Macaque sperm coating protein DEFB126 facilitates sperm penetration of cervical mucus†

Theodore L. Tollner1, Ashley I. Yudin1, Cathy A. Treece1, James W. Overstreet1 and Gary N. Cherr2,3,4

1Center for Health and the Environment, Bodega Marine Laboratory, University of California, Davis, USA; 2Department of Environmental Toxicology, Bodega Marine Laboratory, University of California, Davis, USA; 3Department of Nutrition, Bodega Marine Laboratory, University of California, PO Box 247, Bodega Bay, Davis, CA 94923, USA
4Correspondence address. E-mail: gncherr@ucdavis.edu

BACKGROUND: Sperm coating protein beta-defensin 126 (DEFB126) is adsorbed onto the entire surface of macaque sperm in the caudal epididymis and is retained on viable sperm collected from the cervix and the uterine lumen of mated female macaques. We investigated the role of sperm coating protein DEFB126 in cervical mucus penetration (CMP). METHODS: Cervical mucus (CM) was collected from peri-ovulatory female macaques and loaded into CMP chambers. Sperm were introduced to CMP chambers following treatment with either polyclonal antibodies raised to DEFB126 or seminal plasma proteins (SPPs), 1 mM caffeine 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) (induces release of DEFB126 from sperm surface), neuraminidase (NMase) or poly-L-lysine (PLP). Following removal of DEFB126 or SPPs from the sperm surface, sperm were treated with concentrated DEFB126 or concentrated SPPs prior to being introduced to CMP chambers. The numbers of sperm that penetrated and traversed CM were scored over 6 min. RESULTS: Treatment of sperm with anti-DEFB126 antibodies, 1 mM caffeine 1 mM dbcAMP, NMase, and PLP resulted in similar and significant levels of inhibition of sperm CMP, whereas addition of anti-SPPs antibodies had no effect. In experiments where DEFB126 and SPPs were removed, CMP capability of sperm was restored by addition of DEFB126 back to the sperm surface, whereas treatment of sperm with concentrated SPPs slightly inhibited sperm penetration. CONCLUSIONS: DEFB126 and its high negative charge appears to be critical for the movement of sperm through CM in the macaque, while SPPs adhered to the sperm surface offer no advantage in CMP.

Keywords: DEFB126; sperm surface; sperm glycocalyx; cervical mucus penetration

Introduction

In Eutherian mammals, tens of millions to billions of sperm are deposited in the female reproductive tract at the time of copulation but only a minute fraction of these sperm reach the site of fertilization. Those few sperm, perhaps numbering only in the dozens, are highly competent; they exhibit vigorous motility, have normal morphology, and are enabled through numerous intracellular and membrane alterations (capacitation) to fertilize the oocyte (Yanagimachi, 1994; Suarez and Pacey, 2006; for review). Furthermore, the presence of these sperm in the oviductal ampulla coincides tightly with ovulation (Topfer-Petersen et al., 2002; Hunter and Rodriguez-Martinez, 2004; for review). It is evident from many independent observations that the female tract is not acting as a simple conduit, but rather it greatly restricts sperm numbers, selects for sperm quality, preserves sperm viability and controls the release of sperm in a capacitated state at or near the time of ovulation.

In primates, much of the restriction and selection of sperm occurs at the cervix and is largely the result of sperm interaction with cervical mucus (CM). From studies in humans, it has been shown that a large portion of the semen flows away from the vaginal vault within 30 min of coitus resulting in 35 to nearly 100% of sperm eventually leaking out of the vagina (Baker and Bellis, 1993). The small percentage of sperm that enter the cervix is likely drawn there, at least initially, by uterine contractions (Drobnis and Overstreet, 1992; for review). It is generally viewed that subsequent movement of sperm in the cervix and progression into the upper reproductive tract requires that sperm propel themselves via vigorous flagellar movement (Katz et al., 1989; for review). To ascend the cervix, sperm must first overcome resistance applied by CM, which stems from its unique visco-elastic properties (Cone, 1998; for review). CM is a hydrated gel composed of several forms of filamentous, densely glycosylated proteins (mucins) that appear to cross-link at hydrophobic folded peptide ‘beads’ interspersed along their length (Cone, 1998). Nearing ovulation and under
maximal hydration, CM ‘swells’ to form a matrix with box-shaped interstices filled with mucus plasma (Yudin et al., 1989). The interstices are smaller than the sperm head and therefore it is likely that sperm must continually force their way through the mucus microstructure during migration through the cervix (Katz et al., 1989). Evidence of this relationship has been demonstrated with freeze-substitution electron microscopy which shows distorted and disrupted mucus matrix filaments around and trailing sperm that had penetrated CM (Yudin et al., 1989). The importance of these observations can be weighed in light of carefully controlled and timed studies that demonstrate a good correlation between spontaneous pregnancy rates and the ability of sperm to penetrate CM in vitro and in vivo (Eggert-Kruse et al., 1989; Beltso et al., 1996; Glazener et al., 2000; Hunault et al., 2005).

Extensive studies of sperm–CM interaction in humans reveal that various properties of sperm facilitate penetration of CM. Not surprisingly, the character and vigor of sperm motility has been shown to be a critical factor in sperm–mucus interaction. The numbers of sperm entering mucus in vitro depends on the number of sperm in semen exhibiting linear and progressive trajectories (Aitken et al., 1985; Mortimer et al., 1986). Men whose sperm exhibit greater mean straight-line swimming velocity and amplitude of lateral head movements have greater numbers of sperm that penetrate CM (Aitken et al., 1985). A critical number or concentration of progressively motile sperm appears to be required for sperm to readily penetrate the interface of mucus suggesting that sperm somehow act cooperatively (Cummings et al., 1984). Sperm morphology, especially with reference to sperm head shape, also plays a role in cervical mucus penetration (CMP). Men with semen samples that contain an elevated percentage of sperm with amorphous heads have lower numbers of sperm that penetrate CM (Yudin et al., 1995; Morales et al., 1988). This reduced performance is explained in part by the fact that sperm with amorphous heads have an average lower strait-line velocity in both semen and mucus than normal sperm (Morales et al., 1988). Yet, it has been demonstrated that normal and amorphous sperm in CM have nearly identical flagellar beat frequency and amplitude suggesting that amorphous sperm must experience greater drag or resistance while swimming in CM (Katz et al., 1990). The magnitude of increase in resistance cannot be explained solely by deviations from optimal head shape and size but must also stem from changes in sperm surface properties (Katz et al., 1990). Little is known, however, about the surface properties of sperm that are essential for CMP. Given the close apposition of sperm surfaces with the matrix of mucus microfilaments, we speculate that the properties of the sperm plasma membrane and glyocalyx play an important role in the migration of sperm through CM.

We have recently demonstrated in the cynomolgus monkey (Macaca fascicularis) that a single epididymis-derived protein forms a continuous coat on sperm that is released from the sperm surface during in vitro capacitation (Yudin et al., 2003, 2005a, b; Tollner et al., 2004). This epididymal secretory protein (originally called ESP13.2) is now called beta-defensin 126 (DEFB126) due to its amino acid sequence homology and structural similarity to beta-defensins (Lehrer and Ganz, 2002). DEFB126 appears to be the major component of the macaque sperm glyocalyx (Yudin et al., 2005a). DEFB126 bears multiple sialylated oligosaccharides and therefore imparts a high negative charge to the sperm surface (Yudin et al., 2005b). Sperm recovered from the cervix and uterus of mated female macaques are evenly coated with DEFB126 over the entire surface suggesting that DEFB126 is retained on sperm during migration in the cervix and in the upper female reproductive tract (Tollner et al., 2008). In this report, we demonstrate that DEFB126 is crucial for sperm to penetrate and move efficiently in peri-ovulatory CM. Removal of DEFB126 from sperm or cleavage of its terminal sialic acid residues results in significant reductions in the number of sperm that enter and traverse mucus. Addition of soluble DEFB126 back to the sperm surface completely restores CMP ability of sperm, whereas addition of concentrated seminal plasma proteins (SPPs) has no effect. To the best of our knowledge, this is the only report that describes a specific sperm surface component that is required for sperm transport in the lower reproductive tract of primates.

Materials and Methods

Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. (St Louis, MO) unless stated otherwise.

Antibody production and preparation

Antibodies were developed to the purified DEFB126 as described previously (Yudin et al., 2003). Ejaculated sperm were washed through 80% Percoll, washed by centrifugation (300g, 10 min), resuspended in Delbecco’s phosphate buffered saline (DPBS), and treated with phosho-inositol-phospholipase C (PI-PLC; 3 units/80 x 10^6 sperm) and incubated for 1 h at 37°C. PI-PLC releases a number of protein components from the sperm surface, including DEFB126 (Yudin et al., 2003). Sperm were pelleted by centrifugation (1000g, 10 min) and the supernatant was passed through a 0.22 μm syringe filter, concentrated and electrotheroically separated on an 8–16% gel. The gels were stained with Gel Code Blue (Pierce, Rockford, IL). For DEFB126 collection, the 53 kDa band was cut from the gel and electro-eluted (Yudin et al., 2003). After complete electro-elution, samples were chemically reduced with 0.1 M dithiothreitol and electrophoresed on a 16% gel. The entire 31–35 kDa band was cut from the gel and electro-eluted for immunization.

Antibodies were also developed to DEFB126 on intact sperm as described previously (Yudin et al., 2005b). Briefly, sperm were washed in DPBS by centrifugation (300g, 10 min) and fixed in 3% paraformaldehyde for 30 min. Fixed sperm were washed twice by centrifugation in DPBS (300g, 10 min each), the sperm pellet was mixed with Freund’s adjuvant and injected into rabbits as previously described (Yudin et al., 2005b). Antibodies were also raised to SPPs. Seminal plasma was collected and purified as described below. SPPs were prepared for immunization in rabbits as described previously (Yudin et al., 2003).

The resulting serum samples were initially heat inactivated (56°C/30 min) and then precipitated with ammonium sulfate (0.24 g/ml). The ammonium sulfate was added slowly over a 4 h period at 4°C. The precipitated Igs were pelleted and resuspended in DPBS (Life
DEFB126 facilitates sperm penetration of cervical mucus

Technologies, Rockville, MD) and dialyzed overnight. Total protein concentration of precipitated IgGs was determined with a BCA protein analysis system (Pierce). The IgGs was stored at -20°C. IgGs raised to both isolated DEFB126 and to DEFB126 on sperm only recognized DEFB126 (a single band at 31–35 kDa) on western blots of whole sperm (Yudin et al., 2003, 2005b). IgGs raised to SPPs recognized multiple protein bands on western blots ranging from 14 to 64 kDa (Fig. 8).

Antibodies to sperm protein PSP94 were raised in chickens as described previously (Tollner et al., 2004). PSP94 was isolated and excised from gels as described above for DEFB126. Aliquots of PSP94 (25 µg/each) were mixed with Freunds complete adjuvant and used for the first injection. Three subsequent injections were done with Freunds incomplete adjuvant for a total of four immunizations into three separate chickens. After the fourth injection, a series of eight eggs was collected and the IgY was purified using the reagents and procedures outlined in Eggcellent (Pierce). Antibodies to PSP94 localize to the plasma membrane overlying mid-piece and principal piece of the sperm (Tollner et al., 2004).

Animals

Eight male and 10 female cynomolgus macaques were housed at the California National Primate Research Center (CNPRC) in compliance with American Association of Accreditation of Laboratory Animal Care Standards. The Institutional Animal Care and Use Committee (IACUC) at the University of California, Davis, approved all methods and procedures with animal subjects.

Semen collection and sperm preparation

Semen samples were collected by electro-ejaculation from eight individually caged male cynomolgus macaques (Sarason et al., 1991). Each ejaculate was collected into a 15 ml centrifuge tube containing 5 ml of Biggers, Whitten and Whittingham medium (BWW; Biggers et al., 1971) modified with addition of HEPES buffer (21 mM; Irvine Scientific, Santa Ana, CA), supplemented with 3 mg/ml BSA, and adjusted to a pH of 7.4 with concentrated NaOH (hereafter the modified BWW is referred to as mBWW). After 1 h, the samples were checked for motility and only those samples having >70% motile sperm were used in experiments. Following removal of coagulum, sperm were pelleted by centrifugation (300g, 10 min.) and resuspended to a concentration of 30 x 10⁶/ml in mBWW. Sperm were then treated with various antibodies and compounds to mask, remove or modify DEFB126 prior to CMP experiments as described below. For all sperm experiments, percent motility was determined for all treatment groups. The percentage of progressively motile sperm was scored with a cell counter for 200 sperm each treatment, 8 per treatment were analyzed for straight-line velocity, curvilinear velocity, linearity and amplitude of lateral head displacement.

Treatment with IgGs

Sperm were incubated for 30 min with polyclonal antibodies raised to DEFB126 and PSP94 at a final concentration of 100 µg/ml or polyclonal antibodies raised to SPPs that ranged in concentration from 100 to 200 µg/ml. Controls received equivalent dilution with DPBS. Sperm suspensions were diluted 15-fold by volume with mBWW medium, washed 1 x by centrifugation (300g, 10 min), and resuspended to a concentration of 30 x 10⁶/ml in mBWW.

Removal and ‘add back’ of DEFB126

Sperm were capacitated with activator (ACT), a cocktail of 1 mM caffeine and 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP), for 1 h resulting in the loss of DEFB126 from over the sperm surface (Tollner et al., 2004). Controls were treated with equivalent volume of DPBS. Sperm were washed out of ACT by centrifugation (2 x 300g, 10 min each), resuspended in mBWW and incubated an additional 30 min. Control and ACT-treated sperm were split into two equal volumes each and pelleted by centrifugation (300g, 10 min). The volume of the pellets was brought up to 300 µl with mBWW. One of the pellets of ACT-treated sperm received 100 µl ‘add back’ solution of DPBS containing soluble DEFB126 that was prepared as described by Tollner (2004). The concentration of DEFB126 in the ‘add back’ solution was 29 µg/ml as determined by a BCA protein analysis system (Pierce). The other ACT-treated pellet and one of the control pellets received 100 µl of DPBS containing 30 µg/ml BSA. All samples were then diluted 15-fold by volume with mBWW and washed by centrifugation (300g, 10 min). Sperm pellets were resuspended in mBWW to give a final concentration of 30 x 10⁶ sperm/ml.

Treatment with NMase, PLP

One ml aliquots of sperm were treated with neuraminidase (NMase) (0.5 units/10 x 10⁶ sperm/ml for 30 min, whereas controls received the equivalent volume (30 µl) of DPBS. Sperm were diluted 15-fold with mBWW, washed by centrifugation (300g, 10 min) and resuspended to concentration of 30 x 10⁶/ml in mBWW. In separate experiments, sperm were treated with 1–5 kDa poly-l-lysine (PLP) at 100, 200 and 400 µg/ml in mBWW for 30 min with gently rocking. Control sperm received equivalent volume of mBWW. Sperm were diluted, washed and resuspended as described for NMase treatment.

Removal of DEFB126 and elimination of hyperactivated motility

Sperm were treated with ACT for 1 h. Controls were treated with equivalent volume of DPBS. Sperm were washed by centrifugation (300g, 10 min) and allowed to return to normal (non-hyperactivated) motility over the next 2 h before they were used in CMP assays. Two hours following ACT treatment of the first aliquot, another aliquot of sperm from the same male was treated with ACT. These sperm were washed by centrifugation (300g, 10 min) and after an additional 10 min, while still displaying hyperactivated motility were used for CM penetration experiments.

Hyperactivated motility was verified with computer-assisted sperm analysis (CASA). Videomicrography was performed as described previously (VandeVoort et al., 1994) but with the following changes. For each treatment, 8 µl drops of sperm suspension were loaded into 2-µm cell semen analysis chambers (Fertility Technologies Inc., Natick, MA) with a 10 µ depth. In each chamber, 8–10 randomly selected microscope fields were video recorded, capturing several hundred sperm. Motion characteristics of the recorded sperm were analyzed using the Hobson Tracker V72B (Hobson Tracking Systems, Inc., Sheffield, UK). Sperm tracks were digitally captured using a frame rate of 60 Hz and a minimum track time of 1 s. At least 200 sperm per treatment were analyzed for straight-line velocity, curvilinear velocity, linearity and amplitude of lateral head displacement.

Treatment with SPPs

Semen samples were collected into 1 ml mBWW without BSA and incubated for 15 min at room temperature. Sperm were pelleted at ~2000g for 5 min. Supernatants were filtered to remove cytoplasmic droplets using 100 kDa MWCO Centricron centrifugal filter devices according to manufacturer specifications (Millipore, Bedford, MA). The retentate was discarded and the filtrate was concentrated ~10-fold using a 3 kDa MWCO Centricron device. Total protein concentration of SPPs was determined by BCA protein analysis.
Prior to sperm treatment with SPPs, sperm were washed through a 3 ml column of Percoll (40% with BWW) to remove excess coating proteins (Yudin et al., 2005b). Sperm pellets were resuspended in mBWW and washed again (300g, 10 min) and resuspended to 15 × 10⁶/ml. SPPs were added 1:1 by volume to one aliquot of macaque sperm for a final concentration of ~130 μg SPPs/ml. Soluble DEFB126 was added to another aliquot as described above, and mBWW was added to a third (control). After 30 min of gentle rocking, sperm suspensions were further diluted 15-fold with mBWW, washed by centrifugation (300g, 10 min) and resuspended to concentration of 30 × 10⁶/ml in mBWW.

The purity of SPPs was evaluated using an 8–16% Tris–Glycine gel for electrophoresis (Invitrogen, Carlsbad, CA). The proteins were solubilized in SDS-reducing buffer (Pierce), electrophoresed and stained as described previously (Yudin et al., 2003). After electrophoresis, the gel was electroblotted to nitrocellulose membranes and blocked for at least 1 h in TBS (50 mM Tris–HCl, pH 7.4, 0.3 M NaCl) containing 5% non-fat dry milk and 0.1% NaN₃. After blocking, the blots were incubated with 50 μg/ml of Igs (either anti-DFEB126, anti-PSP94, or anti-SPPs as prepared above, or anti-human PSA polyclonal Ig; Imgenex, San Diego, CA) per 10 ml of TBS with 3% BSA and 0.1% NaN₃. After thorough washing in TBS (50 mM Tris–HCL, pH 7.4, 0.3 M NaCl), blots were subsequently incubated with the appropriate secondary antibody (1:2000), goat anti-rabbit IgG-alkaline phosphatase (BioRad; Richmond, CA) or goat anti-chicken IgG-alkaline phosphatase (Aves Labs, Tigard, OR). After washing in TBS, immune complexes were detected using precipitating alkaline phosphate substrate (1-Step NBT-BCIP, Pierce).

For comparison, nitrocellulose blots of whole sperm were also probed with anti-DFEB126, anti-PSP94, anti-PSA, and anti-SPPs Igs as described above. Methods of solubilization, gel electrophoresis and western blotting of whole sperm have been described in detail previously (Yudin et al., 2003). To provide reference for the positions of sperm coating proteins on blots, proteins released from the surface of sperm with ACT were also solubilized, electrophoresed and stained as described previously (Tollner et al., 2004). Whole ejaculated sperm (washed 1 × in mBWW at 300g for 10 min) were also solubilized and blotted following either an additional 2 × wash in mBWW by centrifugation (300g, 10 min each) or a wash through 40% Percoll as described above. Subsequently, blots were probed with anti-SPPs Igs.

**CM penetration experiments**

CM was collected from 10 peri-ovulatory female cynomolgus macaques into polyethylene catheters. Females were briefly anesthetized with ketamine hydrochloride (10 mg/kg body weight) prior to CM collection. A pediatric proctoscope was inserted into the vagina and a 10 cm long polyethylene catheter (ID = 1.19 mm; OD = 1.70 mm) was guided to the cervical region. A stainless steel stylet fed into the catheter facilitated the insertion of the last 1 cm section of the catheter into the cervical OS. Once the catheter was in position, the stylet was removed and gentle suction applied, recovering several centimeter of CM. CM from a single female was used for all replicates within an experimental set with the exception of experiments using SPPs, in which CM from two different females was used. A small volume of CM corresponding to 0.5 cm length of catheter was expressed onto a glass slide. The drop of CM was surrounded by a ‘U’-shaped continuous column of silicone grease containing 20-μm diameter glass beads. A 22 × 22 mm glass coverslip was carefully pressed down onto the column until the grease was flattened to the limit of the glass beads forming a chamber for the CM that was enclosed on three sides (Fig. 1A). The slide was warmed for 5 min on a microscope stage warmer (Motion Analysis Inc., Santa Clara, CA) set at 39°C prior to the addition of sperm. Following various treatments described above, 10 μl of sperm suspension were introduced to the open side of the CM chamber and were immediately drawn by capillary action to the CM interface. The open side of the chamber was then sealed with a drop of mineral oil warmed to 39°C. Sperm were observed in CM with an Olympus BH2 microscope and a 10 × phase objective. A CCD black and white video camera (Panasonic model vv-BD400), attached to the microscope via an Olympus adapter (with 3.3 × ocular) captured images of sperm at 30 frames per second. The video signal passed in series through a video time generator (For.A.Inc. model 33) and a 0.5 inch video tape recorder (Panasonic model AG6300). After 2 min from the time sperm were introduced to the chamber, video recordings were initiated, capturing a region in the center of the microscope field that was ~2.75 mm from the sperm–CM interface (Fig. 1B). Recordings continued for a minimum of 4 min. CMP was quantified from video recordings by counting the number of sperm in the video field that was paused at the very beginning of the recording (t = 2) and every min thereafter (t = 3–6) of the 4 min recording interval.

CM penetration data was analyzed with a 1-factor (sperm treatment) ANOVA. Response means of treatments were compared using Tukey’s range test. All experiments (replicates) generated values of numbers of sperm/video field (at 1 min interval from t = 0–4) for every treatment. Data reported as mean number of sperm/video field ± SEM. Differences in treatments were considered significant at values of P < 0.05. All ANOVAs met assumptions of factor independence, as well as normality of data distribution. Frequently, sperm from control groups exhibited greater variation in CMP than did treatments that inhibited sperm CMP. When responses did not meet

---

**Figure 1:** Diagram of CM chamber. (A) General configuration of CM chamber with a flattened bead of silicon grease containing 20-μm diameter glass particles (dark gray) establishing both the borders and the depth of the CM chamber. (B) Enlargement of the sperm suspension–CM interface to show distance of video field from the leading edge of CM.
conditions of homogeneity of variance as determined by Levene’s test, data were transformed using square root function before ANOVA. Analyses were conducted with SAS statistical program (SAS Institute, Cary, NC) according to the principles described by Steel et al. (1997).

Results

Treatment of sperm with anti-DEFB126 antibodies consistently resulted in significant inhibition of CMP. Polyclonal antibodies raised to isolated DEFB126 (Ig# 1652) significantly reduced the number of sperm compared with controls that reached the video field at all time intervals after 3 min. This was especially noticeable at 6 min where average inhibition of CMP with 1652 treatment was 89% (Fig. 2). Antibodies to sperm surface protein PSP94 localize to the sperm flagellum (Tollner et al., 2004). These anti-PSP94 antibodies had no appreciable effect on CMP at any time interval. The effect of 1652 on penetration was compared with two other polyclonal anti-DEFB126 antibodies (1459 and 1460), both of which were raised against intact sperm that were fixed prior to injection into rabbits. Treatment of sperm with either 1459 or 1460 appeared to have an enhanced inhibition of CMP when compared with 1652, although this difference was not significant (Fig. 3). All antibody treatments significantly inhibited the numbers of sperm present in the video field at all time intervals after 2 min (Fig. 3). No appreciably different effects on CMP were observed with higher concentrations of Igs (>100 μg/ml) to DEFB126 or PSP94 (data not shown).

Treatment of sperm with ACT, which removes DEFB126 from the sperm surface (Tollner et al., 2004), inhibited CMP significantly over all time intervals after 2 min (Fig. 4). Inhibition was most evident after 6 min with a reduction of the penetration rate by 81.5% (Fig. 4). We demonstrated previously that DEFB126 released from capacitated sperm can be concentrated and added back to the sperm surface (Tollner et al., 2004). Addition of ‘add back’ DEFB126 to sperm following removal of sperm from ACT conditions restored CMP to control levels as assessed at 6 min (Fig. 4).

Approximately 50–60% of sperm undergo hyperactivation following treatment with ACT and retain hyperactivated motility for 20–30 min after being washed out of medium containing ACT. Hyperactivated motility in macaque sperm is characterized by vigorous, non-symmetrical bending of the flagellum resulting in large lateral deflections of the sperm head and a significant decrease in straight-line movement (Vandevoort et al., 1994). In order to test whether motility changes associated with ACT treatment accounted for the inhibition of CMP, we compared penetration ability of ACT-treated sperm immediately after washing into ACT-free medium (sperm remain hyperactivated) with sperm that had been washed free of ACT and incubated for 2 h. Motility patterns of ACT-treated sperm that were incubated for 2 h following washing did not differ significantly from that of controls, while sperm that were video recorded shortly after being washed out of ACT exhibited the decreased straight-line velocity and increased lateral displacement of the head indicative of hyperactivated motility (Table I). Percent motility (as determined by manual counts) and overall progression (as determined by CASA measures of curvilinear velocity) were the same for all three groups. ACT-treated sperm that no longer

Figure 2: Treatment of sperm with antibodies raised to isolated DEFB126 inhibits CMP.

Following treatment of sperm with PBS (control) or antibodies specific to sperm surface proteins DEFB126 (1652) and PSP94, sperm were deposited into slide chambers containing peri-ovulatory CM. After 2 min, sperm were recorded continuously for 4 min as they entered a video field 2.75 mm from the sperm suspension-CM interface (see Fig. 1). From video recordings, numbers of sperm in the video field were counted at 1-min intervals. Points on each curve represent mean numbers of sperm/field ± SEM, experiment conducted with sperm from three different male macaques. Different letters indicate significant differences in mean sperm numbers between treatments within time intervals.

Figure 3: Treatment of sperm with antibodies raised to DEFB126 on intact sperm inhibits CMP.

Following treatment of sperm with PBS (control) or with antibodies raised to either isolated DEFB126 (1652) or DEFB126 on intact sperm (1459, 1460), sperm were deposited into slide chambers containing peri-ovulatory CM. CMP assays were performed as described in Figs 1 and 2. Points on each curve represent mean numbers of sperm/field ± SEM, experiment conducted with sperm from four different male macaques. Different letters indicate significant differences in mean sperm numbers between treatments within time intervals.
Yudin et al. (2005a, b) demonstrated that DEFB126 is highly glycosylated and rich in terminal sialic acid residues. Treatment of non-capacitated macaque sperm with the sialidase NMase greatly shifts the isoelectric focal (IEF) point of DEFB126 but leaves the protein on the sperm surface (Yudin et al., 2005a). Treatment of sperm with NMase inhibited numbers of sperm reaching the video field at all time intervals significantly, except at 5 min where inhibition was not significant (Fig. 6). Over 4, 5 and 6 min, average inhibition of CMP by treatment with NMase was 88.9% (Fig. 6). The highly cationic amino-polymer, PLP binds evenly over the entire surface of macaque sperm, its distribution corresponding to the presence of sialic acid on the sperm surface (Yudin et al., 2005a). Treatment of macaque sperm with PLP significantly inhibits sperm penetration of CM at all time intervals after 2 min (Fig. 7). A maximal inhibition of CMP of 91.2% was achieved with 400 µg/ml PLP (Fig. 7). Doses of PLP > 500 µg/ml could not be used for these studies due to sperm agglutination. No sperm agglutination or changes in percent motility or progression were observed at the doses of PLP used in this report.

Macaque seminal plasma appears to be comprised of at least 9–10 major proteins as determined by the number of bands that focus following gel electrophoresis (Fig. 8, lane F). Antibody probes of nitrocellulose blots indicate that DEFB126 is not among the SPPs (lane I), but that prostatic proteins PSP94 and PSA are represented in seminal plasma (lanes G and H). Antibodies to PSA, PSP94 and DEFB126 all localize to concise bands on blots of whole sperm at the predicted molecular weights for their respective proteins (lanes C–E). The predominant proteins that are released with ACT treatment are DEFB126 and PSP94 (Tollner et al., 2004). A gel of ACT-released sperm proteins shows DEFB126 (~32–35 kDa) and PSP94 (~14 kDa) as reference for the positions of the sperm proteins following electrophoresis (lane B). Western blots of whole sperm washed once after ejaculation were probed with Table I. Motion characteristics of control sperm (non-hyperactivated), sperm washed out of activator and incubated for 2 h post-wash (2 h PW), or for 10 min post-wash (10 min PW; fully hyperactivated) were compared by CASA.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>VSL</th>
<th>ALH</th>
<th>LIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>90.5 ± 4.9a</td>
<td>7.18 ± 0.6a</td>
<td>45.35 ± 3.7a</td>
</tr>
<tr>
<td>2 h PW</td>
<td>78.93 ± 7.3a</td>
<td>7.95 ± 0.6a</td>
<td>34.33 ± 3.3a,b</td>
</tr>
<tr>
<td>10 min PW</td>
<td>51.9 ± 3.4b</td>
<td>11.45 ± 0.5b</td>
<td>23.25 ± 1.3b</td>
</tr>
</tbody>
</table>

Straight-line velocity (VSL) and amplitude of lateral displacement of the sperm head (ALH) are reported in mean ± SEM microns/s. Linearity (LIN) is given by curvilinear velocity/VSL. Different letters within table columns indicate significant differences in mean values between treatments. Experiment repeated four times, each time with sperm from a different male.

Figure 4: Removal of DEFB126 from sperm results in loss of CMP ability which is recovered when DEFB126 is added back to the sperm surface.

Figure 5: Sperm motility changes do not account for the inhibition of CMP observed following sperm treatment with ACT.

Following treatment of sperm with DPBS (control) or with either activator (ACT) to remove DEFB126, or soluble DEFB126 to restore or add back DEFB126 (AB), sperm were deposited into slide chambers containing peri-ovulatory CM. CMP assays were performed as described in Figs 1 and 2. Points on each curve represent mean numbers of sperm/field ± SEM, experiment conducted with sperm from three different male macaques. Different letters indicate significant differences in mean sperm numbers between treatments within time intervals.

exhibited hyperactivated motility had no advantage in CMP over ACT-treated sperm that remained hyperactivated (Fig. 5). To the contrary, more hyperactivated sperm on average reached the video field after 5 and 6 min than did non-hyperactivated ACT-treated sperm (Fig. 5). Both ACT-treatment groups exhibited significantly reduced CMP at both time intervals compared with controls, presumably due to the loss of DEFB126 (Fig. 5).

Yudin et al. (2005a, b) demonstrated that DEFB126 is highly glycosylated and rich in terminal sialic acid residues. Treatment of non-capacitated macaque sperm with the sialidase NMase greatly shifts the isoelectric focal (IEF) point of DEFB126 but leaves the protein on the sperm surface (Yudin et al., 2005a). Treatment of sperm with NMase inhibited numbers of sperm reaching the video field at all time intervals compared with controls, presumably due to the loss of DEFB126 (Fig. 5).
anti-SPPs Igs. At least 6 SPPs that are associated with sperm are highly immuno-reactive (Fig. 8, lane J). Following an additional 2× wash in medium, most of these SPPs have been removed from sperm (lane K). Following washing through 40% Percoll, only one protein is still detectable but is markedly reduced in intensity (lane L).

We addressed the role SSPs in sperm penetration of CM. Treatment of sperm with concentrated SSPs (130 μg/ml) following either washing through Percoll (Fig. 9A) or 2× wash with medium (Fig. 9B) consistently resulted in fewer sperm in the video field at each time interval compared with controls, although the differences in penetrations were only significant when sperm were first washed with medium (Fig. 9B). No significant effect on CMP was observed with addition of SSPs at concentrations < 100 μg/ml (data not shown). In contrast, CMP following treatment of sperm with soluble DEFB126 did not differ from CMP of controls regardless of the washing method used prior to treatment. A relatively large variation in response across all treatments was observed and was likely due at least in part to differences in the properties of CM from the two different females used in this experiment. Treatment of sperm with anti-SPP Igs had no effect on CMP, even when the concentration of Igs was several fold higher than that of anti-DEFB126 Igs (Fig. 10).

Discussion

It has been twenty years since it was initially proposed that a surface component lost at capacitation provided sperm with the surface properties essential for transport through mucus (Katz et al., 1989; for review), yet no specific sperm-associated factors have been identified that impart this function. Given that: (i) DEFB126 remains on sperm following passage through the cervix (Tollner et al., 2008); (ii) it is responsible for the high negative charge on the sperm surface (Yudin et al., 2005b) and (iii) CM is known to consist of negatively charged mucin molecules (Cone, 1998; Lagow et al., 1999), we hypothesized that DEFB126 is critical for sperm passage through CM. Initially, we evaluated the effect of anti-DEFB126 Igs on CMP. All anti-DEFB126 Igs significantly inhibited CMP compared with controls, with antibodies...
generated to motifs exposed on the sperm surface potentially having a greater inhibitory effect.

The effect of anti-DEFB126 Igs on CMP could result from a general increase in drag experienced by sperm rather than a masking or neutralization of surface properties specific to DEFB126. A large number of clinical reports have demonstrated that anti-sperm antibodies can greatly inhibit CMP and is a common cause of infertility in humans (Hjort, 1999; Lombardo et al., 2001; for review). However, the immobilization of sperm in CM in response to surface-directed Igs is mediated by Ig type A and not IgG (Kremer and Jager, 1992; for review; Jager et al., 1981a, b), which constitutes the vast majority of the serosal polyclonal Ig isotypes raised against haptens administered with complete Freund's adjuvant in rodents (Furuichi and Koyama, 1975; Portis and Coe, 1976; Beck and Spiegelberg, 1989). In order to test if antibodies to other sperm surface proteins would also result in reduced CMP, we were limited to using anti-PSP94 Ig, which binds to the flagellum of non-capacitated sperm (Tollner et al., 2004), since other proteins that are firmly associated with the sperm surface appear to be masked by DEFB126 (Yudin et al., 2005a, b). Antibodies raised to three different plasma membrane proteins overlying the sperm head will not localize to the sperm surface until DEFB126 is removed (Yudin et al., 2005b). Treatment of sperm with anti-PSP94 Ig had no effect on CMP, which supports the conclusion that antibodies to DEFB126 may be altering properties of the DEFB126 surface coat. It must be noted that antibodies that attach to the flagellum only may not impede sperm movement in mucus as much as head-directed antibodies of a similar isotype (Wang et al., 1985). The fact that anti-DEFB126 Igs cover the entire sperm surface suggests that maximal drag could be imposed on sperm. Whether by DEFB126-specific or non-specific mechanisms, the finding that anti-DEFB126 antibodies reduce CMP has implications for immunocontraceptive development, whereby vaccine-induced Ig production to DEFB126 may establish an effective blockade to sperm movement through the cervix.

Treatment of sperm with ACT induces capacitation in macaque sperm and is accompanied by an efficient and nearly complete release of DEFB126 from the surface of most sperm (Yudin et al., 2003; Tollner et al., 2004). In the present study, ACT treatment reduced CMP as much as 81% suggesting that the removal of DEFB126 greatly impedes

**Figure 9:** Treatment of sperm with SPPs inhibits CMP. Sperm previously washed through Percoll (A) or washed 2 x in mBWW (B) were treated with either PBS (control), SPPs, or DEFB126 (DEFB). Sperm were washed into mBWW and added to slide chambers for CMP assays. Points on each curve represent mean numbers of sperm/field ± SEM, experiments conducted with sperm from four different male macaques. Different letters indicate significant differences in mean sperm numbers between treatments within time intervals.

**Figure 10:** Treatment of sperm with anti-SPPs Igs has no effect on CMP. Following treatment of sperm with either PBS (control), with anti-DEFB126 antibodies (1652; 100 μg/ml) or with anti-SPPs antibodies (100 and 200 μg/ml), sperm were deposited into slide chambers containing peri-ovulatory CM. CMP assays were performed as described in Figs 1 and 2. Points on each curve represent mean numbers of sperm/field ± SEM, experiment conducted with sperm from four different male macaques. Different letters indicate significant differences in mean sperm numbers between treatments within time intervals.
sperm movement through CM. To determine if this response was specific to DEFB126 removal and not to other changes in surface properties associated with capacitation, we evaluated CMP following the addition of DEFB126 back to the sperm surface. We have demonstrated previously that sperm can be recoated with DEFB126 if they have been washed out of capacitating conditions (Tollner et al., 2004, 2008). Removal of DEFB126 from the sperm surface is required for sperm to recognize and bind the zona pellucida (Tollner et al., 2004). The ‘add back’ of DEFB126 appears to restore the surface properties of sperm to their original state as determined by re-establishment of inhibition of sperm–zona pellucida binding (Tollner et al., 2004). Similarly, following ‘add back’ of DEFB126, CMP was restored to slightly better than control levels although the difference was not significant.

As motility of the sperm in the ACT-treatment group remained hyperactivated even though they were washed similarly to the ‘add back’ treatments, we wanted to evaluate the effect on CMP of changes in motility associated with activation. We compared two ACT-treated groups; one which retained hyperactivated motility shortly after washing and one which was incubated until motility no longer appeared hyperactivated. No advantage for CMP was observed with non-hyperactivated sperm. In fact, hyperactivated sperm were significantly more motile in CM although both ACT-treated groups had significantly reduced CMP compared with controls. Consistent with this observation, it has been demonstrated that an increase in sperm curvilinear velocity and lateral head displacement indicative of hyperactivation provides sperm with greater thrust, and hence mobility in high viscosity solutions (Shier et al., 1984; Pugliese et al., 1989; Steadman et al., 1990). PLP when added to macaque sperm coats the entire sperm surface (Yudin et al., 2005a) and nearly blocked sperm penetration of CM. These data indicate that a high negative surface charge is required by sperm to move relatively un-abated past mucin molecules in CM, which are nearly saturated with negatively charged carbohydrates along most of their segments (Cone, 1998; Lagow et al., 1999).

Numerous studies across many mammalian species have demonstrated that at the time of ejaculation, sperm possess a high net negative charge that is retained until they undergo capacitation. The addition of negatively charged carbohydrates to sperm appears to be a conserved feature of maturation. Bedford (1963) observed that rabbit sperm increased in electrophoretic mobility following transit through the epididymis. The isoelectric point for rabbit sperm extracted from the caudal epididymis was ~4.4, suggesting that mature sperm posses a high net negative charge (Bedford, 1963; Moore, 1979). Measures of total surface anion sites with colloidal iron or gold show that sperm steadily increase in negative surface as they move from the caput to the distal cauda in the hamster (Yanagimachi et al., 1972, 1973), boar (Stoffel et al., 2002), ram, bull (Holt, 1980) stallion (Lopez et al., 1987) and chimpanzee (Gould et al., 1984). The increase in net surface charge corresponds with an increase in sialylation of the sperm surface. Labeling intensity of the sperm surface associated with WGA (weat germ agglutinin; specific for sialic acid residues and N-acetyl glucosamine) increases with epididymal transit in the rat, mouse, hamster, rabbit and goat (Kumar et al., 1990), as well.
as the ram (Magargee et al., 1988), baboon, vervet monkey (Fourie et al., 1996) and human (Arenas et al., 1996). The majority of the WGA-binding was eliminated when human sperm were pretreated with NMase which cleaves terminal 2,6-linked sialic acid residues (Lassalle and Testart, 1994). Similarly, virtually all of the negative surface charge added to ram and bull sperm during maturation was eliminated with NMase treatment (Holt, 1980). Furthermore, treatment of human sperm with NMase reduced net negative surface charge to a fifth its original value as measured shortly after ejaculation (Rosado et al., 1973). During capacitation, about one half of net negative surface charge of ejaculated human sperm is lost (Rosado et al., 1973). Similarly, capacitating conditions dramatically reduced the percentage of bull sperm that migrated electrophoretically to the anode (Iqbal and Hunter, 1995a). The greater the loss of net negative charge, the greater the efficiency of bull sperm penetration of zona-free hamster oocytes (Iqbal and Hunter, 1995a, b). Capacitation of human sperm is associated with a >60% decrease in WGA-binding over the anterior head (Lassalle and Testart, 1994) which coincides with the release of numerous sialylated glycoproteins (Focarelli et al., 1990). As demonstrated with our studies with DEFB126 (Tollner et al., 2004; Yudin et al., 2005a), we speculate that the shift in charge in mammalian sperm with capacitation is due to loss of coating proteins that are rich in sialylated glycoconjugates. This would explain why human sperm that are incubated under capacitation conditions lose the ability for sustained mucus penetration in vitro after 2–4 h despite maintenance of vigorous flagellar motility (Katz et al., 1988, 1989).

Negative surface charge is likely critical for sperm transport and survival in the female reproductive tract. First, the sperm glyocalyx, in particular the negatively charged sialic acid residues, have been shown to play a potential role in immunoprotection. Removal of sperm surface sialic acid exposes surface proteins to detection by antibodies (Czuppon, 1984; Yudin et al., 2005b) and enhances phagocytosis of sperm by macrophages (Toshimori et al., 1991). It has been proposed that sialyl-glycoconjugates of the sperm glyocalyx mask potentially highly immunogenic sperm-specific proteins that are critical to fertilization (Toshimori et al., 1991; Yudin et al., 2005b). This idea is supported by the fact that treatment of non-capacitated human and non-human primate sperm with NMase enables sperm to recognize and bind to the zona pellucida, presumably by unmasking sperm receptors for the zona (Lassalle and Testart, 1994; Tollner et al., 2008). The protective qualities conferred by sialylated oligosaccharides are hardly unique to sperm, having been well-described in an assortment of somatic cells (Schauer, 2004; for review) and in microorganisms that scavenge exogenous sialic acid in order to go undetected in the host (Vimr and Lichtensteiger, 2002; for review). Secondly, sperm of all mammals must move through the secretions of mucosal membranes of the female reproductive tract. Epithelia of the uterus and oviduct of rodents, ruminants and primates secrete several classes of mucins or mucin-like glycoproteins (Lagow et al., 1999; for review). Mucins secreted in the oviduct are particularly acidic (Jansen, 1995) with 50% of molecular mass of some members being contributed by carbohydrates (Lagow et al., 1999). It would not be surprising if movement of sperm into and through the oviduct would be greatly restricted if sperm did not possess an adequate negative surface charge. It is interesting to note that sperm from male mice that are null mutants for the gene that encodes for fertilin b, a major component of the sperm glyocalyx (Schorer et al., 1999), are infertile (Cho et al., 1998), cannot pass through the utero-tubal junction even though they possess normal morphology and motility (Cho et al., 1998). Perhaps reduced surface charge underlies the failure of these otherwise normal-looking sperm to enter into the oviduct.

While the importance of sperm surface charge in CMP has not been reported previously in mammals, several studies have demonstrated the requirement of a negatively charged glyocalyx for successful sperm transport in the chicken. As in mammals, the terminal sugars of the glyocalyx of poultry sperm are highly sialylated to the extent that other carbohydrate components are effectively masked until sperm are treated with sialidase (Pelaez and Long, 2007). Also like mammals, sialylation of the sperm surface appears to occur as a consequence of transit through the avian epididymis and ductus deferens (Esponda and Bedford, 1985; Morris et al., 1987). Unlike mammals, chicken testicular sperm are capable of motility and fertilization (Howarth, 1983), yet fertility is only possible if sperm are inseminated directly into the magnum, bypassing the vagina. Extratesticular maturation in the rooster, therefore, appears to impart to sperm the ability to move through the lower regions of the hen’s reproductive tract. In support of this supposition, NMase-treated rooster sperm inseminated into the vagina are unable to populate sperm storage tubules found at the some 6–8 cm from the site of deposition (Froman and Engel, 1989; Howarth, 1990; Steele and Wishart, 1996). Sperm storage tubules (functionally analogous to the oviductal reservoir in mammals) are invaginations within a narrow band of mucosal tissue at the utero-vaginal margin (Froman and Engel, 1989). Perhaps these observations speak to what may be a conserved feature of internal fertilization, the requirement of sperm to move across mucosal membranes without getting entangled in either negatively charged extracellular matrices or the glyocalyxes of epithelial apical membranes.

In conclusion, we have demonstrated that DEFB126, the dominant component of the macaque sperm glyocalyx, is critically important for the ability of macaque sperm to penetrate and move through CM. The full functional capacity of DEFB126 appears to reside in the negatively charged terminal sialic acid residues, and this negative surface charge of sperm is critical for CMP. Addition of DEFB126 to non-capacitated sperm may offer a slight advantage in CM penetration. On the other hand, SPPs adsorbed onto the sperm surface do not appear to provide sperm with any additional benefit in CMP and may actually inhibit the ability of sperm to migrate through mucus.
References


Czuppon AB. Biochemical characterization of a human spermatozoal sialoglycoprotein with respect to antigenicity masking by its sialic acid moieties. Biochem Int 1984;8:9–18.


Iqbal N, Hunter AG. Comparison of various bovine sperm capacitation systems for their ability to alter the net negative surface charge of spermatozoa. J Dairy Sci 1995a;78:84–90.


Tollner TL, Yudin AI, Treece CA, Overstreet JW, Cherr GN. (2005a) The role of the progaglandin synthesis inducer, prostaglandin release and prostaglandin synthesis in cultured fibroblasts by poly(L-lysine) and other synthetic polycations. Biochim Biophys Acta 1984;793:238–250.


Steele MG, Wishart GJ. Demonstration that the removal of sialic acid from the surface of chicken spermatozoa impedes their transvaginal migration. Theriogenology 1996;46:1037–1044.


Submitted on March 18, 2008; resubmitted on May 20, 2008; accepted on May 28, 2008.