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Changes in the sialylglycoconjugate distribution on the human sperm surface during in-vitro capacitation: partial purification of a 20 kDa sialylglycoprotein of capacitated spermatozoa

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Changes in the distribution of sialylglycoconjugates on the surface of uncapacitated and in-vitro capacitated human spermatozoa were studied by means of two sialic acid specific lectins (*Maackia amurensis* agglutinin and *Sambucus nigra* agglutinin). On the uncapacitated sperm surface, sialylglycoconjugates were found to be localized from the post-acrosomal region of the sperm head to the tail middle piece, whereas after in-vitro capacitation these molecules were only found in a small area of the post-acrosomal region. The surface of capacitated human spermatozoa was also investigated by specifically radiolabelling its terminal sialic acid residues. A 20 kDa glycoprotein, which was partially purified by anion-exchange chromatography, was the main component of the sialylglycoconjugate pattern after in-vitro capacitation.

Key words: capacitation/glycoconjugates/human spermatozoa/lectins/sialic acid

Introduction

Freshly ejaculated mammalian spermatozoa, although motile and structurally mature, fail to fertilize eggs. They only acquire this ability after a maturation process in the female reproductive tract that can be mimicked *in vitro* under selected conditions. The process, which is probably a multistep process, is known as capacitation. The ability to undergo an induced acrosomal reaction or to fertilize zona pellucida-encased or denuded eggs are the two principal assays used to establish the fertile or capacitated status of spermatozoa (see Florman and Babcock, 1991). These events only reflect the end of the process but it would be useful to have parameters identifying various stages of the capacitation process. Different molecular events have been associated with this transition. One of the most important ones is the removal of coating substances derived from seminal fluid. This modification seems to be a prerequisite for almost all the other events, such as changes in membrane cholesterol concentrations and membrane phospholipid structure (see review of Langlais and Roberts, 1985; Benoff *et al.*, 1993), modifications in sperm surface features, such as changes in lectin and antibody binding sites (Schwarz and Koehler, 1979; Cross and Overstreet, 1987; Monroe *et al.*, 1990; Margalioth *et al.*, 1992), the exposure of new, previously hidden, molecules (Berger, 1990) and a decrease in surface negative charge (Rosado *et al.*, 1973). Removal of sialic acid residues from the surface sialylglycoconjugates present on epididymal and ejaculated spermatozoa in several mammals (Holt, 1980; Toowicharanont and Chulavatnatol, 1983; Toshimori *et al.*, 1991) including man (Wongkome and Chulavatnatol, 1984; Lassalle and Testart, 1994) has been indicated as an important step in the capacitation process responsible for reducing the negative charge. Indeed, it has been shown that loss of the net negative charge of the sperm surface (Rosado *et al.*, 1973), capacitation (Srivastava *et al.*, 1988) and enhancement of sperm attachment to the zona pellucida (Lassalle and Testart, 1994) can be achieved by treating spermatozoa with neuraminidase. Neuraminidase activity has been found in the endometrium of some mammals (Srivastava and Farooqui, 1980). In a previous study we radiolabelled sialic acid residues on freshly ejaculated spermatozoa and followed changes in these components during in-vitro capacitation. We studied the kinetics of release and the size of material removed from the sperm surface and the first clear evidence that human sperm capacitation is associated with the removal of two classes of sialylglycoconjugates was obtained (Focarelli *et al.*, 1990).

In this paper we report the distribution pattern of the sialylglycoconjugates detected with two lectins in freshly ejaculated spermatozoa and its changes after in-vitro capacitation. The sialylglycoprotein pattern of in-vitro capacitated spermatozoa was also studied by radiolabelling the surface sialic acid residues at the end of capacitation; a sialylglycoprotein of ~20 kDa was identified on the capacitated sperm membranes.

Materials and methods

Collection and capacitation of spermatozoa

Human ejaculates were obtained from healthy men and allowed to liquefy at room temperature for ~30 min. Motile spermatozoa were separated from seminal plasma by centrifugation on a Percoll gradient essentially as described by Siiteri *et al.* (1988). Briefly, aliquots (500 μl) of liquified semen were stratified over a discontinuous Percoll gradient consisting of an upper layer (1 ml) of 40% Percoll and a lower layer (1 ml) of 80% Percoll and centrifuged at 300 *g* for 20 min, taking care to avoid osmotic shock (Morales *et al.*, 1991). The motile sperm fraction was recovered at the interface between the two Percoll solutions and washed once in phosphate-buffered saline (PBS) at 300 *g* for 10 min. In each experiment, the spermatozoa were divided into two aliquots, one of which was capacitated for 6 h in

Biggers–Whitten–Whittingham (BWW) medium containing 35 mg/ml of human serum albumin (HSA) as previously described (Focarelli *et al.*, 1990). Uncapacitated and capacitated spermatozoa were then used for lectin-binding studies or for radiolabelling of their sialic acid residues as described below. Motile sperm cells always constituted at least 70% of the population before and after incubation under capacitating conditions. Sperm membrane integrity was evaluated by the hypo-osmotic swelling test, as described by Jeyendran *et al.* (1984).

Labelling of terminal sialic acid residues

Exposed terminal sialic acid residues of surface glycoconjugates of capacitated human spermatozoa were radiolabelled essentially as described previously by Focarelli *et al.* (1990), following the method of Gahmberg and Anderson (1977). Briefly, capacitated human spermatozoa were washed in PBS at pH 7.0 by centrifugation at 300 g for 10 min, resuspended in the same buffer containing 1 mM sodium metaperiodate and incubated in the dark with occasional agitation for 10 min at room temperature. After washing with 50 ml PBS, the spermatozoa were labelled by reduction of the oxidized spermatozoa with 500 μCi of NaB_3H_4 (490 mCi/mmol; New England Nuclear, Boston, MA, USA) for 30 min at room temperature. After addition of 50 ml of PBS containing 0.2 mg/ml of unlabelled sodium borohydride, the spermatozoa were centrifuged at 300 g for 10 min. The radiolabelled sperm glycoconjugates were then extracted by dissolving in 50 mM Tris-HCl, pH 7.2 containing 0.1% Triton X-100, 1.25 mM *n*-amino benzamidine, 1 mM phenylmethylsulphonyl fluoride and 1 mM EDTA for 1 h at 4°C with constant shaking. Suspension was centrifuged at 15 000 g for 15 min and the clear supernatant was used for further studies. To confirm that the tritium really was incorporated in sialic acid residues, the radiolabelled material was digested with neuraminidase from *Clostridium perfrigens* and the radiolabelled material released was analysed by thin layer chromatography as previously described (Focarelli *et al.*, 1990).

Electrophoretic analysis

The dissolved sperm membrane components were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS–PAGE) according to Laemmli (1970) on 10–20% gradient slab gels. The gels were stained with Coomassie Brilliant Blue, incubated for 30 min with Amplify (Amersham, Buckinghamshire, UK) and exposed at –80°C. Alternatively, they were sliced into 2 mm segments, each of which was placed in a vial containing 500 ml Solvable (NEN-Dupont, Boston, MA, USA) and warmed at 50°C for 3 h. After the addition of 10 ml Formula-989 liquid scintillation cocktail (NEN-Dupont), the samples were counted in a Beckman scintillation counter.

Ionic exchange chromatography

Proteins from pooled, radiolabelled, capacitated, human sperm membranes (see above) were separated by ionic exchange on a MonoQ column (Pharmacia, Uppsala, Sweden) fitted in a Fast Performance Liquid Chromatography apparatus and eluted at a flow rate of 0.5 ml/min. The mobile phase consisted of a linear gradient produced by mixing 10 mM Tris-HCl pH 8.0 (eluent A) and 1 M NaCl, 10 mM Tris-HCl pH 8.0 (eluent B) as follows: isocritically with A for 4 ml, followed by 0–40% B for 30 ml, 40–100% B for 10 ml, isocritically with 100% B for 4 ml and 100–0% B for 4 ml. Proteins were detected at 280 nm with a Monitor UV-M-II (Pharmacia). Radioactivity was counted in 25 μl aliquots of each fraction. Fractions were pooled according to radioactivity profile, lyophilized and stored for further analysis.

Fluorescence microscopy

Aliquots (100 μl) of uncapacitated and capacitated human spermatozoa were smeared onto ethanol-cleaned glass slides and allowed to attach at room temperature, taking care to keep them in a liquid phase since air-drying has been reported to damage the sperm membrane. The smears were then left unfixed or treated for 10 min with 4% formalin in PBS or with a buffered $HgCl_2$ solution (6% $HgCl_2$, 1% sodium acetate and 0.1% glutaraldehyde solution) according to Schulte and Spicer (1983). After repeated washings in PBS, the smears were blocked for 30 min with 2% bovine serum albumin (BSA) in PBS and then incubated for 1 h in the same buffer containing 10 μg/ml of *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) lectins conjugated with digoxigenin (Boehringer, Mannheim, Germany). After repeated washings, the smears were incubated with an anti-digoxigenin antibody conjugated with fluorescein (Boehringer) diluted according to the manufacturer's instructions, washed, mounted in PBS-glycerol and observed with a Laser Scanning Confocal Apparatus (BioRad Microscience, Cambridge, MA, USA). Control experiments were performed in the presence of a 1 mg/ml concentration of transferrin (a SNA-positive glycoprotein). Membrane integrity was checked in sperm smears using the impermeable nuclear dye propidium iodide (1 μg/ml) with and without permeabilization. Some of the smears were also double-labelled with rhodamine-conjugated PSA (*Pisum sativum* agglutinin) to assess acrosomal status according to the method of Cross *et al.* (1986), with or without ethanol permeabilization.

Quantification of SNA-binding sites by solid phase assay

In order to quantify the SNA binding sites on human spermatozoa before and after incubation under capacitating conditions, Percoll-selected human spermatozoa were resuspended in coating buffer (0.05 M sodium carbonate, sodium hydrogenocarbonate, pH 9.6) to a final concentration of 2×10^7 spermatozoa/ml. The suspension (100 μl) was immobilized in the wells of microtitre plates by incubation overnight at 4°C. After rinsing with PBS containing 0.05% Tween-20, the immobilized cells were blocked for 1 h in the same buffer (100 μl/well) containing 2% BSA and then incubated for 1 h with increasing amounts of SNA-biotin (Oxford Glycosystems, Abingdon, UK). After several washings in the same buffer, wells were incubated with 100 μl of avidin conjugated with horseradish peroxidase at a final concentration of 4 μg/ml. Following several rinses with PBS-Tween, the enzyme reaction was developed with tetramethylbenzamidine (0.3%) and hydrogen peroxide (0.02%) in phosphocitrate buffer (0.1 M sodium dihydrogenphosphate, 0.1 M citric acid, pH 5.0) (150 μl/well). The reaction was stopped after 15 min with 0.1 M HCl (50 μl/well) and the absorbance determined at 450 nm. A separate set of wells was incubated with SNA-biotin in the presence of a 1 mg/ml concentration of transferrin to establish the degree of non-specific binding which was then subtracted from each measurement. All experiments were performed at least three times using three wells per sample per assay.

Results

Distribution of sialyl residues (SNA- and MAA-binding sites) on the sperm surface before and after in-vitro capacitation

Two sialic acid-specific lectins, MAA and SNA, were used to localize the distribution of sialic acid residues on human spermatozoa before and after in-vitro capacitation. The specificity difference between the two lectins is the linkage of the terminal sialic acid with the penultimate sugar of the oligosaccharide chain (i.e. galactose or N-acetylgalactosamine). MAA is specific for the sequence SAa2,3Gal/GalNAc (Knibbs *et al.*, 1991) and SNA is specific for the SAa2,6Gal/GalNAc

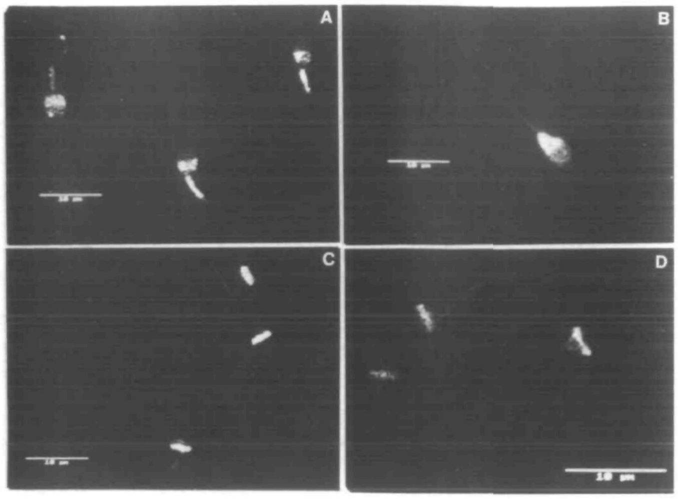

Figure 1. Percoll-selected non-capacitated (**A** and **B**) and capacitated (**C** and **D**) human sperm cells stained with *Sambucus nigra* agglutin (SNA)-digoxygenin (DIG) (**A** and **C**) and *Maackia amurensis* agglutinin (MAA)-DIG (**B** and **D**) followed by anti-DIG antibody conjugated with fluorescein isothiocyanate. In (**A**) the bright fluorescence in the post-acrosomal region and in the tail middle piece of spermatozoa treated with SNA is shown and in (**B**) the fluorescence in the post-acrosomal and in the droplet region of spermatozoa treated with MAA. (**C**) and (**D**) show how the fluorescence, bright after SNA and faint after MAA, only remains in the post-acrosomal region.

sequence (Shibuya *et al.*, 1987). In a first series of experiments the lectin binding analysis was performed on unfixed spermatozoa and spermatozoa fixed with formalin or glutaraldehyde in $HgCl_2$ buffered solution (see above). The status of unfixed Percoll-selected spermatozoa was always checked by PSA-staining the cells after ethanol-permeabilization. A very small number (<5%) of damaged and/or spontaneously acrosome-reacted cells were found in the sperm populations used in the experiments. When unfixed and glutaraldehyde-$HgCl_2$ fixed sperm populations were treated with the lectins and observed by laser confocal microscope, the surface of almost all of them showed a typical and reproducible fluorescence pattern to each of the two lectins, whereas an intriguing and variable pattern was observed when formalin fixed cells were tested. This led us to abandon formalin fixation. The fluorescence pattern of the uncapacitated spermatozoa to the two sialic acid specific lectins is shown in Figure 1. Faint fluorescence in the acrosomal region and very bright fluorescence from the post-acrosomal region up to the neck zona was typical of MAA. Similar bright fluorescence in the post-acrosomal region was also detectable with SNA but with this lectin the neck or cytoplasmic droplet region was negative whereas the tail middle region was positive. When the capacitated cells were tested, the fluorescence distribution to both the lectins was the same, i.e completely restricted to a narrow zone of the post-acrosomal region, but the brightness was completely different. Faint fluorescence was detectable after MAA treatment and very bright fluorescence after SNA. In order to determine whether binding sites to SNA were reduced or merely redistributed we tested the lectin in a solid phase assay. The results, shown in Figure 2, clearly demonstrated that SNA reactivity after capacitation was reduced to ~30%.

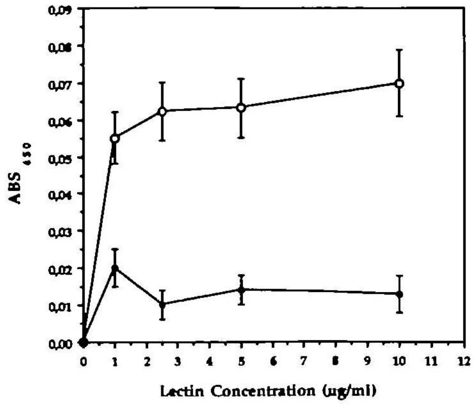

Figure 2. Binding of the *Sambucus nigra* agglutin lectin to immobilized capacitated (●) and uncapacitated (○) human spermatozoa. Bound lectin was detected by a solid-phase assay and expressed as absorbance at 450 nm. Absorbance due to non-specific labelling was determined in the presence of 1 mg/ml transferrin and subtracted from each point.

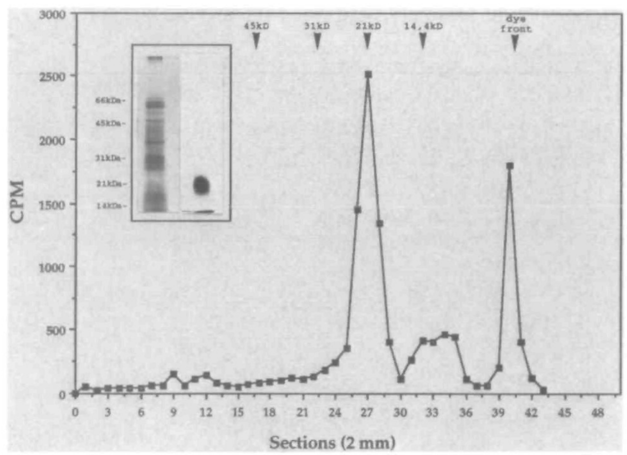

Figure 3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis of Triton X-100 extracts from ^{3}H-labelled membranes of capacitated human spermatozoa. Slices (2 mm) were cut and counted for radioactivity. Inset shows the same material after Coomassie Blue staining on the left and after fluorography on the right.

Analysis of sperm surface sialylglycoconjugates after capacitation

In order to determine whether sialylglycoproteins could be identified biochemically on the surface of capacitated spermatozoa, we radiolabelled the terminal sialic acid residues 6 h after incubation in the capacitating medium. SDS–PAGE of the Triton extract sperm components followed by autoradiography showed a single broad band of radioactivity migrating with an average molecular weight of about 20 kDa (Figure 3). A single sialylglycoprotein species with the same molecular weight was also revealed when the gel was sliced into 2 mm sections as described above (Figure 3). In an effort to isolate and purify the 20 kDa protein, the dissolved radiolabelled sperm components were subjected to different methods of gel filtration, but in all cases the radioactive material was recovered in the excluded volume. Partial purification of the protein was achieved by fractionating the membrane components by anion-

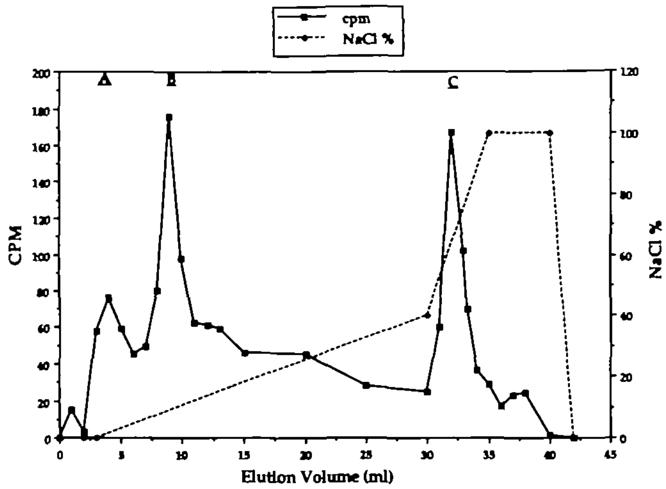

Figure 4. Anion-exchange Fast Performance Liquid Chromatography fractionation of radiolabelled membrane extracts from capacitated human spermatozoa. The solid line shows the radioactivity profile and the dashed line the NaCl gradient. Separation conditions are described in the text. The fractions indicated in the figure (A, B and C) were pooled, dialysed, lyophilized and analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

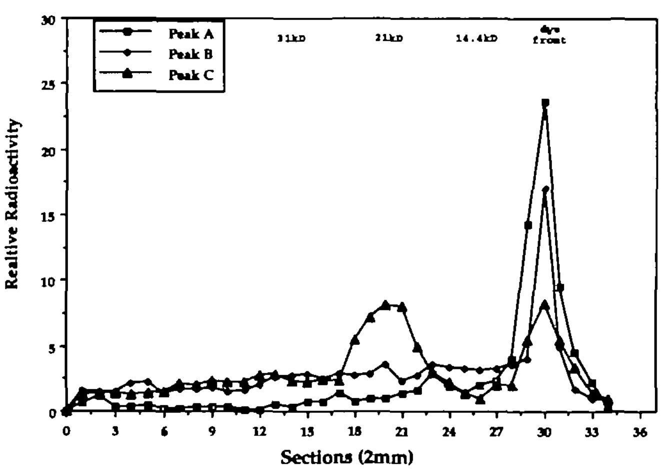

Figure 5. Electrophoretic analysis of the three fractions obtained after anion-exchange fractionation of radiolabelled membrane extracts from capacitated human spermatozoa. The gel was cut into 2 mm slices and counted for radioactivity. The 20 kDa sialylglycoprotein was found exclusively in fraction C.

exchange FPLC. As shown in Figure 4, three radioactive peaks (A, B and C) were obtained using a gradient of increasing concentrations of NaCl. The peaks were dialysed extensively against distilled water and the corresponding fractions loaded on a 10–20% gradient polyacrylamide slab gel. As shown in Figure 5, a 20 kDa protein species was found in peak C which eluted at about 0.8 M NaCl whereas no sialylglycoproteins were associated with the other peaks since all the radiolabelled material ran with the dye.

Discussion

After ejaculation, the mammalian spermatozoon has a hard journey in the female genital tract to the site of fertilization (Hunter, 1988). In the course of this journey, it undergoes capacitation, in which it acquires the properties necessary to undergo the acrosome reaction, a specialized exocytotic process, on interaction with the zona pellucida. The acrosome reaction is a prerequisite for fertilization. The capacitated status can also be acquired *in vitro* by incubating spermatozoa in specific media whose key components are serum albumin and energy substrates. Although the sequential molecular mechanisms of capacitation are far from understood (Florman and Babcock, 1991), different modifications have been described which are associated with capacitating spermatozoa. Removal of coating components, which is probably a prerequisite for other events, is one of the most important of these.

In our work on human sperm capacitation, we have focused on surface sialylglycoconjugates and their changes, if any, during the event. This was performed by radiolabelling surface sialic acid residues (Focarelli *et al.*, 1990). In a previous paper we demonstrated two classes of sialic acid-bearing molecules on the sperm surface; these molecules are completely or partially shed during in-vitro capacitation. Molecules no larger than 5000 kDa belong to the first class and two glycoproteins of ~15.5 and 22.5 kDa to the second (Focarelli *et al.*, 1990).

The results of the present study show where sialylglycoconjugates are localized in the uncapacitated spermatozoa; from where they are removed during in-vitro capacitation and where they remain after this process. The results obtained by means of two lectins specific for sialic acid residues in different configurations suggest that glycoconjugates with terminal sialic acid residues in both the configurations recognized by SNA (α2,6-Gal/GalNAc) and MAA (α2,3-Gal/GalNAc) are components of the post-acrosomal plasma membrane of ejaculated spermatozoa whereas only those with the sialic acid in α2,6-Gal/GalNAc configuration are components of the tail middle region and those with sialic acid in α2,3-Gal/GalNAc configuration are components of the neck region. Incubation in the capacitation medium resulted in the loss of most of the sialylglycoconjugates from the sperm surface, except in a small area of the post-acrosomal region where molecules with sialic acid residues prevalently in the α2,6-Gal/GalNAc configuration seemed to increase. By this method, it is of course impossible to identify whether the sialylglycoconjugates of the capacitated sperm surface are derived from redistribution of the molecules of the uncapacitated spermatozoa or whether they are newly exposed molecules.

Biochemical identification of the sialylglycoconjugates exposed as a consequence of the capacitation was approached by radiolabelling the terminal sialic acid residues on capacitated human spermatozoa. Only a sialylglycoprotein of ~20 kDa was found to be present in the sperm membrane after capacitation. Partial purification of the protein was achieved by anion exchange FPLC, and in fact this protein is eluted by 0.8 M NaCl. Preliminary analysis of the uncapacitated sperm extract by FPLC showed that the 22.5 kDa protein reported on the surface of uncapacitated sperm and half shed during capacitation (Focarelli *et al.*, 1990), eluted in a very similar way to the 20 kDa species (unpublished results). Detailed characterization of both proteins, their amino acid sequence and production of the corresponding antibodies are currently underway to determine what kind of relationship exists between this protein and the 22.5 kDa component. The present results together with our previous findings that molecules <5000 kDa and a 15.5 kDa glycoprotein are completely shed during capacitation (Focarelli *et al.*, 1990) indicate that removal of sialic acid-containing molecules from some surface regions to create a new surface sialylglycoconjugate pattern is certainly one of the parameters characterizing the transition of spermatozoa from uncapacitated to capacitated status. These studies do not provide any direct insight into the role of the surface sialylglycoconjugates in bringing spermatozoa to fertile status but they have many implications for the sperm journey in the female genital tract. In this context, it is interesting that a specific sialic acid-binding protein has been found in the uterus of many mammals including man (Chowdhury *et al.*, 1985; Chowdhury and Bhattacharya, 1988; Banerjee and Chowdhury, 1994). The lectin has been purified and proved to specifically bind uncapacitated but not capacitated spermatozoa (Banerjee and Chowdhury, 1994). The presence of the lectin in the uterus at each menstrual cycle and its absence from post-menopausal uterine homogenate and in some cases of unexplained infertility suggest that this sialic acid-binding protein plays a functional role in sperm attachment and in aiding spermatozoa to shed masking molecules (Banerjee and Chowdhury, 1994). The sperm surface sialylglycoconjugates could therefore be involved in modulating sperm travel in the female genital tract and in the correct transition to fertile status. In this context, it is interesting that loss of sperm surface sialic acid allows phagocytosis of spermatozoa by macrophages (Holt, 1980; Toshimori *et al.*, 1991).

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