

## Beta-Defensin 126 on the Surface of Macaque Sperm Mediates Attachment of Sperm to Oviductal Epithelia<sup>1</sup>

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### ABSTRACT

Beta-defensin 126 (DEFB126) coats the entire surface of macaque sperm until sperm become capacitated, and the removal of DEFB126 from over the head of sperm is required for sperm-zona recognition. Viable sperm collected from cervix and the uterine lumen of mated female macaques had DEFB126 coating the entire surface, suggesting that DEFB126 is retained on sperm en route to the oviduct. DEFB126 plays a major role in attachment of sperm to oviductal epithelial cells (OECs). Following treatment to either remove or alter DEFB126, sperm were coincubated with explants of OECs, which were assessed for sperm binding following rinsing to remove superficially attached sperm. Sperm treated with either 1 mM caffeine + 1 mM dibutyryl cyclic adenosine monophosphate (dbcAMP) (induces capacitation and complete release of DEFB126 from sperm), 2 mM caffeine (removes DEFB126 from over the head and midpiece but does not induce capacitation), anti-DEFB126 immunoglobulin, or neuraminidase (cleaves sialic acid from terminal positions on glycosylation sites of DEFB126) resulted in similar and significant levels of inhibition of sperm-OEC binding. Preincubation of OECs with soluble DEFB126 also resulted in significantly reduced sperm-OEC binding. Furthermore, reduced OEC binding capability of sperm lacking DEFB126 could be restored by addition of soluble DEFB126 to the sperm surface prior to incubation with OECs. Finally, purified DEFB126, infused into oviducts in situ, associated primarily with the apical membranes of secretory-type epithelial cells. In summary, treatments of macaque sperm that result in either removal, masking, or alteration of DEFB126 result in loss of sperm-OEC binding that is independent of changes in sperm motility. DEFB126 may be directly involved in the formation of a reservoir of sperm in the oviduct of macaques.

*gamete biology, fallopian tubes, oviduct, sperm motility and transport, sperm oviductal reservoir*

### INTRODUCTION

In the majority of mammals, the formation of a reservoir of sperm in the oviduct appears to be critical for the regulation of fertilization. Depending on the species, tens of millions to

several billions of sperm are deposited in either the anterior vagina (as in cattle, sheep, rabbits, and primates) or into the uterus (as in pigs, horses, dogs, and many rodents) during coitus [1]. During ascent in the female tract, only a minute fraction of sperm successfully migrate to the site of fertilization, the ampulla, or the ampullar-isthmic junction of the oviduct. It has been demonstrated in rodents that the ratio of the number of sperm to oocytes in the ampulla approaches unity [1]. This fine tuning of the ratio of gametes is accomplished by a gradual release of fertilization-competent sperm from the oviductal isthmus in response to ovulation. The mechanisms associated with this timed release of sperm are not well understood but are likely important to prevent polyspermy, to select for highly competent sperm, and to deliver those sperm in a capacitated state to the ovulated egg(s) before they age excessively [1].

The isthmic sperm reservoir appears to be formed by selective binding of sperm to epithelial cells of the oviduct. In hamsters, binding of sperm to the mucosal surface of oviductal epithelial cells (OECs) could be observed through the semitransparent wall of the intact oviduct excised a few hours after mating [2]. Similarly, binding of mouse sperm to OECs was observed in the oviduct [3]. In both cases, sperm attached to OECs by the rostral surface of the head, rocking back and forth, and in the case of hamster sperm, occasionally detaching and reattaching a short distance away. In vitro, motile sperm have been shown to bind to OECs obtained from cattle [4], pigs [5], horses [6], and humans [7]. From observations with scanning electron microscopy, sperm were shown to make close contact with OECs via the plasma membrane overlying the acrosome [8–11]. The formation of the reservoir appears to result as more and more sperm enter the oviduct and are “captured” by binding to the luminal surface.

In some species, binding of sperm to OECs appears to be mediated by lectin-like proteins adhered to the surface of sperm. PDC-109, a product of bovine seminal vesicles and the most abundant protein in seminal plasma [12], coats the anterior head of ejaculated bull sperm but not epididymal sperm [13]. Purified PDC-109 inhibited sperm-OEC binding by approximately 80%, whereas affinity column passthrough had no effect [14]. Epididymal sperm, lacking PDC-109, bound to OEC explants in very limited numbers compared with ejaculated sperm, but binding ability could be imparted when epididymal sperm were pretreated with free PDC-109 [13]. In porcine sperm, spermadhesins, which are also produced by the seminal vesicles, are the major components of the sperm coat [15]. Of the family of these lectin-like proteins, only purified AQN-1 effectively inhibits sperm-OEC binding [16, 17]. Both PDC-109 and AQN-1 appeared to be lost from the plasma

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membrane overlying the sperm head during capacitation [14, 15, 18, 19]. This capacitation-associated loss results in reduced OEC binding [13, 17] which, in bovine sperm, can be restored when sperm are washed out of capacitating conditions and treated with PDC-109 [13].

We have recently shown that beta-defensin 126 (DEFB126), a previously described epididymis-specific protein (formerly ESP13.2) [20], is the primary surface-coating protein of cynomolgus macaque sperm, and that it has an important function in capacitation and fertilization [21–24]. DEFB126 is a member of the beta-defensin family, a class of proteins that are components of the innate immune system [25, 26]. We have shown that DEFB126 is bound to the entire surface of ejaculated macaque sperm [21] and that it must be released from the sperm surface in order for sperm to bind to the zona pellucida [22]. The release of DEFB126 from the sperm surface occurs as part of capacitation and can be induced by treatment of sperm with caffeine but not dibutyl cyclic adenosine monophosphate (dbcAMP); both are required for complete capacitation leading to the acrosome reaction and zona penetration [27, 28]. Sperm that release DEFB126 acquire the ability to bind to zonae pellucidae, and this ability can be reversed if the released DEFB126 is added back to sperm prior to zona interaction [22].

In this study, we show that DEFB126 is retained on the surface of viable sperm recovered from cervical mucus and uterine fluid of mated cynomolgus macaques, suggesting that the obligatory loss of DEFB126 prior to fertilization likely occurs in the oviduct. Furthermore, we demonstrate that the loss of DEFB126 from over the sperm head with capacitation is associated with a significant loss of the ability of sperm to attach to sheets of OECs obtained from follicular phase oviducts. We also show that antibodies to DEFB126 inhibit binding of noncapacitated sperm to OECs, as does modification of DEFB126 with neuraminidase. Addition of DEFB126 back to the surface of formerly capacitated sperm completely restores sperm-OEC binding to levels associated with non-capacitating conditions. Based on our findings, we conclude that DEFB126 on macaque sperm mediates sperm-OEC binding in vivo and is a key component in the formation of an oviductal reservoir of sperm in the primate.

## 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 various sperm surface proteins. Antibodies were raised in rabbits, as described previously, to purified DEFB126 [21] and to recombinant SPAM1 (formerly PH20) [29, 30]. Antibodies were developed in chickens to sperm protein PSP94, as described previously [22].

For labeling of live sperm recovered from the reproductive tract of mated female macaques, anti-DEFB126 immunoglobulin (Ig) was conjugated to fluorescein Alexa 488. Coupling of Alexa 488-reactive dye to anti-DEFB126 Ig and purification of the Alexa-anti-DEFB126 Ig complex were accomplished using the Alexa 488 Protein Labeling kit (Invitrogen, Carlsbad, CA).

### Animals

A total of 8 male and 16 female cynomolgus macaques (*Macaca fascicularis*) and 16 female rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center (CNPRC) in compliance with the 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.

### DEFB126 on Sperm Recovered from Female Reproductive Tracts

Six periovulatory female cynomolgus macaques were bred, and then, 4 h after mating, were sedated with ketamine hydrochloride (10 mg/kg body weight) prior to cervical mucus collection. A pediatric proctoscope was inserted into the vagina, and a 10-cm-long polyethylene catheter (inner diameter = 1.19 mm; outer diameter = 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 centimeters of mucus. The catheter was cut into several 1-cm-long sections and placed into Biggers, Whitten, and Whittingham medium as originally formulated with 35.7 mM sodium bicarbonate and supplemented with 30 mg/ml BSA (hereafter referred to as BWB). Sperm were allowed to swim out of mucus into BWB for 1 h at 37°C, 5% CO<sub>2</sub>. "Swim-out" sperm were washed in BWB medium modified with addition of HEPES buffer (Irvine Scientific, Santa Ana, CA) and supplemented with 3 mg/ml BSA (hereafter referred to as mBWB). Sperm were then incubated in mBWB containing Alexa-anti-DEFB126 Ig for 30 min. Sperm were washed free of excess Ig and checked for motility and anti-DEFB126 labeling by fluorescence microscopy. A total of 200 sperm per swim-out were scored. The experiment was performed six times, each time with a mucus sample from different females. At 18–24 h later, females were anesthetized again for uterine fluid collection. A total of 20–75 µl uterine fluid was aspirated by ultrasound guidance of a needle transfundally to the uterine lumen [31]. The aspirates were immediately added to 100 µl mBWB and 100 µg/ml Alexa-anti-DEFB126 Ig. Samples were allowed to incubate with anti-DEFB126 Ig for 30–40 min. A total of six aspirates, each from a different female, containing a total of 293 sperm were assessed for motility and DEFB126 labeling.

### Semen Collection and Sperm Preparation

Semen samples were collected by electro-ejaculation from individually housed male cynomolgus macaques using established techniques [32]. Each ejaculate was collected into a 15-ml centrifuge tube containing 5 ml mBWB. After 1 h, the samples were checked for motility, and only those samples having greater than 70% motile sperm were used in the experiments. Following removal of coagulum, sperm were pelleted by centrifugation at 300 × g for further processing. Sperm were then incubated overnight as described previously to enhance responsiveness to activator compounds [22, 27]. Briefly, sperm were washed through a 3.5-ml column of 80% Percoll and suspended in BWB. Sperm were washed two more times at 300 × g and resuspended in BWB medium to a concentration of 10 × 10<sup>6</sup> to 15 × 10<sup>6</sup>/ml. Following overnight incubation at 28°C and 5% CO<sub>2</sub>, sperm were placed into a 37°C incubator with 5% CO<sub>2</sub> for an additional 2 h. Sperm were treated with 2 µg/ml H33348, a fully permeable chromatin dye, during this incubation step to later facilitate scoring numbers of sperm adhered to OECs and to the zona pellucida. For the initial series of sperm-OEC binding experiments (see sections on series 1 and 2, below), sperm concentration was adjusted to 1 × 10<sup>6</sup> to 2 × 10<sup>6</sup> sperm/ml. For later experiments (series 3 and 4), sperm concentration was increased to 5 × 10<sup>6</sup> sperm/ml to account for decreases (15%–20%) in both percentage of sperm motility and progression observed with repetitive washing.

For all sperm-OEC and sperm-zona binding experiments, percentage of motility was determined for all treatment groups. The percentage of progressively motile sperm was scored with a cell counter for 200 sperm per treatment. Sperm were considered progressively motile if they displayed vigorous forward movement. Only sperm samples exhibiting 70% or better progressive motility following overnight incubation were used in these experiments.

### Collection and Preparation of OEC Explants

Oviductal specimens were obtained from 11 female rhesus macaques and 6 female cynomolgus macaques of reproductive age killed with an overdose of pentobarbital according to established CNPRC procedures and IACUC-approved protocols. Oviducts were excised and cut into approximately 5-mm sections, and the luminal epithelial cells were expelled from the proximal half containing the isthmus by "milking" the oviduct with fine-tipped forceps [4]. The epithelial cells either free in medium or in elongated sheets (explants) were washed by centrifugation twice at 300 × g for 5 min each with mBWB. Explants were separated from blood cells and individual epithelial cells by transferring the cells through several large drops of mBWB medium with a Pasteur pipette, and then incubating in BWB medium for 2–3 h at 37°C and 5% CO<sub>2</sub>. In most cases, epithelial cell sheets were found to fold upon themselves, forming inverted tubules with apical surfaces facing outward, and

were thus referred to as explants. In cynomolgus and rhesus macaques, it has been reported that oviductal epithelium exhibits extensive ciliogenesis during the follicular phase [33]. Both the percentage and height of ciliated cells increase to a maximum at midcycle and undergo a rapid regression during the luteal phase [33]. Regardless of cycle day estimates of female macaques at time of killing, only those oviductal explants that exhibited a high percentage (>30%) of vigorously motile, densely ciliated cells that were nearly the height of neighboring secretory cells were used in sperm-oviduct binding studies. Highly ciliated explants were considered to be of a morphology indicative of the late follicular phase or periovulatory interval.

### Sperm-OEC Binding Experiments

**Series 1.** Following washing through Percoll and overnight incubation as described above, capacitation of the majority of sperm was induced with the addition of 1 mM dbcAMP + 1 mM caffeine ("activators") to sperm suspensions for an additional 1 h of incubation at 37°C and 5% CO<sub>2</sub> [28]. Sperm were either activated or treated 1:100 with anti-DEFB126 Ig (~100 µg/ml) for 1 h. Controls received addition of vehicle (10 µl/ml Dulbecco phosphate-buffered saline, DPBS). Prior to being introduced into OEC cultures, sperm were washed out of treatment solutions by centrifugation (300 × g for 10 min) and resuspended in BWB. Sperm suspensions were deposited into drops of medium under oil. Oviductal epithelial cells explants were transferred to the sperm drops and cocultured for 15 min. Oviductal epithelial cell explants then were transferred through three reservoirs of mBWW using narrow-bore (~1-mm diameter) Pastuer pipettes to remove nontightly adhered sperm, and they were deposited into DPBS containing 1% paraformaldehyde. Oviductal epithelial cells were placed on a glass slide between four posts of silicon grease containing 100-µm beads. A glass coverslip was applied to the posts and gently pressed down, compressing OEC explants to a 100-µm depth. The H33342-labeled nuclei of sperm were easily visualized with the 50× fluorescence objective of a Leitz Laborlux S fluorescence microscope (Carl Zeiss Vision) equipped with a BP 340–380 excitation filter, an RKP 0400 dichromatic mirror, and an LP 425 suppression filter. The number of sperm bound to apical surfaces of OECs (determined by the presence of cilia) was scored over uniformly sized fields (100 µm<sup>2</sup>) using a microscope ocular counting grid (Olympus). Sperm were scored over 15 to 25 fields (subsamples) per each treatment chosen at random on a minimum of four different explant tubules per treatment. Within each treatment, the numbers of sperm bound were averaged across subsamples to provide a single value or replicate. Sperm from three males were used across six experiments, two experiments per male. Each experiment used explants from a different female. Sperm in each treatment suspension were labeled immunofluorescently for DEFB126.

**Series 2.** Sperm were washed and incubated as described above. Prior to sperm-OEC binding, explants were incubated with a solution containing soluble DEFB126 (~23 µg/ml) for 1 h at room temperature with gentle rocking. Explants were transferred through three drops of mBWW to remove excess DEFB126 and deposited in sperm suspensions maintained under equilibrated mineral oil as described for series 1. Sperm-OEC binding was assessed as described above in series 1 experiment. Experiments were repeated four times, each time with sperm from a different male.

A solution containing soluble DEFB126 was prepared as described previously [22]. Briefly, 25 sperm samples each were washed through Percoll as described above and were resuspended in 10 ml DPBS without energy substrates and BSA. Sperm were washed by centrifugation, and the pellet was resuspended in DPBS. Sperm suspensions were incubated at 37°C for 1 h and then treated with activators for an additional hour. Sperm were pelleted at ~1000 × g for 5 min, and the supernatants were then filtered, concentrated, and dialyzed into DPBS. The concentration of proteins released from sperm was determined with a BCA protein analysis system (Pierce). The purity of these sperm proteins was evaluated using an 8%–16% Tris-glycine gel for electrophoresis (Invitrogen). The proteins were solubilized in SDS-reducing buffer (Pierce), electrophoresed, and stained as described previously [21]. DEFB126 was identified by probing Western blots with anti-DEFB126 Ig. The purity of DEFB126 in the preparation was estimated from the gel according to the optical density of protein bands in the entire gel as determined by analysis of digital scans using National Institutes of Health ImageJ software and the gel analysis macro. Two proteins constituted 98% of the total protein on the gel, of which DEFB126 represented 79% of the total protein. The other prominent protein in the gel was PSP94, which localizes to the principal piece of the flagellum and does not "add back" to the sperm surface [22].

**Series 3.** Sperm were washed and incubated as described above. Sperm were treated with caffeine (2 mM) only for 1 h, which resulted in the loss of DEFB126 from over the head and midpiece but did not result in capacitation [22]. Controls were treated with equivalent volumes of DPBS. Sperm were washed out of caffeine by centrifugation (300 × g for 10 min), resuspended in BWB medium, and incubated an additional 30 min before soluble DEFB126

was added back to the surface of sperm [22]. Control and caffeine-treated sperm were split into two equal volumes each and pelleted by centrifugation (300 × g, 10 min). One of the pellets of caffeine-treated sperm received "add back" solution containing soluble DEFB126 at an estimated concentration of ~7 µg/ml for 60 min. The other caffeine-treated pellet and one of the control pellets received the equivalent volume of DPBS containing 35 µg/ml BSA. All samples were then diluted 15-fold by volume with BWB medium and washed by centrifugation to remove excess DEFB126. Sperm pellets were resuspended in BWB medium to give a final concentration of 5 × 10<sup>6</sup> sperm/ml. Sperm suspensions were deposited under oil, and OEC binding experiments were performed as described for series 1. Experiments were repeated five times, each time with sperm from a different male.

**Series 4.** Sperm were washed and incubated as described above. Sperm samples were split into three. One sample was treated with 2 mM caffeine, one with neuraminidase (NMase; 0.5 units/10 × 10<sup>6</sup> sperm/ml), and the remaining sample with equivalent volume of solvent (DPBS). Samples were further incubated for 1 h at 37°C, washed by centrifugation out of caffeine, NMase, and control solvent, and resuspended into BWB medium. Sperm samples were cocultured with oviductal explants as described for series 1. Experiments were repeated five times, each time with sperm from a different male.

In a separate set of experiments, sperm treated with either caffeine or NMase were cocultured with intact zonae pellucidae as described previously [22]. Briefly, frozen-stored whole-macaque zonae pellucidae were thawed and washed free of cryoprotectant. Two to three zonae were added for 2 min to sperm suspension drops maintained under equilibrated mineral oil. Zonae were then transferred through three baths of mBWW at 37°C with a fine-bore glass pipette and deposited on a glass slide with a small drop of medium. The drop was covered with a coverslip supported by columns of silicon grease containing 100-µm glass beads. The total number of sperm bound to the zona was scored with fluorescence microscopy as described for sperm attached to OECs above. Experiments were repeated four times, each time with sperm from a different male.

### Fluorescent Immunolocalization of DEFB126 on Sperm and OECs

Sperm from control suspensions in series 1 and from control, caffeine-treated, and add-back suspensions in series 3 were fixed in 1% paraformaldehyde in DPBS for 20 min. Explants with attached control sperm from series 1 were fixed in 3% paraformaldehyde for 2–3 h. After fixation, DEFB126 on free sperm and sperm bound to explants was labeled similarly. Sperm (and explants with attached sperm) were thoroughly washed (two to three times) in blocking solution (1% BSA, 0.1% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 1% gelatin/DPBS). Sperm samples were suspended in anti-DEFB126 Ig (10 µg Ig/ml) or in DPBS (control), gently rolled for 1 h, and then washed three times in blocking solution and resuspended in a solution of 20 µg/ml goat anti-rabbit Alexa 488 (Molecular Probes, Eugene, OR) in blocking solution. The samples were rolled again for 1 h and then thoroughly washed and resuspended in a fluorescent stabilization medium (50% glycerol, 0.2% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>, 1% paraformaldehyde/DPBS). Percentages of sperm that were thoroughly coated with DEFB126 over the head in both sperm suspensions and in association with OECs were determined with epifluorescence microscopy described below.

Images of representative cells were collected using a cooled CCD digital camera (MagnaFire; Optronics, Santa Barbara, CA) mounted on a Leitz Laborlux S fluorescence microscope employing a BP 450–490 excitation filter, an RKP 0510 dichromatic mirror, and an LP 515 suppression filter. Optics included a 3.3× intraocular magnifier (Scientific Instruments, Sunnyvale, CA) and a Zeiss 63× oil immersion fluorescence objective (JH Technologies, San Jose, CA.). Initial images were captured using MagnaFire 2.0 software (Optronics) and processed with Adobe Photoshop (Adobe Systems, San Jose, CA) for production of figures.

Explants treated with soluble DEFB126 in series 2 were fixed and labeled for the presence of DEFB126 as described above for sperm-OEC complexes, or they were labeled for the presence of PSP94 using polyclonal anti-PSP94 Ig (10 µg/ml). Controls involved labeling of explants with anti-DEFB126 Ig and secondary antibody.

Intact oviducts were flushed *in situ* with soluble DEFB126 prior to removal of epithelium. Oviducts were recovered at midcycle from six macaques (four cynomolgus and two rhesus), all proven breeders that had regular menstrual cycles. Females were monitored for first day of menstrual bleeding (Cycle Day 1) and scheduled for killing and specimen collection on Day 13 or 14, depending on prior cycle length. Oviducts were sectioned at midlength, and a blunt 18-gauge needle was inserted approximately 0.5 cm into the distal end of the isthmus region and secured with 4–0 silk suture material (Ethicon Inc.). The proximal end (the utero-tubal junction) was left unobstructed. The isthmus was flushed initially with 1.0 ml mBWW to remove mucus that might interfere with interaction of DEFB126 with the epithelial surface. Flushes were delivered with

a 1-cc syringe attached to the 18-gauge needle. The flush volume appeared to be adequate, as the capacity of the isthmus was, on average, less than 100  $\mu$ l (estimated from the amount of clear oviductal fluid expelled before mBWW containing phenol was infused). Oviducts then were flushed with 0.5 ml DPBS, and 200  $\mu$ l solution containing soluble DEFB126 was pushed into the oviduct. Once approximately 100  $\mu$ l DPBS was expelled, the distal portion of the oviduct was clamped, and slight positive pressure was applied for 30 min. The isthmus region of the contralateral oviduct was used as a control and was clamped, having received only DPBS. Oviducts were flushed with 1.5 ml DPBS to remove unbound DEFB126 and were fixed from the inside by displacing DPBS with DPBS containing either 3% paraformaldehyde ( $n=3$ ) or a combination of 3% paraformaldehyde and 0.1% glutaraldehyde ( $n=1$ ); then, oviducts were clamped, and slight positive pressure was applied for 20 min. Following removal of fixative by flushing with 1.5 ml DPBS, oviducts were sectioned longitudinally, and epithelial lining was scraped free with a stainless steel weigh spatula. The resulting OEC sheets were labeled with anti-DEFB126 Ig and secondary Ig as described above for explants used in sperm binding studies.

The oviducts of two macaques (one cynomolgus and one rhesus) were infused with proteins released from the sperm surface with phosphatidylinositol-specific phospholipase C (PI-PLC). Purification and preparation of PI-PLC-released sperm proteins has been described previously [34]; the most prominent of these proteins as determined by SDS-PAGE are SPAM1 and DEFB126 [21, 35]. Oviducts were processed for in situ labeling as described above, but with the following differences. One oviduct of each pair was fixed (3% paraformaldehyde, 0.1 glutaraldehyde) prior to addition of PI-PLC-released proteins, whereas the contralateral oviducts were fixed (3% paraformaldehyde, 0.1 glutaraldehyde) after the addition of the sperm proteins. Rinses of the oviduct with DPBS preceded and followed fixation. Sheets of OECs were removed as described previously and were treated with anti-DEFB126 Ig, anti-SPAM1 Ig, and secondary Ig, as described for explants.

Explants from binding experiments and OEC sheets from in situ labeling experiments were viewed using an Olympus Fluoview 500 laser scanning confocal microscope (equipped with acousto-optic tunable filter (AOTF) for complete channel separation) mounted on an Olympus BX60WI fixed-stage upright microscope. Samples were imaged using a water immersion 60 $\times$  fluorescence objective lens. Images (both fluorescence at 488-nm excitation and differential interference contrast) were collected as single scans or as Z-series with 2- to 5- $\mu$ m optical sections, followed by Z-series projection using the Fluoview software (Olympus, Melville, NY). Samples double-labeled with Hoechst 33342 were excited with a blue diode laser (405-nm excitation), and separation of emission signals was accomplished with the AOTF.

### Videomicrography of Sperm-OEC Interaction

Oviducts were recovered at midcycle from three additional proven fertile rhesus macaques as described for in situ labeling experiments. The isthmus region of each oviduct was sectioned longitudinally, and the epithelial lining was scraped free with a stainless steel weigh spatula. Explants were mounted on glass slides with a small amount of mBWW and were covered with a coverslip supported by columns of silicon grease containing 100- $\mu$ m glass beads. The slide was warmed for 5 min on a microscope stage warmer set at 37.5°C. Explants were observed with an Olympus inverted phase-contrast microscope with a 40 $\times$  objective and Hoffman modulation optics. A plane midway between the coverslip and slide was prefocused prior to the addition of sperm. A 60- $\mu$ l aliquot of sperm, washed and diluted to  $2 \times 10^6$ /ml in mBWW as described above, was added to the warmed slide at the edge of the coverslip. Sperm were drawn under the coverslip and around the explants by capillary action. A CCD black-and-white video camera (Panasonic model WV-BD400) attached to the microscope via an Olympus adapter (with 2.5 $\times$  ocular) captured images of sperm binding to OECs in real time at 30 frames per second. The video signal passed in series through a video time generator (VTG; For.A.Inc. model 33) and a 0.5-inch video tape recorder (Panasonic model AG6300). After several minutes, 10–15 microscopic fields of explants from each female were videotaped for assessment of sperm binding numbers.

### Statistical Analysis

For series 1 experiments, sperm-OEC binding data were analyzed with a mixed-model, multifactorial ANOVA. The effects of the three sperm treatments (fixed factor) were tested on the sperm from three males (random factor). Nested within each male, the experiment was organized as a random complete block design (RCBD) with two blocks (one block for each oviduct). Response means of treatments were compared to the control using Dunnett means comparison test. This design enabled simultaneous partitioning of the variability due to both male and female factors. For experiments in series 2–4, sperm from different males were sometimes used with explants from the

same female, and therefore a nested RCBD could not be used. Instead, in these experiments a one-factor (sperm treatment) ANOVA was used with different males representing blocks in an RCDB. Response means of treatments were compared using Tukey range test. All experiments (replicates) generated values of numbers of sperm bound per grid for every treatment. Data are reported as mean number of OEC-bound sperm/100- $\mu$ m<sup>2</sup> grid  $\pm$  SEM. All ANOVAs met assumptions of factor independence, as well as normality of data distribution and variability. Analyses were conducted with an SAS statistical program (SAS Institute, Cary, NC) according to the principles described previously [36].

## RESULTS

### DEFB126 on Sperm Recovered from Female Reproductive Tracts

Generally, millions of sperm would swim out of a 1-cm section of catheter containing postcoital cervical mucus into BWB medium over an hour. The percentage of motility of swim-out sperm was high ( $89.7\% \pm 2.4\%$ ). Nonmotile sperm would not readily take up supravital dye H33258, consistent with previous observations, where loss of membrane integrity lagged several hours behind loss of motility in dying macaque sperm [37]. As such, nonmotility in this study was taken as an indicator of nonviability. The vast majority of motile swim-out sperm ( $98.4\% \pm 3.2\%$ ) were heavily coated over the head with Alexa-anti-DEFB126 Ig (Fig. 1A). Loss of DEFB126 from over the head of sperm appeared to be associated with loss of viability, as more than 50% of the nonmotile sperm had no Alexa-anti-DEFB126 Ig labeling over the head (Fig. 1A).

From six mated females, a total of 293 sperm were recovered from the uterus by transabdominal aspiration, with an average of  $48.7 \pm 19$  sperm per aspirate. Only  $43\% \pm 6\%$  of the uterine sperm were motile; of these, 98.1% were labeled brightly with Alexa-anti-DEFB126 Ig over the head (Fig. 1B). As with sperm recovered from cervical mucus, high levels of loss of DEFB126 were observed for nonmotile sperm ( $15.5\% \pm 3\%$  of total “uterine” sperm; or 27% of nonmotile sperm), suggesting that loss of DEFB126 is associated with loss of viability.

Patterns of Alexa-DEFB126 Ig labeling of motile swim-out and uterine sperm were very similar (Fig. 2, B and D, respectively). Labeling was bright over the head, especially over the equatorial segment and acrosomal cap, but was faint over the flagellum (Fig. 2, B and D). A few motile sperm had faint labeling only over the apical ridge of the anterior head region and were scored as DEFB126 “minus” (labeling pattern not shown).

### Sperm-OEC Binding Experiments

Given that the majority of viable macaque sperm recovered from the reproductive tracts of mated females retain DEFB126 over the head, we hypothesized that DEFB126 may be important for interaction with cells of the upper reproductive tract. Initial studies demonstrated that noncapacitated sperm adhered tightly to OEC explants, resisting removal with repeated aspirations into narrow-bore pipettes (data not shown). In series 1 experiments, we investigated the effects of capacitation and the removal of DEFB126 on sperm-OEC interaction. Macaque sperm do not capacitate spontaneously in vitro with incubation in culture medium alone, but require addition of exogenous activator compounds [38]. Treatment of macaque sperm with activator (ACT: 1 mM caffeine and 1 mM dbcAMP) results in synchronized capacitation, including the development of full hyperactivated motility [38–40] and the nearly complete loss of DEFB126 from over the head and flagellum in more than 80% of sperm [22]. In OEC binding experiments, sperm treatment with ACT significantly inhibited

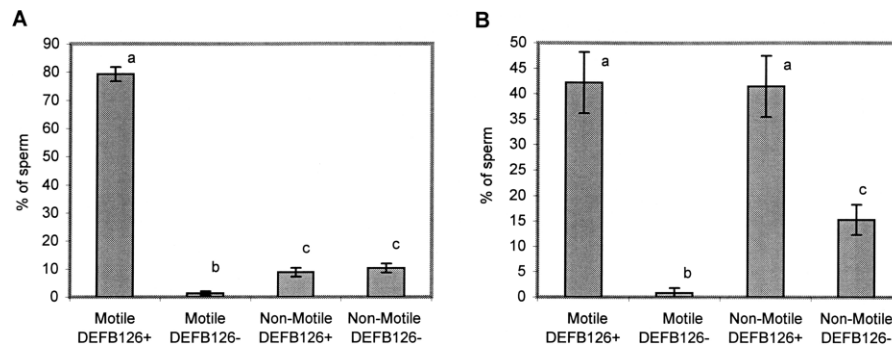


FIG. 1. **A)** Labeling of sperm with Alexa-anti-DEFB126 Ig following swim out of postcoital cervical mucus. Perioviulatory females were bred and then 4 h later were briefly anesthetized for collection of cervical mucus into polyethylene catheters. Over 1 h, sperm swam out of a 1-cm-long section of catheter into BWW medium with 3% BSA at 37°C, 5%CO<sub>2</sub>. Sperm in suspension were washed in mBWW containing Alexa-anti-DEFB126 Ig and incubated for 30 min. Sperm were washed free of excess Ig and checked for motility and anti-DEFB126 labeling by fluorescence microscopy. A total of 200 sperm per swim out were scored. Experiment was performed six times, each time with a mucus samples from different females. **B)** Labeling of sperm recovered from the uterus with Alexa-anti-DEFB126 Ig. At 18–24 h later, females were anesthetized again for uterine fluid collection. A total of 20–75 µl uterine fluid was aspirated by ultrasound guidance of a needle transfundally to the uterine lumen. The aspirates were immediately added to 100 µl mBWW with 100 µg/ml Alexa-anti-DEFB126 Ig. Samples were allowed to incubate with Ig for 30–40 min. A total of six aspirates, each from a different female, containing a total of 293 sperm were assessed for motility and DEFB126 labeling. Data are reported as mean ± SEM. Columns with different letters (a, b, c) are significantly different (ANOVA, alpha = 0.01; Tukey range testing;  $P \leq 0.05$ ).

the ability of macaque sperm to tightly bind to OEC explants compared with controls by approximately 60% ( $P \leq 0.002$ ; Fig. 3).

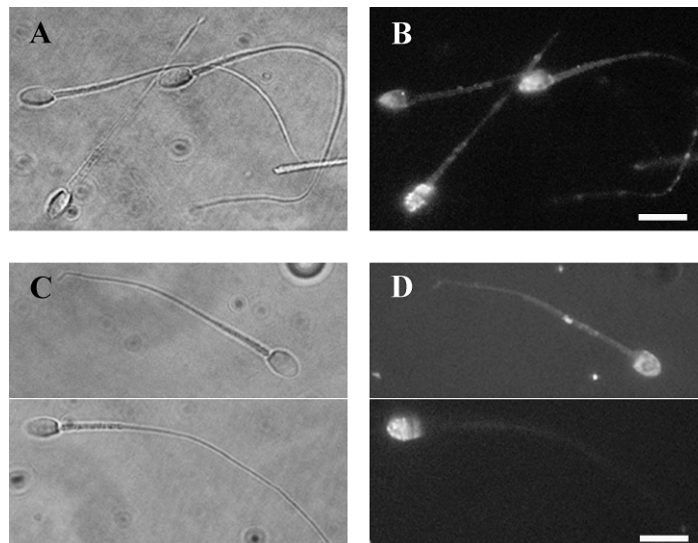
Similar results were obtained with pretreatment of non-capacitated sperm with anti-DEFB126 Ig. Antibody treatment significantly inhibited sperm-OEC binding by approximately 50% ( $P \leq 0.018$ ; Fig. 3). Lower dilutions with antibodies (1:50, 1:25) did not result in significantly lower sperm-OEC binding (data not shown). The fact that DEFB126 on sperm shields other sperm surface proteins from immunological recognition [23] eliminates the possibility of an appropriate antibody control. We used antibodies to sperm surface protein SPAM1 [35] in these experiments. Although sperm treatment with anti-SPAM1 Ig had no effect on sperm-OEC binding, only 20% of noncapacitated sperm were shown labeled with anti-SPAM1 Ig (not shown). The 20% of sperm that label with anti-SPAM1 Ig are likely those sperm that spontaneously lose DEFB126 from their surfaces with overnight incubation [22, 24]. We reasoned that these sperm are not likely to participate in sperm-OEC binding, bringing into question the relevance of the result with anti-SPAM1 Ig (data not shown).

Immunofluorescent labeling of sperm-OEC complexes demonstrated that the vast majority of tightly adhered sperm had DEFB126 over the head. Whereas only 66% of control sperm in suspension were evenly coated with DEFB126 over the head, 97% of sperm attached to OEC explants had labeling of DEFB126 over the head (Fig. 4). Sperm from labeling controls (receiving only secondary antibodies) were not distinguishable from OECs (not shown). Oviductal epithelial cells, therefore, appeared to select for sperm whose heads were well coated with DEFB126.

Confocal images of sperm-OEC interaction demonstrated that sperm attach to apical surfaces of epithelial cells by the rostral portion of the head. The majority of sperm appeared to “embed” their heads into the recesses between more pronounced OECs, whereas only a few sperm associated with the apical surface of a single epithelial cell (Fig. 5). Most sperm appeared to be attached along the sides of nonciliated columnar epithelia, the secretory or “goblet” cells (Fig. 5).

Pretreatment of explants with soluble DEFB126 previously released from the surface of capacitated sperm [22] inhibited the attachment of noncapacitated sperm to OECs (Fig. 6). Due

FIG. 2. Pattern of Alexa-anti-DEFB126 labeling of live sperm was consistent for both mucus swim-out (**B**) and uterine aspirates (**D**). Corresponding phase images are shown in **A** and **C**. Bar = 10 µm.



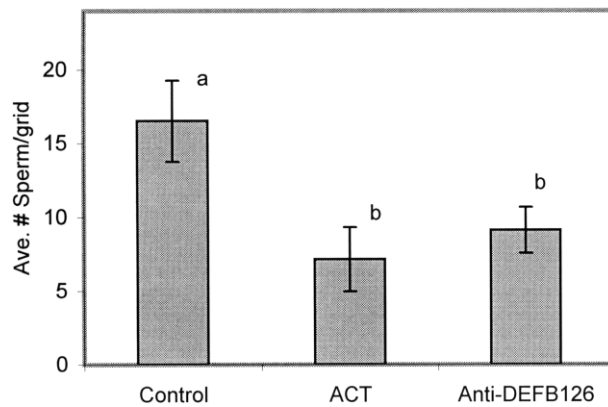


FIG. 3. Sperm binding to OECs following activation or treatment with anti-DEFB126 Ig. Sperm were either activated (ACT; 1 mM caffeine + 1 mM dbcAMP), treated with anti-DEFB126 Ig (100  $\mu$ g/ml), or treated with DPBS (control) as described in series 1. Sperm were washed into BWB, adjusted to  $1 \times 10^6$ /ml, and coincubated with OEC explants for 15 min. Explants were rinsed several times to remove nontightly adhered sperm, fixed in 1% paraformaldehyde, and mounted on glass slides. The number of sperm bound to OECs was scored over uniformly sized fields using a microscope ocular counting grid (100  $\mu$ m<sup>2</sup>). Data are reported as mean  $\pm$  SEM. Experiments were repeated six times, each time with six different females and three different males. Columns with different letters (a, b) are significantly different (ANOVA,  $\alpha = 0.01$ ; Dunnett range test;  $P \leq 0.002$  and  $0.013$  for ACT and anti-DEFB126 Ig treatments, respectively).

to the limitations of concentrating soluble DEFB126 [22], as well as a difficulty in saturating potential binding sites on large OEC sheets, OEC explants were incubated with a single “high” dose of soluble DEFB126. Pretreatment of explants with DEFB126 significantly inhibited sperm-OEC binding by 46.2% ( $P \leq 0.03$ ; Fig. 6).

#### DEFB126-OEC Binding Experiments

Immunofluorescent labeling of explants treated with soluble DEFB126 demonstrated that DEFB126 associates with the apical membranes of specific OEC cell types. Rather than

binding to the entire epithelial surface, DEFB126 appeared to localize to the surface of select epithelial cells (Fig. 7, A: phase-contrast; B: fluorescence). Depending on the field and explants used, the number of OECs that labeled positive for DEFB126 varied from as few as  $\sim 10\%$  of the epithelial surface to as much as approximately 40% (Fig. 7B). PSP94, which is present in DEFB126 solution, did not appear to be associated with OECs, as labeling of explants with anti-PSP94 antibodies resulted in no fluorescence (Fig. 7, C: phase-contrast; D: fluorescence). Likewise, labeling controls (explants receiving anti-DEFB126 Ig and secondary antibodies) showed no fluorescence (not shown). High-magnification confocal microscopy of OEC sheets extracted from oviducts labeled with soluble DEFB126 in situ demonstrated that DEFB126 bound predominantly to secretory cells (Fig. 8, A and B), whereas ciliated epithelial cells lacked labeling of apical membrane (Fig. 8, arrows). In OEC sheets from one midcycle oviduct, nearly 80% of all secretory cells labeled positive for DEFB126 (Fig. 8A). Oviductal epithelial cells from contralateral oviducts (label control) infused with DPBS instead of soluble DEFB126 lacked positive labeling pattern with anti-DEFB126 Ig and Alexa 488-conjugated secondary antibody (Fig. 8C). Labeling patterns of OECs from oviducts infused with PI-PLC-released sperm proteins did not change significantly with fixation protocol. As before, DEFB126 was observed to localize only to apical regions of secretory cells whether it was added (as part of the PI-PLC sperm protein cocktail) to oviducts prior to or after fixative (Fig. 9, A and B). Antibodies to SPAM1 did not localize to OECs, suggesting that SPAM1 does not associate with the oviductal epithelium (Fig. 9C).

Hoffman modulation on the inverted microscope enabled distinction between ciliated and secretory cells of the apical surfaces of oviductal explants to be made while noncapacitated sperm were observed in real time approaching and binding to the epithelium. Few sperm were observed to make contact with ciliated cells. In general, the vigorous beating of cilia appeared to generate currents in the adjacent medium that quickly moved sperm past the ciliated cells. This displacement was particularly notable in short spans of continuously ciliated epithelium. On the other hand, sperm were frequently observed to make

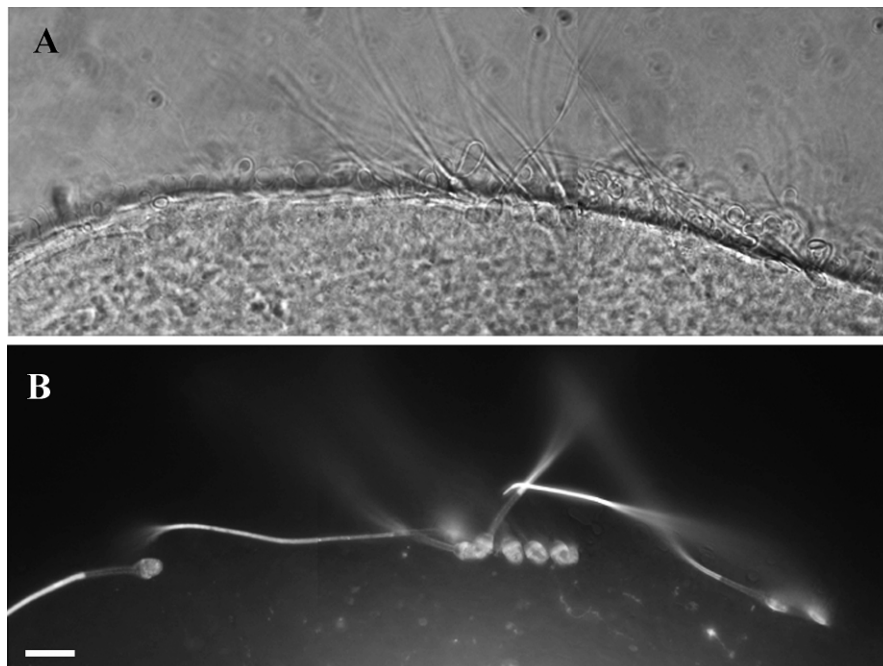
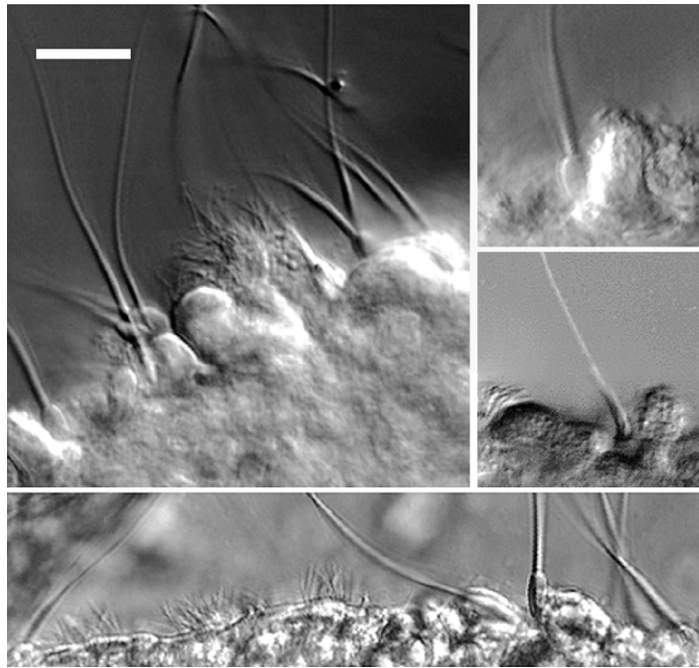


FIG. 4. Antibody labeling of DEFB126 on sperm bound to OECs. Sperm were washed, treated, and coincubated with OECs as described for control sperm in Figure 3. Sperm and sperm-OEC complexes were incubated with anti-DEFB126 Ig, washed in blocking solution, and labeled with Alexa 488-conjugated goat-anti-rabbit IgG. A total of 200 OEC-attached sperm and 200 sperm in suspension were scored for the presence of DEFB126 over the head. Approximately 97% of the OEC-attached sperm were heavily coated with DEFB126 over the head, whereas only 66% of sperm in suspension exhibited bright head labeling with the same antibodies. Observation was made with a sperm sample from a single male. Micrograph shows fluorescence (B) and corresponding phase-contrast (A) images of sperm bound to OECs. Bar = 10  $\mu$ m.

FIG. 5. Images of sperm attached to fixed OEC explants collected with scanning laser confocal microscope using differential interference contrast. Bar = 10  $\mu$ m.



contact with and bind to the tops and sides of secretory cells, especially those cells that protruded well above the apical plane. Of these sperm, many only bound momentarily. The majority of sperm that remained adhered appeared to be wedged in spaces between adjacent secretory cells. The numbers of sperm that remained bound to secretory cells far outnumbered the numbers of sperm that remained bound to ciliated cells ( $5.96 \pm 1.3$  vs.  $0.20 \pm 0.16$  sperm per field;  $P \leq 0.01$ ;  $n = 3$ ).

DEFB126 previously released from capacitated macaque sperm can be added back to the sperm surface following removal of sperm from capacitating conditions [22]. This

property of DEFB126 was exploited for the purpose of defining the role of DEFB126 in sperm-OEC interaction. In series 3 experiments, sperm were treated with 2 mM caffeine to induce the release of DEFB126. Treatment with caffeine alone is as effective as ACT in removing DEFB126 from over the head (Fig. 10A; immunofluorescent labeling of DEFB126 is lost over the head and midpiece of most sperm), but it does not induce capacitation. Caffeine-treated sperm lack hyperactivated motility [27], and although they can bind to the zona pellucida, they do not undergo the zona-induced acrosome reaction [22]. We have determined that DEFB126 adds back more efficiently

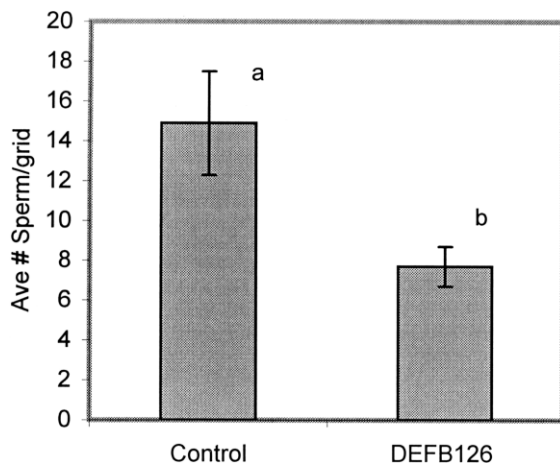


FIG. 6. Effect of pretreatment of oviductal explants with soluble DEFB126 on sperm-OEC binding. Sperm were washed and incubated as described in Figure 3. Prior to sperm-OEC binding, explants were incubated with a solution containing free DEFB126 as described for series 2. Explants were transferred through three drops of mBWW to remove excess DEFB126 and were deposited in sperm suspensions maintained under equilibrated mineral oil, and sperm-OEC binding was assessed as described in Figure 3. Experiments were repeated four times, each time with sperm from a different male. Data are reported as mean  $\pm$  SEM. Columns with different letters (a, b) are significantly different (ANOVA,  $\alpha = 0.01$ ; Tukey range testing;  $P \leq 0.03$ ).

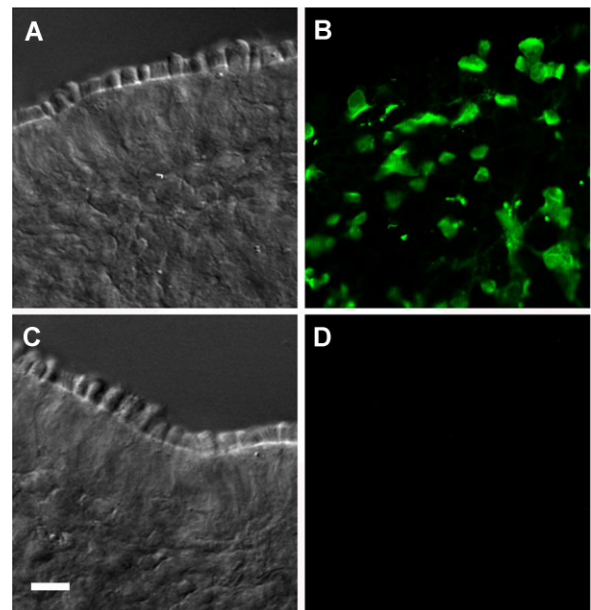


FIG. 7. Confocal images of OEC explants pretreated with free DEFB126 (A, B) and control explants receiving DPBS (C, D). Both DEFB126-treated and controls were labeled with anti-DEFB126 Ig and Alexa 488 secondary antibody. Focal plane includes top and outermost edge of explant. Labeling experiment was repeated with explants from three different macaques. Bar = 20  $\mu$ m.

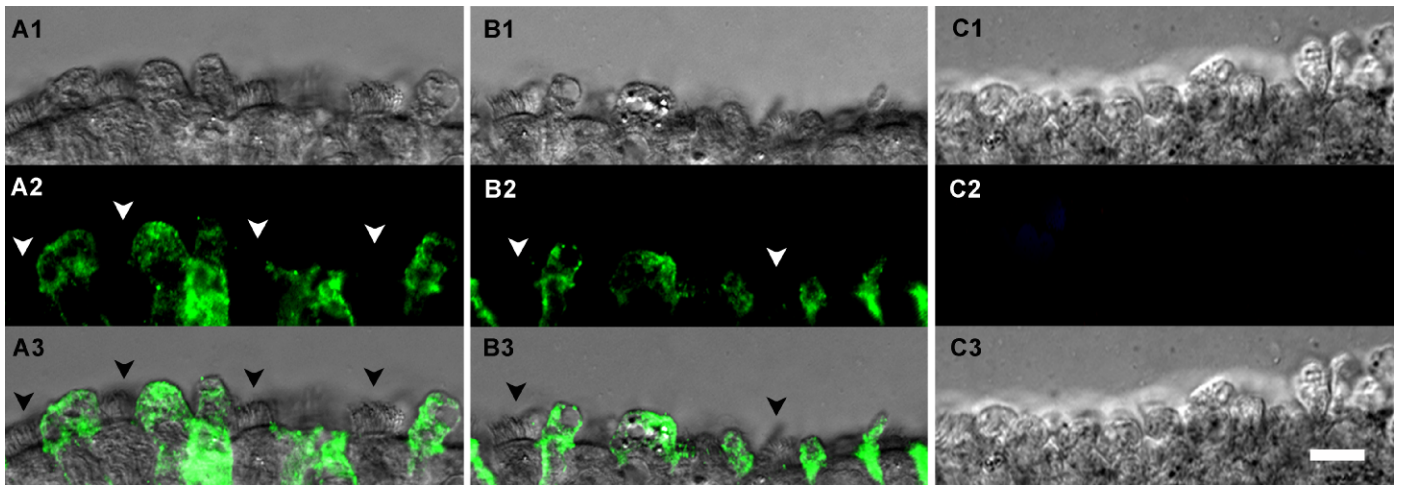


FIG. 8. Confocal images of OEC explants recovered from oviducts that were treated with soluble DEFB126 in situ (**A**, **B**) or with DPBS (**C**). The signal for DEFB126 (green) superimposed on the differential interference contrast image (**A3**, **B3**). Arrows indicate the position of ciliated epithelial cells along the apical surface (**A2**, **A3**; **B2**, **B3**). Contralateral oviducts (controls) were infused with DPBS (**C**). All oviductal explants were labeled with anti-DEFB126 Ig, Alexa 488 secondary antibody, and H33342. Control explants at the same laser exposure intensities as DEFB126-treated explants lacked cell-specific labeling with anti-DEFB126 Ig. The experiment was repeated with oviducts from three different macaques, all of which were at midcycle at time of tissue harvest. Bar = 10  $\mu$ m.

to sperm that were previously treated with caffeine than with ACT [22] using the same conditions described in the present study. Sperm treated with caffeine had a significantly reduced ability to bind to OECs compared with controls ( $13.6 \pm 3$  vs.  $42.1 \pm 8$  sperm/100  $\mu$ m<sup>2</sup>;  $P \leq 0.05$ ; Fig. 10B). The add back of DEFB126 to sperm previously treated with caffeine re-establishes immunofluorescent labeling over the head of sperm (Fig. 10A) and restores sperm-OEC binding to control levels ( $39.9 \pm 9$  sperm/100  $\mu$ m<sup>2</sup>; Fig. 10B).

Sperm treated with caffeine exhibit subtle increases in lateral displacement of the head but do not undergo hyperactivation, as observed with sperm treated with ACT [27]. Following washing to remove sperm from caffeine-treated medium in series 3 experiments, sperm motility was found to be indistinguishable from the controls. The level of inhibition of sperm-OEC binding (68%; Fig. 11) was comparable to inhibition induced by ACT treatment in series 1 experiments in which sperm were hyperactivated at the time of sperm-OEC binding (Fig. 3). Results with caffeine-treated sperm suggest that loss of sperm-OEC binding in these experiments is largely independent of the character or pattern of motility.

We previously demonstrated that DEFB126 is highly glycosylated and rich in terminal sialic acid residues [23, 24]. Treatment of noncapacitated macaque sperm with NMase greatly shifts the isoelectric focal (IEF) point of DEFB126 but leaves the protein on the sperm surface [22]. In series 4 experiments, treatment of macaque sperm with NMase significantly inhibited sperm-OEC binding compared with controls ( $12.2 \pm 5$  vs.  $41.1 \pm 8$  sperm/100  $\mu$ m<sup>2</sup>;  $P \leq 0.03$ ; Fig. 11A). The level of inhibition via NMase modification of DEFB126 was comparable to the inhibition of sperm-OEC binding with DEFB126 removal with caffeine treatment ( $16.7 \pm 2.1$  sperm/100  $\mu$ m<sup>2</sup>; Fig. 11A). Removal of terminal sialic acid residues from DEFB126 also induced sperm-zona binding to levels associated with the removal of DEFB126 with caffeine (Fig. 11B). High numbers of zona-bound sperm suggest that NMase treatment does not alter other sperm proteins, or at least not those involved in zona recognition and binding.

No changes in the percentage of progressively motile sperm were observed with respect to treatment for any of the sperm-

binding experiments. Sperm motility was never seen to drop below 60% during the course of any experiment.

## DISCUSSION

Based on the fact that DEFB126 remains bound to the surface of macaque sperm following rigorous washing through gradient solutions [21, 22], we previously hypothesized that DEFB126 remains on the sperm surface through passage of the cervix and upper female reproductive tract. To investigate this, we collected macaque cervical mucus samples 4–5 h after coitus, allowed sperm to swim out of cervical mucus, and then treated these sperm with Alexa 488-conjugated anti-DEFB126 Ig, which has been shown to recognize DEFB126 on living sperm [22]. Virtually all of the swim-out sperm were brightly labeled over the head. Only the occasional nonmotile sperm did not label with the fluorochrome-conjugated Ig. Sperm collected from the uterus of mated females 18–24 h after coitus by ultrasound-guided transabdominal aspiration [31] contained both motile and nonmotile sperm and labeled similarly. More than 99% of motile uterine sperm labeled over both the anterior aspect of the head overlying the acrosome as well as the principal piece of the flagellum, whereas most of the nonmotile sperm retained labeling over the head. Given the likelihood that DEFB126 remains attached to sperm in the upper female reproductive tract, we evaluated a potential role for DEFB126 in sperm-oviduct interaction using short-term cultured OECs.

As described previously for sperm-oviduct interactions in other species, macaque sperm bind with high affinity to OECs. The ability of sperm to maintain attachment to OECs following rigorous washing of explants could vary depending on a number of factors. The status of DEFB126 on sperm (either removed, added back, or modified) or on OECs (following treatment with soluble DEFB126) was the single most influential variable on sperm-OEC binding. In series 2–4, we used a one-factor ANOVA with an RCBD treating males as blocks. We found that male-to-male variation was minimal, however, relative to other effects (random error, treatment, and female factors), and that all treatment effects were still significant ( $P \leq 0.05$ ) when data were not blocked. We also found differences in sperm-OEC binding when comparing

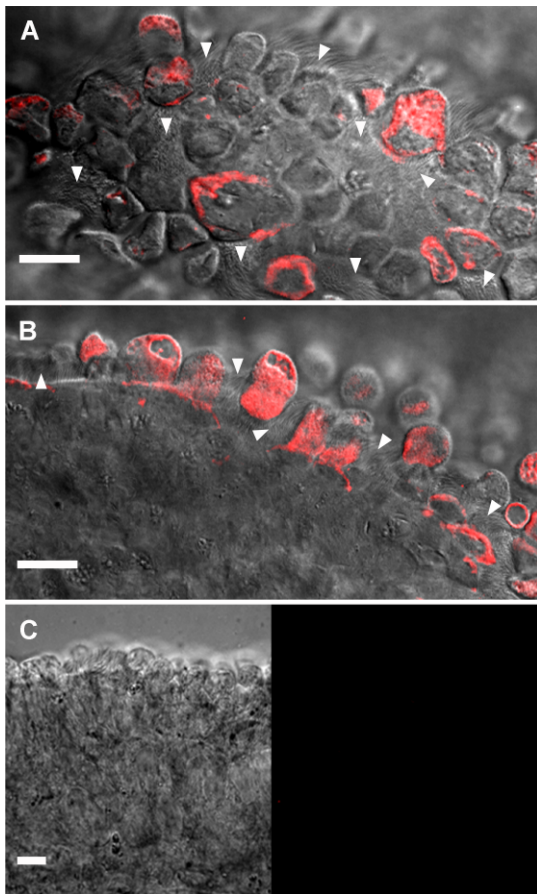


FIG. 9. Confocal images of OEC explants recovered from oviducts that were treated with PI-PLC-released sperm proteins in situ. Oviductal explants were labeled with either anti-DEFB126 Ig (A, B) or anti-SPAM Ig (C) followed with Alexa 488 secondary antibody. The signal for DEFB126 (red) was superimposed on the differential interference contrast image (A, B). Arrows indicate the position of ciliated epithelial cells along the apical surface (A, B). Explants treated with anti-SPAM Ig lacked the cell-specific labeling observed with anti-DEFB126 Ig, even at twice the laser exposure intensities (C). This experiment was repeated with oviducts from two different macaques, both of which were at midcycle at time of tissue harvest. Bars = 10  $\mu$ m.

explants from different females, although no significant differences in sperm-OEC binding were observed when comparing rhesus to cynomolgus monkeys (data not shown). The mixed-model ANOVA used for series 1 experiments explained roughly 83% ( $R^2$ ) of the total amount of variation of all measurements. A considerable portion, or 24.3%, of this variance was found to be due to differences between individual females. In assessing females (explant source) as statistical blocks, some of the variability associated with the potentially heterogeneous tissue (differences in animal hormonal status can affect oviductal histological profile and OEC differentiation; [33]) was removed from the total experimental error. Partitioning of the variation in response due to both male and female effects from the experimental error revealed the inhibitory effects of anti-DEFB126 Igs on sperm-OEC binding (Fig. 3). The variability associated with oviductal explants potentially presents limitations for the detection of more subtle treatment effects. In future studies of sperm-OEC interaction in the macaque, we hope to use OECs that are far more uniform in their sperm-binding properties. Long term, fully differentiated and hormonally responsive OEC cultures have recently been developed for this purpose [41].

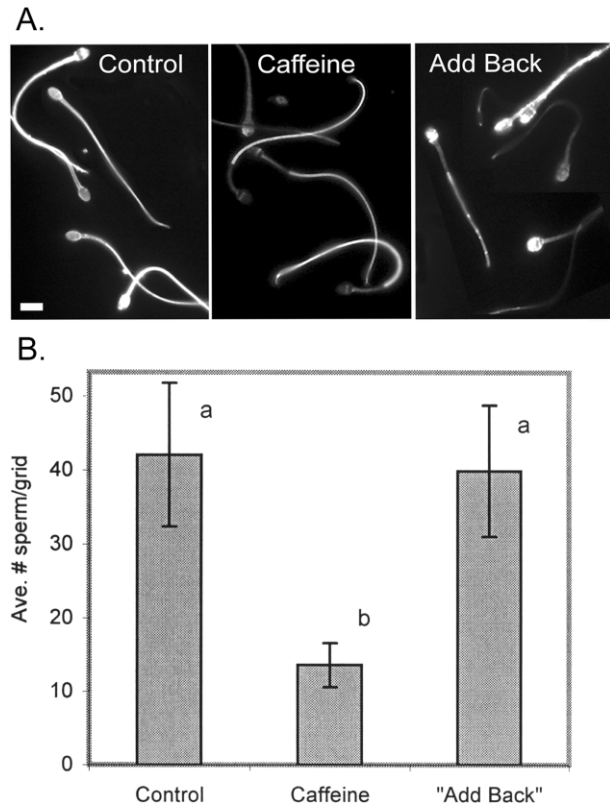


FIG. 10. Adding back DEFB126 to caffeine-treated sperm restores sperm-OEC binding. As described for series 3, sperm were treated with caffeine or with equivalent volume of PBS (control). Sperm were washed into BWB and resuspended into three drops containing about 2 million sperm each. Phosphate-buffered saline was added to a drop of control sperm and a drop of previously caffeine treated sperm (control and caffeine, respectively). DEFB126 solution was added to another drop of previously caffeine treated sample (add back). Drops were incubated for 60 min. Sperm were washed into BWB to remove excess add-back DEFB126. Sperm from each treatment were labeled with anti-DEFB126 Ig and Alexa 488 secondary antibody. Bar = 10  $\mu$ m (A). Sperm-OEC binding was performed and scored as described in Figure 3 (B). Data are reported as mean  $\pm$  SEM. Experiments were repeated five times, each time with sperm from a different male. Columns with different letters (a, b) are significantly different (ANOVA,  $\alpha = 0.01$ ; Tukey range test;  $P \leq 0.05$ ).

With overnight incubation, 70%–80% of sperm respond to activator compounds (caffeine or the combination of caffeine and dbcAMP) with a near-complete release of DEFB126 from over the head [22]. The remaining sperm retain a modest to heavy coat of DEFB126. The less-than-total synchrony of capacitation may explain, in part, why inhibition of sperm-OEC binding never exceeded 70% with treatments that remove DEFB126 from sperm. Yet it remains a possibility that other sperm surface factors could be involved in promoting sperm attachment to the oviductal epithelium. Given that DEFB126 and PSP94 represent 95% of the total protein released from macaque sperm during capacitation [21], it seems unlikely that another “releasable” factor could play a substantial role in OEC binding. Perhaps there are sperm surface components unmasked during capacitation that bind with relatively low affinity to OECs. Such bonds may keep less vigorous sperm adhered to the epithelium. Some sperm may also become wedged in folds of the epithelium, reinforcing weak sperm-OEC bonds and making sperm more resistant to removal by rinsing.

Direct evidence for a sperm reservoir in the oviduct has yet to be reported in primates, yet numerous in vitro studies in

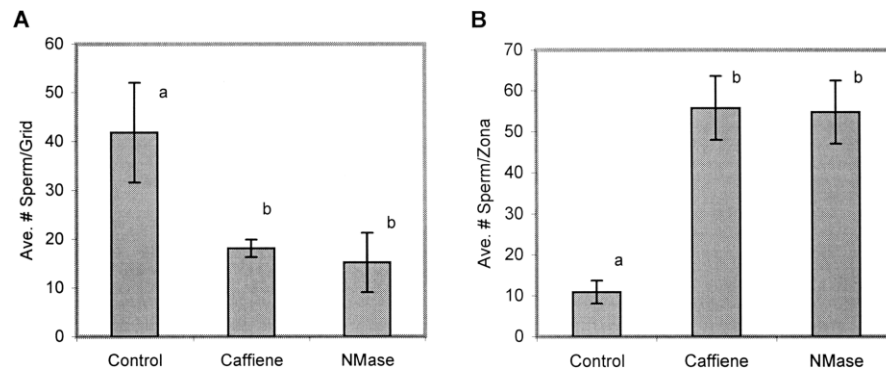


FIG. 11. **A**) Sperm-OEC binding following treatment of sperm with caffeine or NMase. Sperm were treated with caffeine (2 mM), NMase (0.5 units/ $5 \times 10^6$  sperm/ml), or with an equivalent volume of DPBS (control) as described in series 4. Sperm then were coincubated with oviductal explants, which were assessed for sperm attachment as described in Figure 3. Experiments were repeated five times, each time with sperm from a different male. Data are reported as mean  $\pm$  SEM. Columns with different letter superscripts (a, b) are significantly different (ANOVA,  $\alpha = 0.01$ ; Tukey range testing;  $P \leq 0.03$ ). **B**) Sperm-zona pellucida binding following treatment of sperm with caffeine or NMase. Sperm were coincubated with intact zonae pellucidae. Zonae were added for 2 min to sperm suspension drops maintained under equilibrated mineral oil. Zonae then were transferred through drops of BWB at 37°C with a fine-bore glass pipette and mounted on a glass slide. The total number of sperm bound to the zona was scored with phase microscopy at approximately 400 $\times$ . Data are reported as mean  $\pm$  SEM. Experiments were repeated four times, each time with sperm from a different male. Columns with different letters (a, b) are significantly different (ANOVA,  $\alpha = 0.01$ ; Tukey range testing;  $P \leq 0.025$ ).

humans suggest that sperm-OEC attachment is a critical step in the regulation of primate fertilization. Unlike rodents and domestic farm animals, which have easily recognized regions of the oviduct (infundibulum, ampulla, and isthmus) with notable interfaces between them, the primate oviduct lacks such clear anatomical distinctions [1]. As a result, a reservoir of sperm in a specific location of the oviduct may not exist but, rather, sperm could be distributed sparsely along the entire length of the oviduct, a possibility supported by the only two *in vivo* observations reported in women following carefully timed inseminations [42, 43]. It should be noted that in both of these reports, all inseminations were performed after the midcycle estradiol peak, and most oviducts were observed in a span of 48 h bracketing ovulation, a time in which conditions of the oviduct may not support the formation of a reservoir. Furthermore, human sperm do appear to bind in higher number to oviductal epithelial explants extracted from the isthmus than to explants from the ampulla [7]. Human sperm-OEC binding is firm, resisting gentle rinsing with medium. The intimate nature of this interaction was confirmed ultrastructurally where both sperm and epithelial membranes were seen in close apposition [44]. Oviductal epithelial cells appear to select for higher-quality human sperm, as the population of attached sperm had significantly better motility, fewer membrane disruptions, and fewer abnormalities in chromatin structure than sperm that were not attached [45]. Human sperm released from OECs also exhibited enhanced progressive motility, reduced spontaneous acrosome reactions, and increased zona pellucida binding ability [46–48].

The release of sperm from the oviduct appears to signal the completion of sperm capacitation. DeMott and Suarez [49] observed through the wall of the oviduct that only hyperactivated mouse sperm detached from the OECs. Similarly, human sperm at the moment of release from cultured OECs exhibited hyperactivated motility [50]. Increases in cytosolic  $Ca^{++}$ , a universal indicator of capacitation, were observed in horse sperm released from OEC monolayers. The  $Ca^{++}$  concentration of released sperm was three times higher, on average, than that of sperm that remained bound over the same time interval [51]. Once sperm complete capacitation, sperm-OEC binding does not appear to be possible. Hamster sperm capacitated and hyperactivated *in vitro* do not bind to

epithelium when infused into hamster oviducts [2]. Similarly, macaque sperm have significantly reduced sperm-OEC binding when capacitated with ACT (Fig. 3). Some studies have suggested that capacitation results in the removal or loss of sperm surface components with an affinity for OECs, thereby promoting the release of sperm from oviductal surfaces [52]. Consistent with this supposition, treatment with ACT has been shown to be associated with the loss of the dominant surface-coating protein DEFB126 [21, 22], which has been implicated in mediating attachment of sperm to OECs in the studies described in this report. However, ACT treatment has also been shown to induce hyperactivated motility, which may contribute to a decrease in sperm-OEC binding. The increased force generated by hyperactivation could be responsible, at least in part, for disrupting bonds formed between the head of sperm and OECs [53]. This hypothesis, however, is not supported by our data with respect to the formation of sperm-OEC bonds mediated by DEFB126. Treatment of macaque sperm with caffeine, which does not induce hyperactivation but does trigger the release of DEFB126, was shown to result in reductions of sperm-OEC binding comparable in magnitude to reductions observed following treatment with ACT (Figs. 8 and 9A). Although we observed that very few sperm without DEFB126 remain attached to OECs after rinsing (Fig. 4), we speculate that “uncoated” sperm can still bind to OECs, but with lower avidity. Vigorous sperm motility may play a role in breaking weaker sperm-OEC bonds that form after the removal of DEFB126, enabling capacitated sperm to advance in the direction of the egg.

In the macaque, capacitation *in vitro* results in the release of DEFB126 from the sperm surface [21, 22]. We speculate that completion of capacitation *in vivo* results in the release of macaque sperm from the oviductal reservoir. Completion of sperm capacitation in the oviduct likely occurs in response to hormone-regulated changes in the composition of oviductal fluid. Following the preovulatory gonadotropin surge in primates, the rapid decrease of estradiol secretion by the ovarian follicle and the subsequent shift to progesterone secretion [33, 54] represent pivotal events in the preparation of maternal tissues for fertilization and implantation [55–57]. Among the target tissues of reproductive steroid hormones, oviductal epithelium exhibits significant increases in secretory

activity in most mammals during this periovulatory period [55]. The secretory activity results in changes in the oviductal milieu that are characterized by increases in concentration of oviduct-specific glycoproteins [58], amino acids,  $K^+$ , and  $HCO_3^-$  [55, 59] and decreases in overall osmolarity and the concentration of glucose and pyruvate [55]. Furthermore, follicular fluid released into the ampulla at ovulation contains high concentrations of progesterone [60] and glycosaminoglycans [61, 62]. Many of these factors to which sperm are potentially exposed while in the oviduct have been shown to play a role in inducing capacitation in vitro [1, 58, 63]. We speculate that some combination of factors that are only present at or near the time of ovulation triggers the completion of capacitation, the loss of DEFB126 and, therefore, the release of macaque sperm from the oviductal epithelium. Released sperm would then be able to move toward the site of fertilization, aided by peristalsis-generated currents [64], hyperactivated motility [53], and thermotactic and chemotactic gradients [65].

Our experimental results suggest that macaque sperm DEFB126 shares many characteristics with PDC-109, a well-characterized surface-associated sperm protein that mediates bovine sperm-OEC attachment. Both DEFB126 and PDC-109 are released from the surface of sperm during capacitation [13, 14, 19, 21] and with salt extraction [14, 21]. The release of DEFB126 from the head of sperm is crucial for sperm-zona pellucida binding [22]. Capacitated bovine sperm lose the capacity to bind fucose and gain the capacity to bind mannose [66], which is present in terminal positions on bovine zona glycoproteins [67], suggesting that PDC-109 may also mask sperm zona receptors. The removal of DEFB126 and PDC-109 from the head of sperm following capacitation was shown to result in the reduction of sperm-OEC attachment (Figs. 3, 8, and 9; Gwathmey et al. [13]). Oviductal epithelial cell binding can be restored when sperm of either species are washed out of capacitating conditions and treated with their respective coating proteins (Fig. 8; Gwathmey et al. [13]). This capacity for soluble DEFB126 and PDC-109 to readily reassociate with the surface of previously capacitated sperm speaks, perhaps, to a similar mechanism of sperm surface attachment.

Terminal carbohydrates on DEFB126 appear to mediate sperm-OEC bond formation. DEFB126 is likely highly glycosylated, with seven potential *O*-linked glycosylation sites [21]. Following treatment of sperm with *O*-glycosidase, the apparent molecular weight of DEFB126 shifts from 36 kDa to 10 kDa, in line with an expected molecular weight for a 126-amino acid protein [24]. Treatment of macaque sperm with NMase results in a shift in the IEF of DEFB126 from 3.5 to 6.4; the selective removal of terminally positioned sialic acid residues does not appear to affect the mobility of other sperm proteins on a 2D gel [23]. Sperm treated with NMase retain DEFB126 on their surfaces [23]. In the present study, treatment of sperm with NMase resulted in inhibition of sperm-OEC binding comparable to the level of inhibition achieved when DEFB126 was removed from sperm with caffeine (Fig. 9A). The terminal sialic acid on DEFB126, therefore, appears to impart "function." Removal of terminal sialic acid residues with NMase also removes immunoprotection of sperm surface proteins [23], presumably exposing receptors that enable sperm to recognize and attach to the zona pellucida (Fig. 9B). Furthermore, NMase treatment results in significant changes in lectin recognition of the surface of noncapacitated sperm as well as lectin detection of DEFB126 on blots [24]. We speculate that lectin-like proteins are expressed on the surface of OECs and that these proteins recognize terminal sialic acid residues on the *O*-linked carbohydrates of DEFB126 on sperm. This finding appears to be in contrast with observations in

some nonprimate species where sperm recognition of terminal carbohydrates associated with oviduct appears to be the underlying mechanism behind the formation of sperm-OEC "bonds" [52].

At present, the identity of binding molecule(s) for DEFB126 on OECs is not known, but expression of DEFB126 binding sites appears to be consistent with the location and pattern of sperm attachment. In OEC binding experiments, sperm were not evenly distributed across the epithelial surface. Instead, sperm frequently bound in close proximity to one another in small groups, with somewhat larger spaces between groups (data not shown). Such a distribution of sperm is consistent with the observation that DEFB126 binds selectively to specific secretory cells that are "spaced" apart by ciliated epithelial cells and other secretory cells that do not express a DEFB126-binding site (Fig. 7). In support of this notion, both confocal images and videomicrographs of OEC-attached sperm (data not shown) demonstrate that the heads of sperm tend to bind to and between secretory cells.

Selective binding of sperm to secretory cells may be a distinctly primate phenomenon. Scanning electron microscopy revealed in both bovine and porcine systems that sperm bound predominantly to the cilia of epithelium extracted from the isthmus [4, 5]. Scanning electron microscopy of oviductal epithelium recovered from naturally mated animals demonstrated, however, that the sperm of these species also attached to the microvilli of nonciliated oviductal cells [8, 9, 11]. Perhaps in these species, sperm attachment to microvilli is lower in avidity compared with sperm-cilium attachment, but is enabled in vivo by both viscous oviductal secretions and tight grooves formed by extensive secondary epithelial folding [52, 53]. In contrast, horse sperm seem to bind equally well to both ciliated and secretory cells on oviductal explants both in vitro [6] and on epithelium extracted from the oviducts of mated mares [68].

Differences in the site of sperm docking likely reflect differences in oviductal morphology between these hooved species and primates. In cows, sows, mares, and ewes, the morphology of the epithelium of the isthmus, the purported sperm reservoir, shows little change throughout the estrus cycle [4, 6, 69–71]. In cows, scanning electron microscopy studies clearly show that ciliated epithelium dominates the isthmus, and the occasional secretory cells are nonprotruding and partially concealed by the cilia of neighboring cells [4, 69]. In ewes, sows, and mares, the two cell types are more evenly distributed but, as in the cow, the secretory cells exhibit only a slightly convex apex [6, 70, 71]. On the other hand, the morphology of the isthmus changes dramatically throughout the reproductive cycle of macaques [33]. During the follicular phase, the secretory cells are the dominant cell type, and the raised apical projections of secretory cells become more pronounced as the follicular phase progresses, reaching a maximum around the time of ovulation [33, 72, 73]. Similar observations have been reported in oviducts of women [74–76]. In both human [75] and nonhuman primates [72, 73], the apices of the secretory cells extend well beyond the tips of the cilia of ciliated cells at midcycle. The height of these secretory cells appeared to be greatest in the isthmus segment [33, 74]. It is tempting to speculate that these cells, with projections advanced well beyond the apical plane, help impede sperm movement, thereby ensuring contact and attachment via DEFB126. Sperm attachment may, therefore, be promoted by a combination of physical and chemical properties of OEC membranes, both of which may be hormonally regulated.

In conclusion, we have provided several lines of evidence that suggest that DEFB126 on the surface of macaque sperm

mediates sperm attachment to follicular phase oviductal epithelium. Treatment of sperm to remove DEFB126 (with ACT or caffeine), block DEFB126 (with antibodies), or alter DEFB126 (with sialidase) significantly inhibited sperm ability to tightly bind to OECs. Following removal of DEFB126, the OEC binding ability could be restored by adding DEFB126 back to the sperm surface. Furthermore, soluble DEFB126 localized to the apical surface of secretory epithelial cells, consistent with the pattern and location of sperm attachment. It follows that pretreatment of OECs with DEFB126 inhibited sperm-OEC binding. Based on these findings, we speculate that DEFB126 attaches sperm to OECs in vivo, resulting in the formation of a reservoir of sperm in the nonhuman primate oviduct.

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