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# Beta-Defensin 126 on the Cell Surface Protects Sperm from Immunorecognition and Binding of Anti-Sperm Antibodies[1]

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## ABSTRACT

**Beta-defensin 126 (DEFB126), formerly known as epididymal secretory protein 13.2 (ESP13.2), coats the entire primate sperm surface until completion of capacitation, and it is a candidate for providing immune protection in the female reproductive tract. To further examine the potential role of DEFB126 as a means of protection from immune recognition, cynomolgus macaque sperm were exposed to a number of treatments that are known to alter sperm surface coats, including capacitation. We used a novel in vivo assay to determine immune recognition: aldehyde-fixed whole sperm injections into rabbits. Following booster injections, immunoblot analyses of whole sperm prepared in various manners was conducted. On Days 60 and 80 post-initial immunization, the antisera showed a remarkably strong reaction to a single 34–36 kDa protein, which was shown to be DEFB126. Sera from rabbits that were immunized with sperm washed more rigorously using Percoll gradients showed an increase in the number and intensity of proteins recognized on whole sperm Western blots, although DEFB126 was still the major immune response. When capacitated sperm, from which most DEFB126 had been released, were used as the immunogen, there was a dramatic increase in the immune recognition to a variety of protein bands. Sperm treated with neuraminidase to remove sialic acid on DEFB126 before fixation were shown to still possess DEFB126, but lacked the sialic acid component of the glycoprotein. These sperm were as immunogenic as capacitated sperm even though the desialylated DEFB126 still covered the entire cell surface. These sperm lost their highly negative charge (the isoelectric point of DEFB126 shifted from pI 3.0 to pI 6.4). Experiments using different sperm plasma membrane protein-specific Igs showed that recognition did not occur when DEFB126 was present, but following capacitation these Igs readily recognized the exposed sperm membrane. Our data suggest that DEFB126 protects the entire primate sperm surface from immune recognition and that the sialic acid moieties are responsible for the cloaking characteristic of this unique glycoprotein.**

*beta-defensin, capacitation, gamete biology, glycocalyx, immunogenic, immunology, sperm, sperm maturation*

## INTRODUCTION

One of the greatest mysteries of the reproductive system surrounds the mechanism by which sperm reach the oocyte and at the same time evade immune surveillance within the hostile environment of the female reproductive tract [1, 2]. Austin [3] and Chang [4] were the first to point to the need for sperm to reside in the female for an extended period of time before they are able to fertilize eggs. The discovery of sperm capacitation led researchers to investigate events within the sperm cell and the female tract that collectively contribute to this final sperm maturation [5]. One well-recognized component of capacitation is the removal or the loss of the sperm surface coat(s), which exposes specific receptors on the plasma membrane that are critical for zona pellucida recognition [6–8]. In primates, sperm are deposited into the vagina and subsequently move into the cervix en route to the upper oviduct, the site of fertilization [5]. It has been suggested that the few sperm reaching the site of fertilization are protected by a barrier that enables evasion of the immune system [1, 2]. It seems possible that sperm could be enveloped with a protective coat that prevents the female's immune system from recognizing these foreign cells, and that this coat could be the same as that involved in capacitation.

In the male reproductive tract, sperm plasma membrane components are continuously overlaid with a variety of glycoproteins, which are either glycosylphosphatidylinositol (GPI)-linked or applied to the external surface as peripheral investment coats [9, 10]. The composition of this thick glycocalyx coat is still largely unknown [11, 12]. In general, GPI-anchored glycoproteins are inserted into the plasma membrane during sperm residence in the caput of the epididymis, whereas the surface-associated glycoproteins continue to be added until the time of ejaculation [13–17]. The epididymis has been shown to produce peptides classified as "defensins," which were originally thought only to contribute to the defense of the reproductive system from pathogen invasion [18]. More recently, these defensins have also been shown to be associated with specific sperm functions, including initiation of motility and capacitation [19, 20]. Sperm are highly differentiated cells with five unique morphological regions (acrosome, posterior-head, midpiece, principal piece, and distal flagellum), and the plasma membrane of each region is as diverse as the regions themselves [21]. Most, if not all, of the five different structural regions have unique plasma membrane epitopes [22]. DEFB126 is a β-defensin which appears to be unique among sperm surface coating proteins, in that it uniformly spans the entire sperm surface and is not exclusive to a specific domain [23]. CD52 has also been reported to coat the whole sperm, but there is an elevated presence over the equatorial segment and variable labeling depending on where the sperm resided in the male reproductive tract and the state of capacitation [14, 24, 25]. The question of whether the uniform distribution of

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DEFB126 on the macaque sperm surface can serve as an immunoprotective shield was initially proposed by Tollner et al. [20] and is the focus of this study.

## MATERIALS AND METHODS

### Reagents

All chemicals and reagents were purchased from Sigma Aldrich Chemical Co. unless stated otherwise.

### Semen Collection

Cynomolgus macaques were housed at the California National Primate Research Center in compliance with American Association of Accreditation of Laboratory Animal Care Standards. The Institutional Animal Care and Use Committee at the University of California, Davis, approved all methods and procedures with animal subjects. Semen samples were collected by electro-ejaculation from eight individually caged macaques [26]. Each ejaculate was collected into a 15-ml centrifuge tube containing 5 ml of Hepes-buffered Biggers, Whitten, and Whittingham (BWW) medium (Irvine Scientific, Santa Ana, CA). Within 1 h the samples were checked for motility, and those displaying less than 70% vigorous motility or having a high proportion of sperm with any morphological defect were discarded. The 5-ml sperm samples were filtered through 100-μm mesh to remove the coagulum and debris. The samples were centrifuged at 300 × *g* for 10 min and the resulting pellet was resuspended in fresh medium (0.5–1 ml).

### Sperm Preparation

There are numerous sperm washing techniques, but five were chosen to represent known strategies to remove surface coats and select the greatest population of actively motile sperm. Neuraminidase (Type V: *Clostridium perfringens*) was used as a sperm preparation technique (described later) because it was previously shown to expose plasma membrane components in a way similar to that observed with capacitation [24]. For the first preparation, sperm (250 μl) were layered under fresh medium (3 ml) and incubated for 1 h at 37°C. The top 2 ml (swim-up sperm) were removed from each 3-ml column and were centrifuged for 10 min at 300 × *g*. The resulting pellet was suspended in 2 ml of fixative (described below).

In another preparation, sperm (250 μl) were layered on top of a 3 ml column of medium containing 40% Percoll. The sperm samples were centrifuged through the column for 20 min and the resulting pellet was suspended in fresh medium (10 ml) and centrifuged for an additional 10-min wash. The resulting pellet was suspended in 2 ml of fixative. This preparation is termed 40% Percoll-washed sperm. For the third preparation, sperm (250 μl) were layered on top of a 3-ml column of medium containing 80% Percoll. These sperm samples were centrifuged through the column for 30 min and the resulting pellet was suspended in fresh medium (10 ml) and centrifuged for an additional 10 min wash. The resulting pellet was suspended in fixative and this preparation is termed 80% Percoll-washed sperm.

For the fourth preparation, sperm that had been washed through an 80% Percoll column were suspended in fresh medium containing 30 mg/ml BSA and buffered with 35.7 mM sodium bicarbonate. This medium has previously been shown to support full capacitation of macaque sperm [20]. To achieve capacitation, sperm samples (10–15 × $10^6$ /ml) were incubated overnight at 28°C in 5% $CO_2$ in air. After overnight incubation, sperm were transferred to 37°C and 5% $CO_2$. Sperm were allowed to incubate for 2 h before exposure to 1 mM dibutyrl cAMP and 1 mM caffeine, which are collectively referred to as activators and are known to stimulate synchronous capacitation of macaque sperm [27]. After 1 h incubation with activators, the sperm were layered over a 3-ml column of 40% Percoll in medium containing no BSA. After 20 min centrifugation at 300 × *g*, sperm were suspended in 10 ml of medium (no BSA) and centrifuged for an additional 10 min. The resulting pellet of sperm was suspended in fixative, and this preparation was termed capacitated sperm [27].

In the final preparation, sperm were exposed to neuraminidase to remove the terminal sialic acid(s) from DEFB126. Washed swim-up sperm (50 × $10^6$/ml) were incubated for 1 h at 37°C in Dulbecco PBS (DPBS; Life Technologies) containing 1 U of neuraminidase. At the end of the incubation, sperm were centrifuged (300 × *g*) and the resulting pellet was suspended in fixative. Prior to fixation, sperm were assessed for motility and any sign of morphological defects. All five of the sperm preparations had >95% motility and no evidence of morphological abnormalities at the time of fixation.

### Sperm Fixation and Injection

A 16% solution of paraformaldehyde was prepared in DPBS. A 25% solution of glutaraldehyde (EMScience) was diluted into DPBS to obtain a final concentration of 0.1% glutaraldehyde and 1.6% paraformaldehyde. Fixation was conducted in a siliconized 1.5-ml centrifuge tube for 2 h at room temperature. After 15 min of fixation, the sperm samples were centrifuged (500 × *g*/5 min) and the fixed sperm were suspended in fresh fixative for an additional 1 h 45 min. After 2 h, the samples were thoroughly washed overnight, with multiple changes of DPBS to remove any trace of fixative. The subsequent day, washed sperm were diluted into either Freunds complete or incomplete adjuvant and injected subcutaneously (in five separate locations) into the backs of rabbits. All of the rabbit injection procedures were preapproved by the Research Animal Care Committee, which strictly adhere to the *Guide for the Care and Use of Laboratory Animals*. Swim-up and neuraminidase treated sperm were each given to two rabbits as immunogen, and the 40% Percoll-washed, 80% Percoll-washed, and capacitated sperm were each injected into four separate rabbits. Each rabbit received a total sperm count of 1 × $10^6$, divided equally among all of the injection sites. There were two rounds of injections: an initial immunization of sperm with complete Freunds adjuvant, followed 30 days later with a boost using the same concentration of sperm, same number of injection sites, but with incomplete Freunds adjuvant. A 5-ml test bleed was conducted on days 30, 60 and 80 following initial immunization. The immune responses, when analyzed with Western blots. were very similar, if not identical, within each different sperm preparation.

Five female cynomolgus macaques were immunized with fixed swim-up sperm as previously described, except that Montanide was used as the carrier adjuvant [28]. Serum samples were collected from each of the females at 60 and 80 days following immunization and analyzed for recognition of DEFB126.

### Electrophoretic Analysis

An ejaculate was suspended with 5 ml of Hepes-buffered BWW and allowed to disperse at room temperature for 30 min. The sperm suspension was filtered (100 μm) to remove the coagulum. The filtered ejaculate was centrifuged at 300 × *g* for 10 min; the resulting pellet was layered under a 3-ml column of buffer, and sperm were allowed to swim up for 1 h at 37°C. The top 2 ml were diluted into 10 ml of buffer and centrifuged for 10 min at 300 × *g*. The resulting pellet was placed in a 1.5 ml microfuge tube, mixed with buffer, and pelleted at 1000 × *g* for 5 min. The pellet was solubilized for either SDS-PAGE or high-resolution two-dimensional (2-D) gel separation using isoelectric focusing and SDS-PAGE. Standard SDS-PAGE protein separation was accomplished with the washed sperm pellet, solubilized in boiling (100°C) SDS-solubilizing buffer (Pierce) for 3 min. The sample was allowed to cool at room temperature and was centrifuged at 5000 × *g* for 10 min. The supernatant was chemically reduced with 0.1 M dithiothreitol (DTT) and boiled an additional 2 min. This sample was termed whole reduced sperm.

Approximately $10^4$ solubilized sperm were placed in each of 10 wells of an 8%–16% gradient Tris-glycine gel (Invitrogen). Western blots were prepared as described previously [23]. Nitrocellulose membrane blots were blocked for 2 h in Tris-buffered saline (TBS; 50 mM HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20), in which 1% BSA, 1% nonfat dry milk, and 1% gelatin were added. Blots were dried overnight and each well/lane of whole reduced sperm was individually cut into strips to be probed with the appropriate antiserum. 2-D gel separation was accomplished with 40% Percoll-separated sperm as previously described, except that the final wash was in 0.9% NaCl. The washed sperm were solubilized in 2 ml of 5% Nonidet P-40, 8 M thiourea, and 100 mM DTT at 4°C for 2 h with constant agitation. After 2 h the sperm extract was centrifuged at 2000 × *g* for 10 min and the resulting supernatant was equilibrated with 1% ampholines (pH 3–10; Invitrogen). The immobilized pH gradient (IPG) strips were incubated in 250 μl of the sperm extract. After absorption, the IPG strips were focused overnight at 1200 V. Once focused, the IPG strips were soaked in 5% SDS and 100 mM DTT and placed into the running slot of a 10% SDS-PAGE. The sample was run in the second dimension at 4 mA until the tracking dye reached the bottom of the gel. One of the 2-D gels was fixed in 50% methanol/5% acetic acid and prepared for silver staining (Bio-Rad). The second gel was blotted onto nitrocellulose using a semidry transfer cell (Bio-Rad). The blot was incubated in blocking solution for 2 h and allowed to dry overnight.

### Immunoblot Analysis

After electrophoretic transfer, all of the blots were washed to remove any gel material, allowed to dry, and placed in blocking solution as previously described. After blocking for 2 h, the blots were washed briefly in TBS with 0.1% Tween 20 and allowed to dry overnight. The blots were incubated for 2 h at room temperature in serum from the different immunized rabbits. The serum

(preimmune and immune) was diluted 1:100 in blocking solution and allowed to sit overnight at 4°C. Individual strips were incubated for 2 h at room temperature with constant rotation. Each rabbit and time was represented by a separate tube. After 2 h, the strips were washed in TBS plus 0.1% Tween 20 (10 ml/tube, 3 × 10 min washes). After washing, the strips were incubated for 1 h with secondary antibody (goat anti-rabbit alkaline phosphatase, Bio-Rad) diluted 1:2000 in blocking solution. Each strip was thoroughly washed in blot-washing solution before being placed in NBT/BCIP (Pierce) at room temperature for 4 min and then immediately transferred to 100 mM EDTA to stop the development reaction at exactly 4 min. Identification of DEFB126 was conducted with the same whole sperm blots, in which the antibody was anti-DEFB126 specific Ig [23].

### *Immunolocalization of Anti-DEFB126 on Sperm*

Sperm were prepared as previously described for each of the different washing techniques, except that the same ejaculate was used for all the washing. At the completion of each of the washing procedures, sperm were resuspended in BWW with 30 mg/ml of BSA, except for the sperm in capacitation medium, which already contained BSA. Anti-DEFB126 Ig (20 μg/ml) was added to each of the sperm samples and allowed to incubate at 37°C for 30 min, at which time 2% paraformaldehyde was added (1:1) and the samples were centrifuged (300 × *g*). The resulting pellet was placed into fresh fixative (1.6% paraformaldehyde/0.1% glutaraldehyde in DPBS). After 15 min the sperm were thoroughly washed in blocking solution (1%BSA/1% gelatin in DPBS). After a thorough washing, the sperm were fluorescently labeled with goat anti-rabbit Alexa488 (20 μg/ml) for 30 min. The samples were thoroughly washed before viewing and photomicrography, as previously described [20].

### *Sperm Plasma Membrane Antigen Recognition by Antisperm Antibodies*

Three separate macaque sperm head-specific antibodies were used to define their ability to gain access to the sperm surface before and after capacitation. One of the antibodies is directed against SPAM1, which is well characterized as the macaque sperm hyaluronidase that is GPI-linked to the plasma membrane of the sperm head [29, 30].

The second antibody recognized the head-specific antigen, a disintegrin and metalloproteinase domain 30 (ADAM30), which is found in a complex with SPAM1. To obtain this antibody, initially washed sperm ($50 \times 10^6$) were incubated with antibodies specific to SPAM1 (50 μg/ml) for 1 h and then washed through 40% Percoll before solubilization in buffer (25 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1% octyl-glucoside; and 1% triton X-100) with a cocktail of protease inhibitors (Calbiochem) and 10 mM EDTA. Solubilization and incubation were done at 4°C for 1 h before centrifugation at 10,000 × *g* for 1 h. The supernatant was incubated overnight with 50 μg of Protein A beads at 4°C and constant rolling. The beads were thoroughly washed (10×) with the previous incubation medium, but without the detergents. The washed beads were placed in 2 ml of 10 mM Tris-HCl (pH 3), 150 mM NaCl, and protease inhibitors for 10 min; the beads were then removed by centrifugation, and 10 μl of 1M Tris were added to bring the pH above 7. The sample was concentrated and solubilized with electrophoretic buffer (Pierce). Electrophoresis revealed two bands, one of which was SPAM1 (64 kDa). The other band, a 84-kDa protein, was cut out of the gel, digested, and sequenced by the Molecular Structure Laboratory as previously described [20]. An 11-amino-acid sequence revealed a 100% homology with ADAM30, which has a theoretical MW of 79 kDa. [31]. Antibodies (anti-ADAM30 Ig) were raised in a rat to the eluted protein, and it was found to recognize a single band with Western blotting.

A third sperm head-specific antigen was isolated by solubilizing 80% Percoll-washed sperm ($20 \times 10^6$/ml) in the previously described extraction buffer at 4°C in 1.5-ml low-retention tubes. Proteins with a high degree of hydrophobic character will often adhere to low-retention tubes. Sperm suspensions were kept agitated and cooled for 1 h, at which time the solution was discarded and the tubes were washed with buffer three times before adding 300 μl of SDS solubilizing buffer (Pierce). The sample was boiled and 100 μM DTT was added. When the sample was electrophoretically analyzed, there were a small number of bands, two of which run at 240 kDa and appear as a dimer. Antibodies to this complex were developed in rabbits, and the antibodies recognized only the 240-kDa dimer.

Sperm were washed by two separate techniques as previously described for obtaining swim-up and fully-capacitated sperm. In each case sperm were incubated in media containing one of the three antisera (1:100) for 1 h, then washed and fixed as previously described and incubated with blocking solution and goat anti-rabbit/rat Alexa488 (1:200) for 1 h with continuous rolling. Samples were thoroughly washed and viewed with fluorescent microscopy. Sperm first were visualized and photographed for evidence of SPAM1 labeling (after capacitation) and these settings were then used throughout the experiment to standardize the exposures.

## RESULTS

### *Western Blot Analysis of Antisera Obtained Following Whole Sperm Injections*

Whole sperm were originally used for immunizations in initial studies of various sperm surface domains [32, 33]. In the present study, rabbits immunized with whole, fixed, swim-up sperm showed an immune response by 30 days, at which time a boost was given at the same sperm concentration as the original injection. Thirty days following the whole sperm boost, the rabbit sera were examined for any sign of immunoreactivity to whole sperm blots. When whole sperm blots were exposed to each of the preimmune sera, there was little or no evidence of immune reactivity, but on Day 60 there was significant antibody recognition of specific antigens, most notably to a broad band at the 34–36 kDa region (Fig. 1 lane C). This broad band corresponds to DEFB126, which was localized by specific antibody to the same 34–36 kDa region of the whole sperm blot (Fig. 1 lane B). Both rabbits that received whole swim-up sperm as the immunogen showed the same electrophoretic profile, in which there was strong immune recognition of the 34–36 kDa DEFB126 band as well as minor recognition of a high-molecular-weight region (Fig. 1 lane C). Although only one blot is shown for each of the different sperm preparations, it should be noted that the immune sera from the different rabbits receiving a given preparation were very similar if not identical in their immune reactions on a Western blot. Following immunization with sperm that were washed through either 40% or 80% Percoll, there was also evidence of an immune response to the 34–36 kDa regions, but in each case a number of other immunoreactive bands were also apparent (Fig. 1, lanes D and E). More specifically, there was increased recognition of antigens with apparent molecular weight above 100 kDa, the most obvious of these being the region around 240 kDa (Fig. 1, lanes D and E).

When capacitated sperm were used as the immunogen, there was still some recognition of the 34–36 kDa region, but multiple immunoreactive bands appeared to be recognized (Fig. 1 lane F). Not only were the higher molecular weight proteins (>100 kDa) recognized, but also a number of lower molecular weight proteins (Fig. 1 lane F). Capacitated sperm and Percoll-washed sperm each elicited a potent and broad range of immune reaction to a spectrum of sperm antigens from 10 to 300 kDa. The fact that both of these preparation methods result in loss of DEFB126 from the sperm surface [20, 27] suggested that DEFB126 provided immune protection of other sperm antigens. However, it was not clear whether this protection was attributable to the DEFB126 protein itself or to its sialic acid residues. Neuraminidase treatment of swim-up sperm before use as an immunogen resulted in some degree of DEFB126 recognition, but more striking was the strong immune recognition of a variety of other protein bands (Fig. 1 lane G). In fact, this recognition by sera following immunization with neuraminidase-treated sperm was similar to that seen when capacitated sperm was used to elicit the immune response, although capacitating did expose a 20-kDa antigen that was quite apparent (compare Fig. 1, lanes F and G).

To examine the higher molecular weight proteins that were immunologically recognized, whole sperm samples were subjected to electrophoresis in such a manner that the profile of the larger proteins could be more easily distinguished (Fig. 2 lane A). The same 60-day serum samples from animals

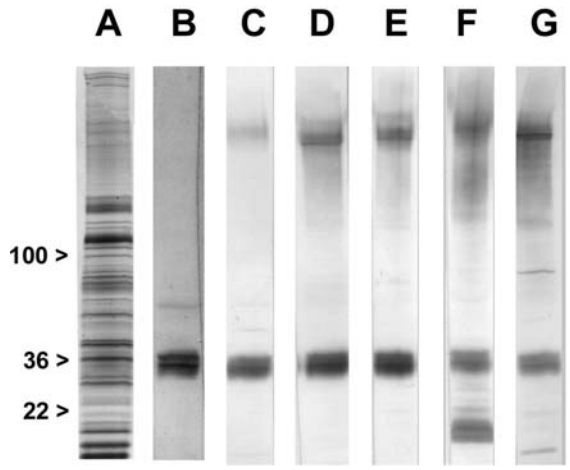


FIG. 1. Western blots of whole macaque sperm on an 8%–16% gel. Sera were obtained from rabbits 60 days after initial immunization with whole fixed sperm prepared using different techniques. Silver staining of whole sperm preparations shows many protein bands ranging from 10 kDa to over 300 kDa (lane A). A nitrocellulose blot of the whole sperm preparation probed with serum from a rabbit immunized with whole swim-up sperm shows strong immune recognition of a 34–36-kDa region (lane C) which corresponds exactly with the 34–36-kDa band recognized by anti-DEFB126 (lane B). The serum from a rabbit immunized with whole swim-up sperm showed additional recognition of the 240-kDa region, although this was a minor band (lane C). Both 40% (lane D) and 80% (lane E) Percoll washing of sperm before fixation and immunization resulted in a number of antigens being recognized, especially above 100 kDa (lanes D and E). Although there were obviously many recognition sites above 100 kDa, there was still a potent immune response to the 34–36-kDa band (lanes D and E). Capacitated sperm, on the other hand, stimulated an immune response to a host of sperm surface antigens from 10 kDa to over 300 kDa (lane F). Immunization with swim-up sperm that were treated with neuraminidase also recognized an increased number of bands, many of which were above 100 kDa (lane G). Sera raised by capacitated and neuraminidase-treated sperm still showed some degree of DEFB126 recognition (lanes F and G). Each blot (lanes C–G) represents a single rabbit, but the serum from all of the rabbits within a preparative technique gave almost identical immunological results on Western blots.

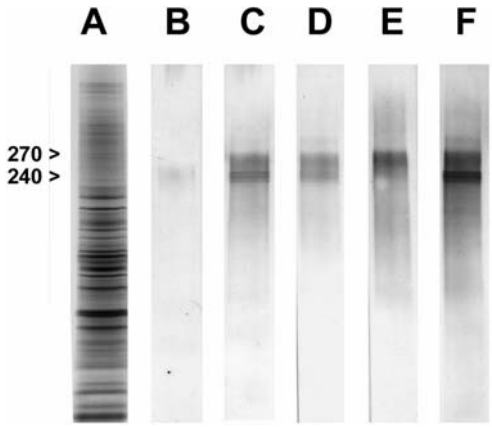


FIG. 2. Whole macaque sperm preparations were run on an 8%–16% gel until the 64-kDa tracking band reached the gel exit. This gave a slightly clearer view of the high molecular weight range, as shown by the silver-stained gel (lane A). Sera obtained from rabbits immunized with swim-up sperm showed evidence of a minor immune response to the 240-kDa region, but immunization with sperm washed through 40% and 80% Percoll resulted in a much more potent immune reaction and a strong recognition of the 240–270-kDa region (lanes B–D). Immunization with capacitated sperm resulted in a strong reaction to a host of antigens above 100 kDa (lane E). Neuraminidase treatment of swim-up sperm clearly exposed the 240–270-kDa region, along with a number of other proteins above 100 kDa (lane F).

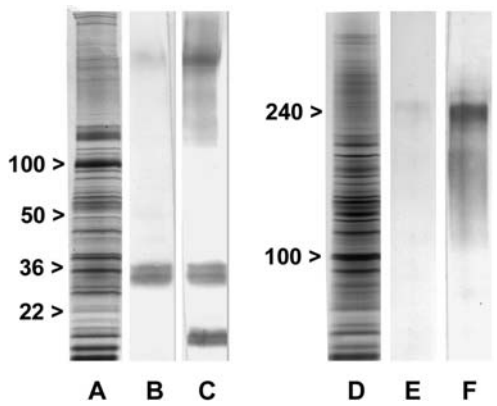


FIG. 3. Silver stained gels of whole macaque sperm (lanes A and D). Western blots of whole macaque sperm were probed with antisera obtained 80 days post-initial immunization. At 80 days postinjection, the antisera against swim-up sperm still had the greatest immune response to DEFB126 and minor recognition of a 240-kDa component (lane B). Antisera to capacitated sperm obtained at 80 days were very similar to the 60-day antisera in that there were a number of immune recognition sites with molecular mass >100 kDa (lane C). The high-molecular-mass region showed a similar response to antisera obtained at the 60- and 80-day time points, at which the sera raised against swim-up sperm showed only a minor reaction to 240 kDa, whereas those raised against capacitated sperm showed a major immune response to a number of antigens >100 kDa (lanes E and F).

immunized with swim-up sperm, when incubated with blots of the higher molecular weight profiles, clearly showed a minor immune recognition of the 270-kDa region (Fig. 2 lane B). Percoll washing appeared to expose more of the 240–270-kDa complex, because each preparation (40% and 80% Percoll wash) resulted in a very strong immune reaction to this region (Fig. 2, lanes C and D). Sera from animals immunized with capacitated sperm showed the same strong recognition of the high molecular weight complexes, as well as recognition of a broad and diffuse region from 100 kDa to well over 300 kDa (Fig. 2 lane E). Neuraminidase treatment of sperm resulted in a profile of immune recognition similar to that seen with capacitated sperm (Fig. 2 lane F).

Sera obtained from animals 80 days following initial immunization with swim-up sperm still had evidence of a strong recognition of the 34–36-kDa (DEFB126) region (Fig. 3, lanes A and B). Even after 80 days the primary immune recognition was to the 34–36-kDa region, although, as seen at 60 days, there was minor recognition to the 240-kDa region. Even with closer examination of the higher molecular weight region, there was little recognition by sera obtained at the 80-day time point following immunization with swim-up sperm, but many bands were recognized following immunization with capacitated sperm (Fig. 3, lanes C and F).

When female macaques were immunized with fixed swim-up sperm and serum samples were obtained 60 and 80 days later, there was no evidence that a 34–36-kDa band was recognized on whole sperm blots (data not shown).

### *DEFB126 on the Sperm Surface after the Washing Procedures*

DEFB126 is a major component of the cynomolgus macaque sperm surface coat [23]. By incubating sperm that have been prepared with a variety of washing techniques and then using anti-DEFB126 to visualize the different profiles, it was clear that sperm preparation by the swim-up technique resulted in a heavy coat of DEFB126 on the entire sperm

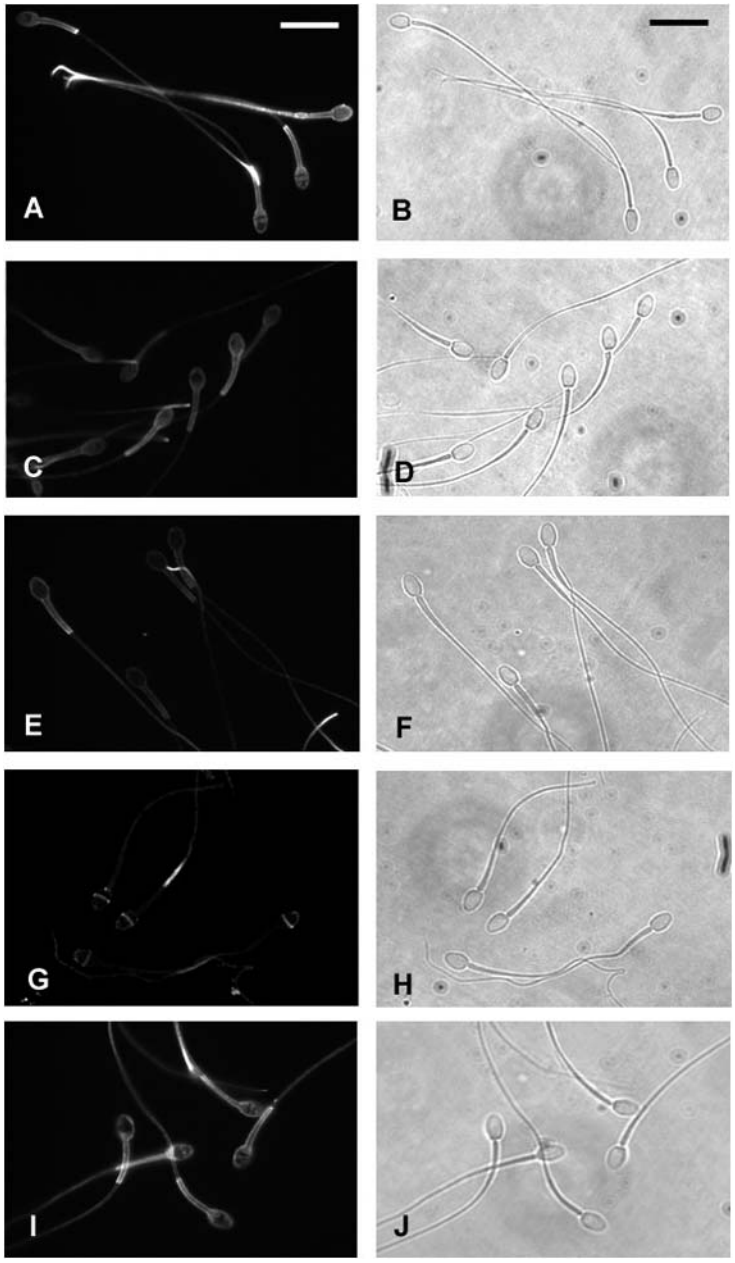


FIG. 4. Sperm were washed or treated in all of the ways used for immunization experiments, but before fixation sperm were incubated for 30 min in anti-DEFB126 Ig. Following secondary antibody labeling, sperm were viewed for immunolocalization of DEFB126. Swim-up sperm had an extensive coating of DEFB126 over the entire sperm surface (**A** and **B**). Sperm washed through 40% and 80% Percoll also had DEFB126 on the entire surface, but in each case there was a distinct reduction in the intensity of the label (**C**–**F**). Capacitated sperm clearly had a dramatic loss of DEFB126, although there was some retention of DEFB126 over the equatorial segment and along the flagellum (**G** and **H**). Neuraminidase treatment did not remove DEFB126 from the sperm surface, as was clearly visible when anti-DEFB126 recognized the entire sperm surface (**I** and **J**), which was quite similar to the labeling of swim-up sperm (**A**). Bar = 10 μm.

surface (Fig. 4A). Sperm that were washed through 40% Percoll before exposure to anti-DEFB126 had the same pattern seen in swim-up sperm, but there was an overall reduction in the amount of DEFB126 on the sperm surface (Fig. 4C). After 80% Percoll washing, sperm still maintained a coat of DEFB126 on the surface, but it was greatly reduced in magnitude (Fig. 4E). Sperm that were incubated overnight in high-BSA medium and also activated to stimulate capacitation showed a dramatic loss of DEFB126, although the equatorial segment and some flagellar labeling were retained (Fig. 4G). Exposure of swim-up sperm to neuraminidase showed a very similar anti-DEFB126 labeling pattern (Fig. 4I) to that shown for swim-up sperm (Fig. 4A).

### *Effect of Desialylation on DEFB126*

The effect of neuraminidase on the DEFB126 glycoprotein was examined using both standard SDS-PAGE and 2-D protein separation. Western blot analysis of DEFB126 from neuraminidase-treated sperm showed a minor, but significant, elevation in molecular weight (Fig. 5). There was an upward shift in molecular weight of about 4 kDa, such that the broad 34–36-kDa band of DEFB126 shifted to 38–40 kDa (Fig. 5). Solubilized swim-up sperm that were separated using standard 2-D technology as previously described for human sperm [34] showed a number of proteins after silver staining (Fig. 6A). The ampholines for the 2-D gel covered a broad range (isolectric point [pI] 3–10) and most of the protein spots were clustered between pI 4.5 and pI 8 (Fig. 6A). Very few spots were observed below pI 4.5, but remarkably, when the whole sperm sample was blotted over to nitrocellulose and probed with anti-DEFB126 Ig, DEFB126 was located at the edge of the gel indicating the lowest pI of 3.0 (Fig. 6B). When sperm were pretreated with 1 U of neuraminidase for 30 min before solubilization, there was a shift in the apparent molecular weight of DEFB126 (Fig. 5) and a shift in pI from 3 to a broad spot close to 6.2–6.4 (Fig. 6D).

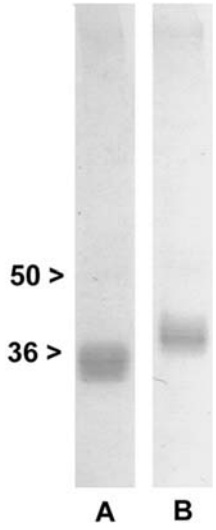


FIG. 5. Macaque sperm exposed to 1 U of neuraminidase/ejaculate were run on an 8%–16% gel. Western blots of the same sperm sample that were and were not exposed to neuraminidase and then probed with anti-DEFB126 Ig showed that there was a noticeable increase in molecular weight from 34–36 kDa to about 38–40 kDa, or about a 4-kDa increase (lanes A and B).

### *DEFB126 Protects Sperm from Antibody Recognition In Vitro*

To determine whether DEFB126 protects sperm surface antigens from being recognized by antisperm antibodies, we exposed sperm to antibodies raised against three specific sperm membrane proteins (SPAM1, ADAM30, and a 240-kDa dimer), chosen because they are directed against antigens on the plasma membrane of the macaque sperm head. When swim-up sperm were incubated for 1 h in each antibody before fixation, there was little or no fluorescent labeling (Fig. 7). Sperm that were fully capacitated before exposure to the antibodies showed evidence of strong antibody recognition of all sperm, indicating that epitopes were exposed for antibody recognition following DEFB126 release (Fig. 8, B, D, and F). Because sperm were not fixed before incubation with the different Igs, the sperm surface antigens showed some degree of aggregation (Fig. 8, B, D, and F). Western blots of whole sperm were probed with the three antibodies to each of the sperm surface components—SPAM1, ADAM30, and the 240-kDa dimer—and all were specific to the single antigen (Fig. 9, lanes A–C).

## DISCUSSION

Mature sperm must reside in the male and female reproductive tracts for extended periods of time without detection by the immune system. To accomplish this, sperm are cloaked with a surface coating that protects these foreign cells from being recognized as nonself. The ability of sperm to masquerade as friend, not foe, in both the male and female reproductive tracts has puzzled researchers for years, because sperm deposition in the female tract is tantamount to the transplantation of foreign cells in the body. How sperm traverse the hostile female reproductive tract and reach the oocyte in such a hostile environment must involve a number of factors, one of the most important being the sperm surface coat. The results of this study suggest that the epididymal secretory protein DEFB126 forms a coat on primate sperm that provides protection from recognition by immunocompetent cells in

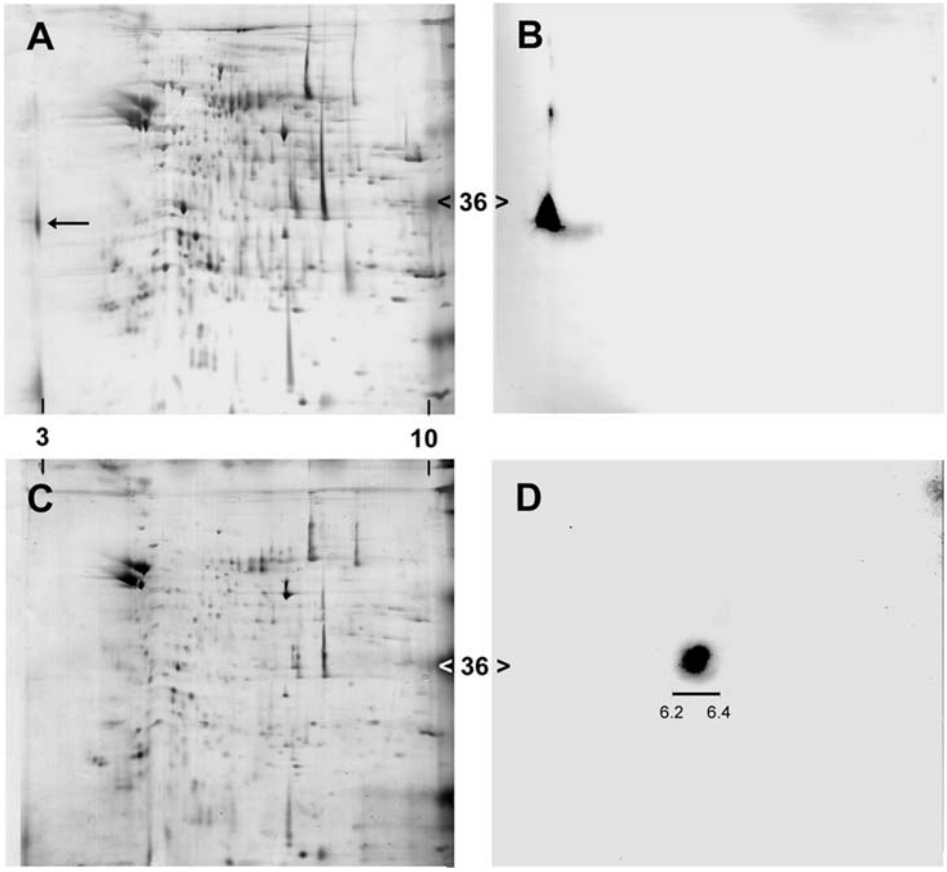


FIG. 6. 2D-gel electrophoresis of whole macaque sperm before and after neuraminidase treatment revealed a number of proteins when the gel was silver stained (**A** and **C**). The whole macaque sperm sample was used for Western blotting and probed with anti-DEFB126 Ig, which revealed a broad band at around pI 3. Exposure to neuraminidase dramatically changed the pI from around 3 to around 6.4 (**B** and **D**). As seen with isoelectric focusing and the standard gel separation, there was a shift in molecular weight following neuraminidase treatment to about 38 kDa (**C**, **D**, and Fig. 5 lane B).

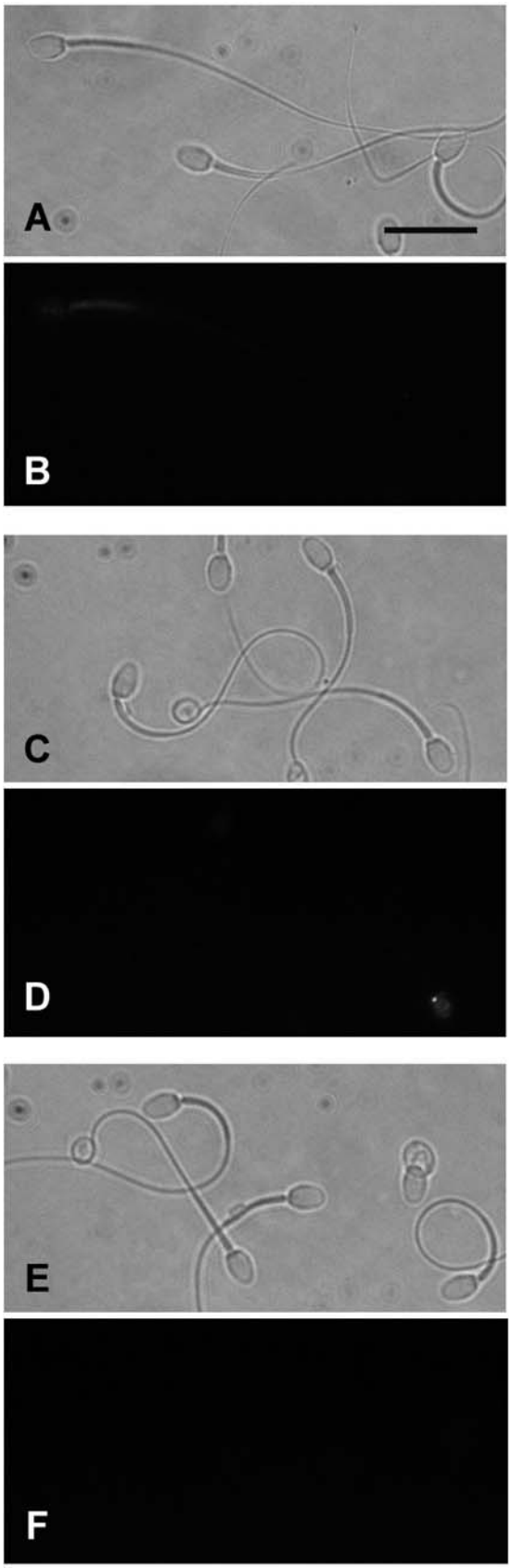


FIG. 7. Swim-up sperm were exposed for 1 h to antibodies raised against specific antigens (SPAM1 [**A** and **B**], a 240-kDa dimer [**C** and **D**], and ADAM30 [**E** and **F**]) on the head plasma membrane. Following fixation and labeling with secondary antibody there was little evidence of antibody binding to any of the target antigens. When time, concentration and exposure were held constant almost no label was detected, although there were occasional sperm that showed some specific label with the antibody directed to the 240-kDa antigen (**D**). Bar = 10 μm.

a novel in vivo model system, as well as when challenged with antisperm antibodies in vitro.

DEFB126 has a high negative charge because of its sialic acid moieties, and this sialic acid appears to be responsible for the immune protective function of DEFB126. The idea that sialic acid residues in some way shield sperm antigens and thereby contribute to the immune protection of exposed antigens has been proposed [35, 36]. It has been known that the protective coats of other cells are often effective because sialic acids are the terminal sugars of the coating glycoproteins [37]. It is fascinating that, for the most part, the immune response of rabbits was directed to a single 34–36-kDa protein band following immunization with lightly washed sperm, given that Schroter et al. [11] and Naaby-Hansen et al. [34] proposed that the human and nonhuman primate sperm surfaces have hundreds of (glyco)proteins.

In recent years, numerous cell coats have been characterized, and sialic acid is still regarded as the primary element of the glycocalyx complex [38]. The molecular sophistication of these coats becomes more complex in cells from higher organisms, which is most likely because of the fact that there are over 40 known derivatives of sialic acid [37]. They are often the terminal sugar in many of the oligosaccharides and are thought to provide protection to the subterminal region of the sugar [38]. Sialic acids are known to function as antirecognition molecules, and microorganisms have been shown to scavenge exogenous sialic acid for their cell surfaces to masquerade as "self" [39–41].

Our data show that when DEFB126 was exposed to a sialidase it lost its cloaking ability. This change in function coincided with a dramatic shift in its isoelectric point from pI 3 to about pI 6.5, and an increase in molecular weight from 34 to 36 kDa to 38–40 kDa; this MW shift may be caused by the removal of the negative hydration shell [42]. DEFB126 has no site for *N*-glycosylation, but it does have numerous threonines and serines along the carboxyl tail, which indicates that the large difference in MW (10 kDa–34 to 36 kDa) is caused by *O*-linked oligosaccharides. The threonines and serines are congregated into clusters, which are reported to create a large hydration shell for protection [43]. It is clear from this study that DEFB126 may behave similarly to other glycocalyx extracellular coats in providing for cell protection [44]. The masking of existing testicular nonself antigens has been thought to be critical for sperm survival, and we propose that DEFB126 is the major, if not the only mechanism for sperm protection in the macaque female reproductive tract. Protection from enzymatic attack is also thought to be a major function of the external cell surface glycocalyx. The rat sperm glycocalyx component DEFB126, previously designated as 2D6 and E-3, the antibodies that recognize this protein, has been shown to be very resilient to protease activity [45].

Recently, Zhou et al. [19] linked SPAG11, a mouse epididymis-specific β-defensin, to the initiation of sperm motility. SPAG11, a cationic antimicrobial peptide, was previously shown to function as one of the numerous epididymis-specific antibacterial peptides that protect the male

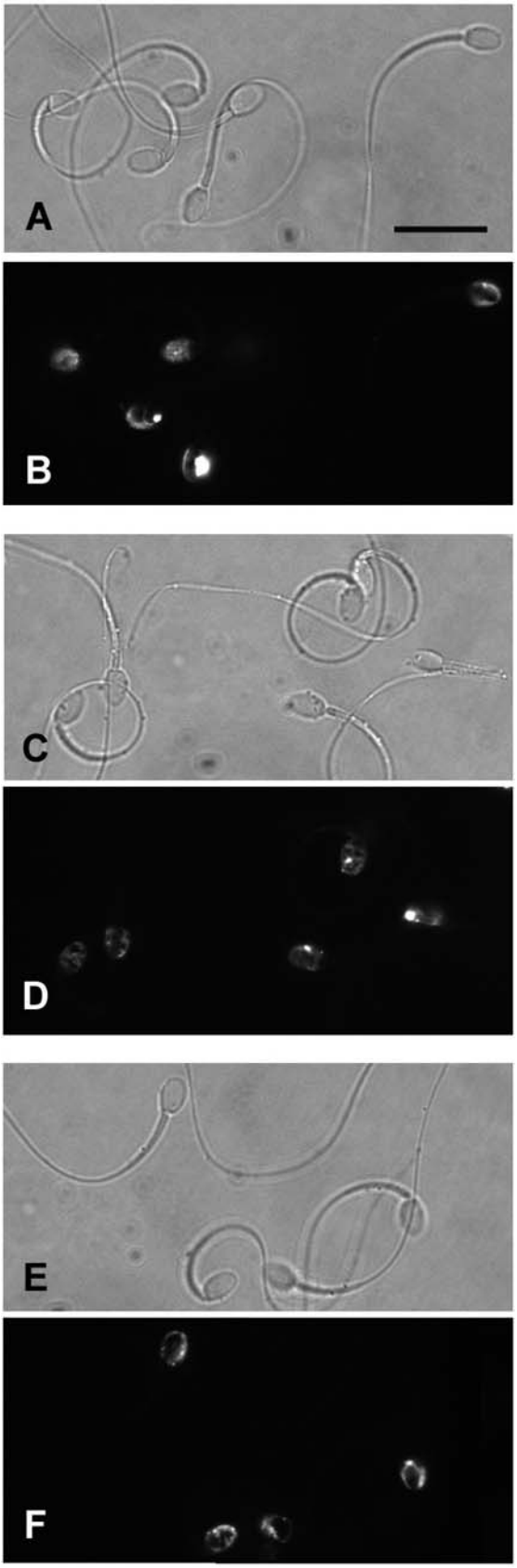


FIG. 8. Capacitated sperm that were incubated with the same sperm specific, head-directed antibodies to SPAM1, a 240-kDa dimer, and ADAM30 (**A** and **B**, **C** and **D**, and **E** and **F**, respectively) for 1 h and then fixed had strong labeling on the head of each sperm. Each antibody caused some degree of aggregation of the antigens along the sperm head. All of the capacitated sperm had some degree of sperm head labeling. Bar = 10 μm.

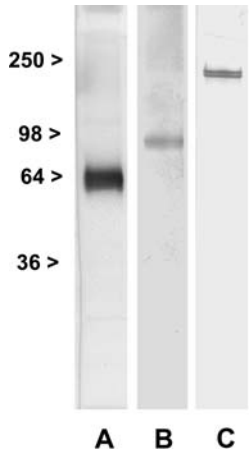


FIG. 9. A Western blot of whole reduced sperm, which were probed with the three different antibodies that were used in Figures 7 and 8. The antibodies to SPAM1 recognize a single 64-kDa band (lane A). Antibodies raised to the ADAM30 antigen recognizes a single protein at approximately 84 kDa (lane B), whereas the antibodies to the highly hydrophobic 240-kDa complex appear to label the 2 closely related bands (lane C).

reproductive tract from infection [18, 46, 47]. Thus, β-defensins can have functions other than antimicrobial activity, and may have dual or multifunctional characteristics. The proposed role of the macaque DEFB126, to cover the entire sperm surface and render protection from the female immune system, may also apply to the rat orthologue DEFB126 [48, 49]. Another defensin, DEFB118, also is found on the macaque sperm surface, possesses antimicrobial activity, and, like DEFB126, has an extended carboxyl tail with a number of *O*-glycosylation sites [50]. To date, a number of defensins have been described in the male reproductive tract, and they are proposed to be critical for the health of the system by providing a rapid and broad response to invading microorganisms. However, secretion of β-defensins in the epididymis is under androgen control, which suggests a role for these glycoproteins in sperm physiological functions in the female tract instead of, or in addition to, their possible antimicrobial activities [18].

At this time it is not known whether DEFB126 has antimicrobial functions in the epididymis, but it is clear that it is readily adsorbed to the entire macaque sperm surface and remains anchored through some unknown mechanism until the final stages of capacitation [20, 23]. The vast majority of epididymal (glyco)proteins are secreted in the caput, and by the time sperm arrive in the caudal region only a few coating proteins remain to be added. Recently, we showed that when macaque sperm were washed through a column of Percoll, many of the sperm surface components were removed, leaving two major glycoproteins (DEFB126 and MSMB) to be released at completion of capacitation [20]. It would appear that density gradient washing substantially reduces the sperm glycocalyx, and/or that most epididymal secretory proteins do not become associated with the sperm [51, 52].

Epididymal secretions previously have been proposed to in some way establish a protective barrier against the enzyme activity of body fluids [53, 54]. Following immunization of rabbits with whole fixed sperm, the immune response was confined mainly to DEFB126, strongly suggesting that this glycoprotein blankets a major portion of the sperm surface and is the most external component of the glycocalyx. When sperm were washed through higher viscosity solution, surface coatings, including DEFB126, were removed or reduced, and as a consequence other sperm surface antigens were exposed for immune recognition.

After washing, DEFB126 was still present over the entire sperm surface, but apparently sufficient amounts are removed to expose the higher molecular weight components of the sperm plasma membrane. Removal of DEFB126 after capacitation exposes a host of other antigens to immune recognition. Antisperm antibodies have been recognized as a major cause of immunological infertility [55]. Antibodies directed toward specific antigens are thought to disrupt the fertilization process in some way [56, 57]. Unfortunately, the number and types of antibodies required to induce infertility are still unclear [58, 59]. One aspect of this mystery may be resolved by our demonstration of a coating protein on the sperm surface that protects the sperm from immune recognition and antibody attack. Such a possibility has been suggested by others [1, 2], but it has not been demonstrated previously.

Mammalian sperm at the time of ejaculation enter the female and are almost immediately attacked by leukocytes [60]. Eisenbach [61] hypothesized that sperm that have completed capacitation may be targets of these leukocytes, whereas noncapacitated sperm go undetected. There has been much discussion of the apparent immunosuppressive factors found in human seminal fluid that may create a more favorable environment for sperm survival as they enter the cervix [62, 63]. This mechanism would serve as only a localized benefit to sperm, because they must be able to move through the entire reproductive tract without immune recognition. In primates, sperm reside in the mucus-filled cervix for several days and then proceed up the tract to the site of fertilization in the ampulla of the oviduct, where capacitated sperm encounter the egg [64].

Sperm found in the upper oviduct at the time of fertilization are likely to be capacitated, because capacitation is considered a requirement for successful fertilization [65]. The loss of sperm surface coat(s), also regarded as a major event in the capacitation process, may be responsible for the exposure of zona receptors, but this loss also puts the sperm at risk of detection by immunocompetent cells and antibodies; therefore, noncapacitated sperm are likely those that are stored in the hostile female environment. It has been reported that animals with high antibody titers as a result of immunization with sperm proteins have sperm in the oviduct with no Ig on their surfaces, which is consistent with sperm being concealed from immune attack by a surface coat [2].

When fixed washed macaque sperm were used as an antigen to immunize female macaques, there was no sign of anti-DEFB126 Ig (Western blot analysis) after 3 injections of whole sperm. This finding may suggest that DEFB126 goes undetected in the homologous female, even though it is a male-specific glycoprotein. Montanide, the adjuvant used for the immunization of macaques with whole sperm, was also used in the rabbit immunizations and was found to be an effective adjuvant; therefore, the lack of response by the female macaques was not caused by the adjuvant (data not shown). If true, such a phenomenon would explain why females do not produce antibodies to noncapacitated sperm.

Our data show that sperm coated with DEFB126 are not recognized by antisperm antibodies. The brief exposure of sperm membrane proteins that takes place when DEFB126 is released at the completion of capacitation may not be sufficient to allow antibody attack or to induce future antibody production. The protection from antisperm antibodies that DEFB126 provides may have led to the problems encountered by researchers attempting to develop immunocontraceptive vaccines to sperm plasma membrane components [28]. The use of monospecific antibodies in targeting tumor cells, sometimes called the "magic bullet" concept, has had limited success, but a number of cell-surface molecules specific to breast cancer have been developed and are currently being used therapeutically [66–68]. Gaining access to the cell surface is critical before antibodies are able to recognize targets on the plasma membrane. This has been a problem in the monoclonal antibody approach to cancer treatment, and similar problems appear to have frustrated the development of antibodies that can recognize sperm and cause infertility.

The differentiation between self and nonself antigens is a critical property of the immune system and is the cornerstone for defending the body against invasion by pathogens [69, 70]. On the other hand, the ability of the female to tolerate male gametes is essential for the continuation of a species. Because sperm antigens have been shown to elicit a potent immunological response in the female, it stands to reason that sperm must have a shield that conceals or masks unique testicular and epididymal antigens on the sperm surface. Our evidence strongly suggests that DEFB126 acts as such a shield.

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