared to controls (4.5 ± 1 sperm/zona vs 25.5 ± 7 sperm/zona, respectively; mean ± SEM; $P < .01$).

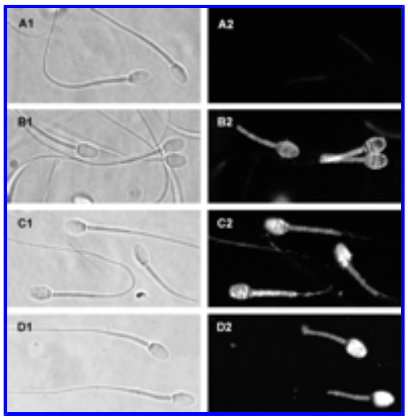

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Figure 4. Fluorescence micrographs displaying labeling patterns of control sperm **(A2)**, sperm labeled with LSA-b before washing (PW; **B2** and **C2**), and sperm labeled with LSA-b after capacitation (PC; **D2**). Prewashed labeled sperm are shown shortly after Percoll separation **(B2)** and after activation **(C2)**. The corresponding phase contrast images are also shown **(A1-D1)**.

Discussion

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Lignin-derived macromolecules are highly sulfonated and negatively charged compounds that exhibit a number of biological activities. LSA and other LDMs are potent inhibitors of HIV in vitro, possibly through interference with the CD-4 receptor/HIV interaction (Suzuki et al, 1989b,c). In vitro microbicidal activity of LDMs has been demonstrated with several other viruses as well (Sorimachi, 1990). This antiviral and antipathogen activity is cell surface—mediated and is likely related to similar inhibitory effects of other sulfonated and sulfated macro-molecules (Zaretzky et al, 1995; Pearce-Pratt and Phillips, 1996; Zacharopoulos and Phillips, 1997a; Anderson, 2000). Selective effects of LDMs on various cell types have also been observed. For example, and LDM has been shown to dramatically inhibit the growth of fibroblast and sarcoma cells (Sorimachi, 1992), but yet activate murine macrophages and induce proliferation of bone marrow cells (Suzuki et al, 1989a). LDMs have also been shown to inhibit fertilization in echinoderms without showing cytotoxic effects (Higashi et al, 1992; Cherr et al, 1993) through a mechanism that involves inhibition of the sperm acrosome reaction (Pillai et al, 1997). Another polysulfonated compound has recently been shown to have sperm-mediated inhibition of fertilization in nonprimate species and is proposed as a potential human contraceptive (Anderson, 2000).

Similar biological properties have been described for common sulfated polysaccharides, some of which affect sperm-oocyte interaction. Dextran sulfate has been shown to inhibit cell fusion (Krumbiegel et al, 1992) and also appears to have a high affinity for mammalian sperm surface proteins (Peterson et al, 1985). This association with the sperm surface inhibits sperm fusion with mouse and hamster oocytes (Ponce et al, 1994), and inhibits human sperm attachment to the zona pellucida (Oehninger et

al, 1991). Similarly, fucoidin, a large polysulfated and negatively charged polysaccharide, has been shown to disrupt the organization of cell culture monolayers in a specific manner (Glabe et al, 1983), as well as inhibit sperm-oocyte interaction in both sea urchins (Glabe et al, 1982; Deangelis and Glabe, 1987, 1988) and mammals (Peterson et al, 1984; Oehninger et al, 1990, 1992).

The mechanism of bioactivity of sulfated macromolecular anions is unclear, but evidence suggests that they act on specific cell surface receptor conformation rather than acting nonspecifically through charge-related effects (Yoshida et al, 1988; Suzuki et al, 1989b,c; Miller and Ax, 1990). Huang and Yanagimachi (1984) showed that fucoidin had a high affinity for the inner acrosomal membrane of guinea pig sperm and was a potent inhibitor of sperm-zona binding. Because fucoidin is a heteropolymer of L-fucose much of this inhibition was originally attributed to the sugar moieties. Subsequently, fucoidin has been shown to be an effective inhibitor of both primary and secondary binding of mammalian sperm to zonae pellucidae, and sulfation of the molecule has been implicated in this bioactivity (Mahoney et al, 1993). The extent, orientation, and distribution of the sulfation charge along anionic polymers are believed to be determining factors in their relative affinities during complementary docking to ligands (Deangelis and Glabe, 1990; Kim et al, 1997). Our previous studies on an LDM related to LSA have shown that it inhibits the sea urchin sperm acrosome reaction (Pillai et al, 1997), and we have preliminary evidence that LSA competes with the natural sulfated ligand, egg jelly, on the sperm surface (Salinas and Cherr, 2000). Furthermore, we have observed that LSA binds to the head of capacitated macaque sperm (Figure 4, B2 and D2), a location consistent with its biological activity.

Because of our previous observations of an antifertility effect of LSA on sea urchin sperm, we carried out the present experiments to determine the effects of this material on the interaction of primate gametes. LSA significantly inhibited the binding of macaque sperm to macaque zonae pellucidae both when the compound was added to sperm after capacitation and when it was added to sperm before Percoll separation and capacitation (Figure 1). Changes in binding properties were not the result of differences in the acrosomal status of sperm. Treatment of sperm with LSA before washing or after capacitation resulted in fewer acrosome-reacted sperm as compared to controls. Presumably, LSA remains with the sperm surface even after thorough washing and long-term incubation. The experiments were repeated with fucoidin, which was previously demonstrated to inhibit human sperm—zona pellucida binding (Oehninger et al, 1990, 1991, 1992). In contrast to LSA, fucoidin, which inhibited sperm-zona binding when added after capacitation, had no significant effect on binding when added before capacitation (Figure 2). The reason for the apparent difference in avidity for the sperm surface of the 2 macromolecules is not known but it may be related to differences in the nature of the sulfur moieties, sulfate (fucoidin) or sulfonate (LSA). Furthermore, the concentration and orientation of anionic charges may differ between the 2 compounds. LSA in particular has a complex, highly branched structure (Pearl, 1967; Higashi et al, 1992) that may promote "binding" to a large number of sperm surface receptors that recognize anionic domains. The fact that non-capacitated sperm bind LSA and are rendered incapable of zona binding suggests that some form of one or more sperm receptors for the zona pellucida is exposed on the surface of noncapacitated sperm. This evidence appears to be in conflict with the results of other studies that have suggested sperm receptors for zona ligands are not exposed until after capacitation (Iborra et al., 1996; Youssef et al, 1997; Fraser, 1998). LSA may provide a useful tool for elucidating the relationship between capacitation and the sperm functions involved in binding to the zona pellucida.

From localization studies, LSA-b appears to remain on the sperm surface through Percoll washing, overnight incubation, and activation. Based on consistent label intensity observed at several stages of the capacitation process, little if any LSA is lost. LSA appears to be distributed over the entire surface of sperm but is more concentrated over the head and midpiece (Figure 4, B2 and C2).

This pattern does not appear to change with a sperm's capacitation state. Labeling is consistent with functional studies that suggest that LSA acts irreversibly on noncapacitated sperm. When added after capacitation, LSA binds primarily to the head in most sperm and, judging from the increase in fluorescence intensity, binds in greater quantities than on precapacitated sperm (Figure 4, C2 and D2).

Due to its lack of cytotoxicity and its antifertility effect on noncapacitated sperm, LSA is a strong candidate for development as a vaginal contraceptive. In addition, formulations containing LSA have been shown to prevent infection with sexually transmitted herpes simplex virus in the mouse without causing vaginal irritation (Ward and Tankersley, 1980). Other polyanionic macromolecules, both sulfated and sulfonated, have been shown to prevent sexually transmitted infections when used in vaginal applications (Ward and Tankersley, 1980; Maguire et al, 2001). Given the ability of LSA to inhibit HIV and other retroviruses, it is plausible that LSA may be capable of functioning both as a contraceptive and as a microbicide.

Footnotes

This work was supported by grants U45-HD-29125 and P51-RR00169 from the National Institutes of Health, and by the University of California Toxic Substances research and Teaching Program (Coastal Toxicology Component).

This is Bodega Marine Laboratory Contribution 2166.

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