Glycoconjugates on the surface of epididymal spermatozoa in a marsupial, the brushtail possum, *Trichosurus vulpecula*

N. J. Cooper*, R. V. McClean†, C. M. Leigh and W. G. Breed‡

Department of Anatomical Sciences, Adelaide University, SA 5005, South Australia

Variation in localization and distribution of saccharides on the sperm surface of a marsupial, the brushtail possum, *Trichosurus vulpecula*, was compared between spermatozoa from the caput and cauda epididymides. Spermatozoa were subjected to the following treatments: (i) unfixed and fixed spermatozoa were stained with fluorescein-labelled lectins; (ii) unfixed spermatozoa were incubated with lectins for determination of agglutination; and (iii) spermatozoa were incubated with detergent to remove the plasma-membrane, the glycoproteins were separated on SDS-PAGE and western blots were stained with biotinylated lectins. Many of the fluorescein isothiocyanate (FITC)-labelled lectins bound selectively to the sperm surface, and marked differences were found in lectin staining affinity between caput and cauda epididymal spermatozoa. Incubation of spermatozoa from the cauda epididymidis with neuraminidase reversed many of the differences in staining of the cauda epididymal spermatozoa, indicating masking of

some terminal saccharides by sialic acid. Agglutination of spermatozoa from the caput epididymidis occurred after incubation with Concanavalin A (ConA) and soybean agglutinin (SBA), but agglutination was less extensive for spermatozoa from the cauda epididymidis. Western blot analysis indicated several ConA-positive bands in caput sperm extracts, but fewer positive bands in the cauda sperm extracts, whereas SBA stained four bands from caput but none from the cauda epididymal spermatozoa. These results demonstrate extensive glycosylation of the surface proteins of spermatozoa from the caput epididymidis and significant differences in spermatozoa from the cauda epididymidis. In general, the findings indicate similar glycosylation of the surface of marsupial spermatozoa to those from eutherian mammals despite marked differences in their morphology and early divergence of marsupials from eutherian mammals. It would appear that this situation differs markedly from that in sub-mammalian vertebrates.

Introduction

In eutherian mammals, spermatozoa are immature when they leave the testis, but undergo maturation as they travel along the epididymis. During this time spermatozoa undergo marked alterations in molecular composition of the cell surface, which include: (i) a redistribution of the intra-membranous protein molecules within the cell membrane; (ii) processing and glycosylation of some of the cell surface proteins and lipids, in part due to the presence of glycosyltransferases within the epididymal environment; and (iii) attachment to the sperm surface of additional molecules synthesized and secreted by the epithelial lining of the epididymis (Bedford, 1975; Orgebin-Crist *et al.*, 1975; Bedford and Cooper, 1978; Jones, 1998; Tulsiani *et al.*, 1998; Schroter *et al.*, 1999, for reviews). The changes in molecular composition of the sperm surface eventually

result in sperm maturation such that cauda, but not caput, epididymal spermatozoa have the potential to fertilize an egg.

Marsupials have a considerably different sperm morphology from that of eutherian mammals (Harding, 1987; for a review, see Temple-Smith, 1987; Bedford and Hoskins, 1991) and some of the processes of sperm-egg interaction at fertilization may also differ significantly (Bedford, 1991, 1998). In some of these species, considerable changes in morphology occur in spermatozoa as they travel along the epididymis, including a reorganization of the acrosomal material, shedding of the cytoplasmic droplet and a re-orientation of the sperm head on its axis with the tail (Harding *et al.*, 1979; Harding, 1987; Temple-Smith, 1987; Bedford and Hoskins, 1991; Lin and Rodger, 1999). Despite these structural changes, only a few studies to date have investigated the molecular organization of the sperm surface. This situation is surprising as in reptiles and birds, unlike in eutherian mammals, post-testicular sperm maturation appears to involve only minimal glycosylation of the surface proteins (Bedford, 1979; Esponda and Bedford, 1985, 1987). Two studies in marsupials have indicated some differences in the molecular masses of proteins in the cell membrane of cauda compared with caput epididymal spermatozoa (Chaturapanich *et al.*, 1992;

*Present address: Nerve-Gut Research Laboratory, Department of Gastroenterology, Hepatology and General Medicine, Royal Adelaide Hospital, Adelaide, South Australia

†Present address: School of Animal Studies, University of Queensland, Gatton, Queensland, Australia

*Correspondence

Email: bill.breed@adelaide.edu.au

Lamont *et al.*, 1998), whereas freeze-fracture studies have indicated a considerable change in the arrangement of intramembranous particles of the sperm plasmalemma during epididymal migration in both the New World opossums (Olson, 1980) and the Australian brushtail possum (Cooper *et al.*, 1998). However, apart from a few observations by Temple-Smith and Bedford (1976) and a preliminary study by Cooper *et al.* (1998), no investigations have been carried out on the surface saccharide composition of spermatozoa of any marsupial, or on the epididymal changes that occur.

Thus, the aim of the present study was to determine the extent of extratesticular glycosylation of sperm proteins in marsupials. Such findings may provide an insight into the uniqueness, or otherwise, of the complex nature of extensive glycoprotein composition of the eutherian sperm plasma membrane and the changes that take place during epididymal maturation.

The distribution of saccharides on the surface of brushtail possum spermatozoa was compared between spermatozoa obtained from the caput and cauda epididymides. Lectins, which have specific binding affinity for particular saccharide groups (for an example, see Goldstein *et al.*, 1997), have demonstrated changes in the distribution of sugars on the surface of eutherian spermatozoa under various physiological conditions (Kinley and Koehler, 1978; for a review, see Koehler, 1978, 1981; Schwartz and Koehler, 1979; Hammerstedt *et al.*, 1982; Margargee *et al.*, 1988; Eddy and O'Brien, 1994). Firstly, fluorescent-labelled lectins were used to determine the distribution and localization of sugars on caput and cauda epididymal spermatozoa of the brushtail possum, *Trichosurus vulpecula*. Secondly, experiments were conducted to determine whether sperm agglutination occurs after incubation with lectins. Finally, western blots were stained with biotinylated lectins after separation of sperm membrane glycoproteins by electrophoresis.

Materials and Methods

Animals

Approximately 20 sexually mature male brushtail possums (*Trichosurus vulpecula*) were obtained from the Division of Animal Services, Adelaide University. These animals were asphyxiated with carbon dioxide and subsequently injected intracardially with sodium pentobarbital to ensure the animals were dead (Adelaide University Animal Ethics Committee approval S/63/98).

Preparation of sperm suspension

After the animals were killed, an incision was made in the scrotum, testes and epididymides were removed, and spermatozoa were expressed from the caput and cauda epididymal ducts. The tissue was placed in Tris-buffered saline, pH 7.6, minced with fine scissors and agitated gently to extrude the spermatozoa, which were aspirated and placed into Eppendorf tubes. Sperm motility was determined

by phase-contrast microscopy after placing a drop of the sperm suspension on a glass slide. Only samples with a high percentage of motile spermatozoa were used for subsequent analysis.

Fluorescent labelling of caput and cauda epididymal spermatozoa with lectins

Caput and cauda epididymal sperm suspensions (200 μ l) were placed into Eppendorf tubes to which 20 μ l propidium iodide (100 μ g ml⁻¹ in Tris-buffered saline) and 200 μ l fluorescein isothiocyanate (FITC)-lectin (10 μ g ml⁻¹ in Tris-buffered saline; Sigma, Cable Hill, NSW) were added, and the solution was incubated for 3 min. The FITC-lectins used included WGA, GSLII, ConA, PSA, RCA, MPL, HPA, DBA, SBA and UEA1 (see Table 1). The sperm-lectin solutions were centrifuged at 300 *g* for 1–2 min, the supernatant was removed and the spermatozoa were resuspended in 200 μ l Tris-buffered saline. Twenty microlitres of each solution containing the incubated spermatozoa was placed on to a slide, and viewed with an epifluorescent and phase-contrast microscope using emission and absorbance wavelengths for FITC. All experiments were repeated three times, each with material from a different animal, except for those experiments in which spermatozoa were incubated with ConA, which were repeated on material from five different animals. The presence of propidium iodide allowed differentiation of live from dead spermatozoa (Harrison and Vickers, 1990). Only fluorescence of live spermatozoa was recorded. Control experiments were carried out by pre-incubation of the lectin with the sugar to which the lectin has strong binding affinity (Table 1).

In a second series of experiments, spermatozoa were fixed in 2.5% (w/v) paraformaldehyde made up in Tris-buffered saline, pH 7.6, for 30 min at room temperature and subsequently stored at 4°C. Aliquots (200 μ l) of fixed spermatozoa were centrifuged at 300 *g* for 1–2 min, resuspended in 200 μ l FITC-lectin solution and treated as described above.

In a third set of experiments, 200 μ l live unfixed cauda epididymal spermatozoa in suspension was incubated in 0.1 U neuraminidase μ l⁻¹ for 30 min. The sperm suspension was centrifuged at 300 *g* for 5 min and resuspended in Tris-buffered saline, pH 7.6. FITC-lectin (200 μ l; 10 μ g ml⁻¹ in Tris-buffered saline) and 10 μ l propidium iodide (100 μ g ml⁻¹ in Tris-buffered saline) were added to each sample and the sample was incubated for 3 min. The sperm-lectin solutions were centrifuged at 300 *g* for 5 min and resuspended in 200 μ l Tris-buffered saline. Sperm suspension (20 μ l) from each solution was placed on to a slide, and viewed by epifluorescent and phase-contrast microscopy.

For recording the localization of the fluorescence on the surface of the spermatozoon, the region of the cell membrane was categorized into that over (i) the acrosome, (ii) the rest of the sperm head, (iii) the mid-piece and (iv) the principal piece of the sperm tail. The intensity of staining with FITC-lectins was recorded as weak, moderate or strong

Table 1. Sugars used to determine specificity of lectin binding to oligosaccharides on the sperm surface of the brushtail possum, *Trichosurus vulpecula*

Lectin	Sugar ^a
WGA ^b	<i>N</i> -acetyl- β -D-glucosamine; sialic acid
GSLII	α - or β - <i>N</i> -acetyl-glucosamine
ConA	α -methyl-mannoside; α -methyl-glucoside
PSA	α -methyl-mannoside; α -methyl-glucoside
SBA	α - and β - <i>N</i> -acetyl-galactosamine; galactose
DBA	α - <i>N</i> -acetyl-galactosamine
HPA	α - <i>N</i> -acetyl-galactosamine
PNA	Galactosidyl(β -1,3)- <i>N</i> -acetyl-galactosamine
RCA ₁₂₀	Terminal β -D-galactose, α -galactose, <i>N</i> -acetyl-galactosamine
RCA ₆₀	α - and β -galactose, <i>N</i> -acetyl- β -D-galactose
MPL	α -D-galactose, <i>N</i> -acetyl-galactosamine
UEA1	α -L-fucose

^aBlocking with 0.2 mol sugar l⁻¹ was used in each case for fluorescein isothiocyanate (FITC) investigations and 0.4 mol sugar l⁻¹ was used for agglutination and western blot analysis.

^bSpecificity of WGA for *N*-acetyl-glucosamine and sialic acid was demonstrated by selective blocking with either *N*-acetyl-glucosamine or sialic acid.

fluorescence. In unfixed spermatozoa, only the fluorescence of live motile spermatozoa was recorded, which were differentiated from dead spermatozoa by the absence of staining with the propidium iodide. The specificity of lectin binding to particular sugars was determined by prior incubation of the lectin with the relevant sugar (Table 1).

Agglutination studies of caput and cauda epididymal spermatozoa with lectins

Caput and cauda epididymides were placed into separate dishes containing Tris-buffered saline, pH 7.6, and protease inhibitors. The tissue was minced gently to release spermatozoa into the media. The sperm suspension was placed into a 10 ml tube and centrifuged at 200 *g* for 2 min to remove tissue debris. The supernatant containing the spermatozoa was aspirated and placed into a second tube, which was centrifuged at 500 *g* for 10 min to pellet the spermatozoa. The supernatant was removed and the spermatozoa were resuspended in 1–2 ml Tris-buffered saline to which protease inhibitors had been added. The number of spermatozoa in the suspension was determined using a haemocytometer, and approximately 1×10^5 spermatozoa were placed into each well of a 24-well tissue culture tray. ConA, PNA, DBA, SBA or UEA1 (Vector Laboratories, Burlington, CA) was added at a concentration of 50 μ g ml⁻¹. Controls included the addition of 0.4 mol l⁻¹ of the relevant sugar. The sperm–lectin solutions were agitated by rocking for 60 min in a humid environment at room temperature. The contents of each well were inspected after 60 min to determine the degree of sperm agglutination and were given a score of 0 to 4 as described by Nicolson *et al.* (1977). For verification of sperm agglutination, an

aliquot of spermatozoa from each well was placed on a microscope slide; a coverslip was placed gently on top and the sample was viewed by Nomarski microscopy. From these samples, head–head, head–tail, and tail–tail agglutination were determined. Replicates of spermatozoa from three different animals were carried out.

Determination of molecular masses of glycoproteins by western blot analysis

Sperm treatment with IGEPAI CA-630 (NP-40) and SDS-PAGE. Caput and cauda epididymal spermatozoa were squeezed from the epididymis into PBS containing protease inhibitors (300 μ g EDTA ml⁻¹, 700 μ g pepstatin A ml⁻¹, 500 μ g leupeptin ml⁻¹, 1 μ g aprotinin ml⁻¹).

The tissue and spermatozoa in solution were centrifuged at 300 *g* for 2 min to remove tissue debris. The sperm solution was removed, placed into a clean tube and centrifuged at 300 *g* for 10 min to pellet the spermatozoa. The supernatant was removed and the spermatozoa were resuspended in 1 ml PBS plus protease inhibitors. An equal volume of 0.1% (v/v) IGEPAI CA-630 (NP-40; Sigma) was added to the sperm suspension and the spermatozoa were incubated on ice for 10 min.

After incubation with the detergent, the sperm solution was centrifuged at 500 *g* for 15 min to pellet any insoluble components. The supernatant, containing plasmalemma and acrosomal extracts, was removed and placed into a new tube. The protein concentrations were determined using Bradford Protein Assay (BIO-RAD, Hercules, CA), with BSA as standards. The protein samples were concentrated by adding nine volumes of ice-cold acetone and were maintained overnight at –20°C. The samples were centrifuged at 500 *g* for 20 min to pellet the proteins, and the supernatant was removed. Samples were washed with five volumes of ice-cold acetone and centrifuged at 500 *g* for 10 min. The supernatant was removed and the samples were allowed to dry overnight.

Protein samples were solubilized in loading sample buffer under denaturing and reducing conditions.

For SDS-PAGE a ten lane 10% (v/v) acrylamide–bis-acrylamide separating gel with a 4% (v/v) stacking gel was assembled. Samples were boiled for 10 min and 5 μ g of each sample was loaded into the appropriate wells. The molecular weights of the bands on the gels were calibrated using commercially available broad range biotinylated standards (BIO-RAD). The gels were run under denaturing and reducing conditions using the buffer system described by Laemmli (1970). Gels were run at a constant current of 45 mA for 45 min.

Western blot analysis. Protein samples in the poly-acrylamide gel were transferred on to nitrocellulose using Towbin's transfer buffer. Transfer was carried out at a constant current of 300 mA for 50 min. After transfer, the nitrocellulose was stained with 1% (w/v) Ponceau S in 5% (v/v) acetic acid which resulted in visualization of the proteins, enabling the

nitrocellulose to be cut into appropriate strips for subsequent labelling with lectins. Stain was removed from the strips with distilled water and the strips were rinsed in Tris-buffered saline, pH 7.4. For labelling, the strips were blocked for 2 h with 3% (w/v) BSA in Tris-buffered saline, pH 7.4, with gentle rocking and then washed once with Tris-buffered saline for 10 min to remove excess BSA. The appropriate strips were incubated with either 5 μg lectin ml^{-1} in 0.2% (w/v) haemoglobin in Tris-buffered saline or 5 μg lectin ml^{-1} + 0.4 mol sugar l^{-1} in 0.2% (w/v) haemoglobin in Tris-buffered saline together with a control of 0.2% (w/v) haemoglobin in Tris-buffered saline for 1 h, and agitated by gentle rocking. All strips were washed (2×10 min) with Tris-buffered saline in 0.2% (v/v) Tween 20 (TBST). After washing, strips were incubated with streptavidin-alkaline phosphatase (1:3000; Amersham) in TBST for 1 h, with gentle rocking, and washed (2×10 min) with TBST. The strips were incubated separately for 5–15 min with 5-bromo-4-chloro-indolylphosphate–nitro blue tetrazolium (BCIP–NBT) liquid substrate system, a substrate for alkaline phosphatase, washed with distilled water and left to dry overnight on filter paper.

Results

WGA and GSLII

For fresh caput epididymal spermatozoa, FITC–WGA showed bright fluorescence over the acrosome and weak fluorescence over most of the rest of the sperm surface, except for the principal piece of the sperm tail, which did not stain. Prior incubation with *N*-acetyl-glucosamine reduced, but did not completely block the fluorescence, indicating that much of it was due to *N*-acetyl-glucosamine, but that some sialic acid may also be present (Table 2). In cauda epididymal spermatozoa, fluorescence was also usually evident over both the head and mid-piece of the sperm tail (Table 2).

Fixed caput epididymal spermatozoa bound FITC–WGA strongly over most of the surface and, in particular, over the sperm tail (Table 3); this fluorescence decreased when *N*-acetyl-glucosamine was added. Cauda epididymal spermatozoa bound FITC–WGA weakly over the head, acrosome and mid-piece, and the staining was not decreased after prior incubation with *N*-acetyl-glucosamine, indicating that most of the staining of the cauda epididymal spermatozoa may be due to the presence of sialic acid.

In unfixed caput spermatozoa, GSLII generally only stained the acrosomal region of the sperm head and there was weak staining of the cytoplasmic droplet and mid-piece. Cauda epididymal spermatozoa generally did not stain, except occasional weak staining over the mid-piece (Table 2). No staining was observed after prior incubation with *N*-acetyl-glucosamine, indicating that the fluorescence was due to the presence of *N*-acetyl-glucosamine.

RCA₁₂₀, RCA₆₀ and MPL

RCA₁₂₀ usually stained the plasmalemma of caput epididymal spermatozoa over the acrosome and there was

weak staining over the mid-piece and the cytoplasmic droplet (Table 2), but no fluorescence was observed after prior incubation with galactose. However, cauda epididymal spermatozoa generally did not stain, except for occasional dull fluorescence over the mid-piece, although prior incubation with neuraminidase resulted in weak fluorescence over the acrosome and mid-piece.

RCA₆₀ showed staining over the head and mid-piece of caput epididymal spermatozoa (Table 2), but staining was not observed after prior incubation with α - or β -galactose. For cauda epididymal spermatozoa, no binding with RCA₆₀ occurred over any region of the spermatozoon (Table 2), although prior incubation of cauda epididymal spermatozoa with neuraminidase resulted in fluorescence over the sperm head, acrosome and mid-piece, indicating that galactose residues on the surface of caput epididymal spermatozoa had been masked by sialic acid during epididymal transit.

Caput epididymal spermatozoa stained with MPL showed fluorescence over the acrosome and mid-piece (Table 2). In addition, the annulus at the junction between the mid- and principal piece stained brightly. Cauda epididymal spermatozoa generally did not show bright fluorescence over any region, although after incubation with neuraminidase, some fluorescence over both the sperm head and tail was evident, indicating that galactose residues on the sperm surface had been masked by the addition of sialic acid during epididymal transit.

Fixed spermatozoa from the caput epididymidis stained with RCA₁₂₀, RCA₆₀ and MPL showed fluorescence over nearly all of the sperm surface, but fluorescence was not observed in spermatozoa from the cauda epididymidis (Table 3).

ConA and PSA

With either α -D-glucose or α -D-mannose as inhibitors, it was found that ConA fluorescence was mostly inhibited by prior incubation with methyl mannoside, indicating that ConA may bind largely to the mannose residues.

In caput epididymal spermatozoa, ConA generally stained the acrosomal region of the sperm head, as well as the cytoplasmic droplet and occasionally the mid-piece of the sperm tail. After prior incubation with methyl-mannoside, fluorescence over the acrosome and mid-piece decreased in intensity. For cauda epididymal spermatozoa, there was generally weak fluorescence over the sperm head, and mid-piece, and prior incubation with both or either sugar eliminated the fluorescence. Incubations with neuraminidase had no effect on the binding pattern of ConA to cauda epididymal spermatozoa. Binding of FITC–ConA to fixed spermatozoa showed similar results to those obtained for unfixed spermatozoa, except that considerably brighter fluorescence was observed (for a comparison, see Tables 2 and 3).

In unfixed caput epididymal spermatozoa, PSA stained all regions of both the head and tail, and the most intense fluorescence occurred over the acrosome (Table 2). For

Table 2. Binding of fluorescein isothiocyanate (FITC) lectins to various regions of the plasma membrane of the head and tail of unfixed spermatozoa from the caput and cauda epididymides of the brushtail possum, *Trichosurus vulpecula*

	Plasmalemma of sperm head		Plasmalemma over sperm tail	
	Acrosome	Rest of head	Mid-piece	Principal piece
WGA caput	++	+	+	—
WGA cauda	+	+	+	—
WGA cauda + N	+	+	+	—
GSLII caput	+	—	+	—
GSLII cauda	—	—	+	—
GSLII cauda + N	—	—	+	—
Con A caput	++	+	++	—
Con A cauda	+	+	+	—
Con A cauda + N	+	+	+	—
PSA caput	++	+	+	+
PSA cauda	+	+	++	—
PSA cauda + N	+	+	++	—
RCA ₁₂₀ caput	++	—	+	—
RCA ₁₂₀ cauda	—	—	—	—
RCA ₁₂₀ cauda + N	+	—	+	—
RCA ₆₀ caput	+	+	+	+
RCA ₆₀ cauda	—	—	—	—
RCA ₆₀ cauda + N	+	+	+	—
MPL caput	+	—	+	—
MPL cauda	—	—	—	—
MPL cauda + N	+	+	+	—
HPA caput	+++	++	+	+
HPA cauda	++	—	—	—
HPA cauda + N	+++	+	++	—
DBA caput	+	—	—	—
DBA cauda	—	—	—	—
DBA cauda + N	+	—	—	—
SBA caput	+	+	+	—
SBA cauda	—	—	—	—
SBA cauda + N	—	—	—	—
UEA1 caput	—	—	—	—
UEA1 cauda	—	—	—	—

Fluorescence was observed with an epifluorescence microscope and scored quantitatively (—: no fluorescence; +: weak fluorescence; ++: moderate fluorescence; +++: strong fluorescence).

Caput: spermatozoa from the caput epididymidis; cauda: spermatozoa from the cauda epididymidis; N: neuraminidase incubation before staining with lectin.

cauda epididymal spermatozoa, although some variation was evident, fluorescence was generally less apparent, although some fluorescence was observed over the acrosome and mid-piece, but not over the principal piece. When both D-glucose and D-mannose were added, no

fluorescence was visible. Neuraminidase had little or no effect on the fluorescence of PSA of cauda epididymal spermatozoa. The binding of FITC-PSA to fixed spermatozoa was generally similar in distribution to that of unfixed spermatozoa, although it was greater in intensity (Table 3).

Fig. 1. Matching (a) phase-contrast and (b) fluorescent light micrographs of unfixed spermatozoa from the caput epididymidis of the brushtail possum (*Trichosurus vulpecula*) showing localization of fluorescein isothiocyanate (FITC)–DBA to the region over the acrosome (arrow). Scale bars represent 2.7 μm .

DBA, HPA, SBA and UEA1

FITC–DBA bound to the plasmalemma of caput epididymal spermatozoa over the acrosome but generally not to other regions of the sperm surface (Table 2; Fig. 1). No fluorescence occurred after prior incubation with this sugar. In some cases the lectin did not bind to any region of cauda epididymal spermatozoa, but in others the region over the acrosome stained weakly. After incubation in neuraminidase, fluorescence was evident over the acrosomal region of the sperm head. The results of fixed spermatozoa were similar to those of unfixed spermatozoa (Table 3).

HPA stained most regions of the caput sperm plasmalemma: intense fluorescence was observed over the acrosome, and moderately intense fluorescence over the rest of the sperm head, cytoplasmic droplet and, to a lesser extent, the mid-piece of the sperm tail (Table 2; Fig. 2). Prior incubation with sugar eliminated this staining. Differences

Fig. 2. Matching (a,c) phase-contrast and (b,d) fluorescent light micrographs of unfixed spermatozoa from the caput (a,b) and cauda (c,d) epididymides of the brushtail possum (*Trichosurus vulpecula*) after staining with fluorescein isothiocyanate (FITC)–HPA. Note that in caput epididymal spermatozoa all of the head and mid-piece fluoresce with lectin, but when the spermatozoa reach the cauda epididymidis, only the regions over the acrosome (arrow) and mid-piece fluoresce. Scale bars represent 2.7 μm .

in staining were discerned for spermatozoa from the cauda epididymidis, in which staining was observed only over the acrosome, but not over the rest of the sperm head (Table 2; Fig. 2), and the mid-piece showed dull fluorescence in some spermatozoa. After prior incubation with neuraminidase, strong staining over the acrosome and mid-piece was observed, which was blocked by prior incubation with galNAc (Table 2). These results indicate that the decrease in staining of cauda epididymal spermatozoa compared with

Table 3. Binding of fluorescein isothiocyanate (FITC) lectins to various regions of the plasma membrane of the head and tail of fixed spermatozoa from the caput and cauda epididymides of the brushtail possum, *Trichosurus vulpecula*

	Plasmalemma of sperm head		Plasmalemma over sperm tail	
	Acrosome	Rest of head	Mid-piece	Principal piece
WGA caput	+	+	++	++
WGA cauda	+	+	+	–
GSLII caput	+	–	–	–
GSLII cauda	–	–	–	–
Con A caput	+++	++	+++	++
Con A cauda	+	++	++	–
PSA caput	+++	++	++	+
PSA cauda	++	+	++	–
RCA ₁₂₀ caput	++	+	+	+
RCA ₁₂₀ cauda	–	–	–	–
RCA ₆₀ caput	++	++	++	++
RCA ₆₀ cauda	–	–	–	–
MPL caput	++	+	+	+
MPL cauda	–	–	–	–
HPA caput	+++	++	++	+
HPA cauda	+	–	–	–
DBA caput	+	–	–	–
DBA cauda	–	–	–	–
SBA caput	++	–	+	–
SBA cauda	–	–	–	–

Fluorescence was observed with an epifluorescent microscope and scored quantitatively (–: no fluorescence; +: weak fluorescence; ++: moderate fluorescence; +++: strong fluorescence). Caput: spermatozoa from the caput epididymidis; cauda: spermatozoa from the cauda epididymidis.

caput epididymal spermatozoa is due to masking of *N*-acetyl-galactosamine by sialic acid. Fixed spermatozoa stained with FITC–HPA showed a similar binding pattern to that of the unfixed spermatozoa (Tables 2 and 3).

SBA staining of spermatozoa from the caput epididymidis showed weak fluorescence over the head, mid-piece and cytoplasmic droplet (Table 2), and very weak staining of the principal piece. Fluorescence was eliminated after prior incubation with the sugar. Either weak or no staining of the cauda epididymal spermatozoa occurred, and prior incubation with neuraminidase had no effect on SBA binding. Fixing of the spermatozoa increased the fluorescence over the acrosome and cytoplasmic droplet of caput epididymal spermatozoa, whereas cauda epididymal spermatozoa did not show any fluorescence (Table 3). UEA1 did not result in any fluorescence of either the head or tail of spermatozoa from either the caput or cauda epididymides.

Agglutination of caput and cauda epididymal spermatozoa with lectins

Incubation of caput epididymal spermatozoa in 50 µg ConA ml^{–1} resulted in agglutination of spermatozoa into large clumps, whereas incubation of the lectin and spermatozoa with 0.4 mol glucose l^{–1} and 0.4 mol methylmannoside l^{–1} inhibited agglutination (Fig. 3). Incubation of spermatozoa from the cauda epididymidis in ConA also resulted in clumping (Fig. 3), but these agglutinated clumps of spermatozoa were not as large as those of caput epididymal spermatozoa. In both caput and cauda epididymal spermatozoa, agglutination was by way of both head–head and head–tail attachment (Table 4), indicating the presence of glucose or mannose over both of these regions of the spermatozoon.

Incubation of caput epididymal spermatozoa with DBA and SBA also resulted in clumping of spermatozoa by way

Fig. 3. Nomarski microscopy of brushtail possum (*Trichosurus vulpecula*) spermatozoa from (a) caput epididymidis and (b) cauda epididymidis after incubation with ConA, and (c) spermatozoa from cauda epididymidis after incubation with glucose and mannose before incubation with ConA. Note clumping of caput and cauda epididymal spermatozoa after incubation in the lectin, but absence of clumping after prior incubation with sugars. Scale bars represent 22 μm .

of head-head or head-tail attachment (Table 4); incubation of spermatozoa and lectin together with the sugar inhibited agglutination. In cauda epididymal spermatozoa, agglutination was less marked and only small clumps occurred by way of head-head interactions; incubation with sugar blocked agglutination.

Incubation of caput epididymal spermatozoa in 50 μg PNA ml^{-1} resulted in clumping of spermatozoa via head-tail interactions, whereas incubation of PNA and caput epididymal spermatozoa with 0.4 mol *N*-acetyl-galactosamine l^{-1} inhibited agglutination. Incubation of cauda epididymal spermatozoa with this lectin did not result in agglutination (Table 4).

Incubation of either caput or cauda epididymal spermatozoa with UEA1 did not result in agglutination.

Determination of molecular masses of glycoproteins stained with WGA and SBA

Several bands were present in sperm plasma membrane extracts of both caput and cauda epididymal spermatozoa that were stained with biotinylated-ConA, some of which had the same molecular masses as serum proteins. However five bands (105, 86, 53, 47 and 39 kDa) were present in caput sperm extracts, whereas two bands (> 200 and 77 kDa) occurred in cauda epididymal sperm extracts (Fig. 4a,b) possibly reflecting, in at least two cases, changes in the amount of glycosylation of proteins during epididymal transit. Three bands (57, 55 and 49 kDa) were common to both caput and cauda epididymal spermatozoa. Incubation of ConA with 0.4 mol glucose l^{-1} and 0.4 mol mannose l^{-1} eliminated binding to all of the observed glycoproteins.

When the sperm extracts were stained with SBA, four bands (116, 103, 82 and 63 kDa) were present in caput epididymal sperm plasma membrane extracts (Fig. 4c), whereas no staining was detected in the cauda epididymal sperm plasma membrane extracts. Incubation of SBA with 0.4 mol *N*-acetyl-galactosamine eliminated binding to all observed bands.

Discussion

In eutherian mammals, numerous studies have demonstrated that, as spermatozoa travel along the epididymis, changes in the distribution and localization of proteins occur within, and on, the plasmalemma. Furthermore, it is also apparent that many of the proteins, and possibly lipids, which are exposed on the cell surface are glycosylated and that the staining for these saccharides changes during epididymal transit (Nicolson *et al.*, 1977; Olson and Danzo, 1981; Hammerstedt *et al.*, 1982; Margaree *et al.*, 1988; Srivastava and Olson, 1991; for a review, see Eddy and O'Brien, 1994). Changes in lectin staining by the time spermatozoa reach the cauda epididymidis indicate a marked alteration in saccharide distribution and abundance during epididymal transit. These changes may be brought about by: (i) the presence of glycosidases and glycosyltransferases within the epididymal fluid; (ii) the secretion

Table 4. Agglutination of spermatozoa of the brushtail possum, *Trichosurus vulpecula*, after incubation with lectins

Lectin	Caput epididymal spermatozoa		Cauda epididymal spermatozoa	
	Agglutination	Sperm region showing agglutination	Agglutination	Sperm region showing agglutination
ConA	4 (large clumps)	Head-head, head-tail	3 (small clumps)	Head-head, head-tail
SBA	4	Head-head, head-tail	2	Head-head
DBA	3	Head-head, head-tail	1	Head-head
PNA	3	Head-tail	0	–
UEA1	0	–	0	–

Extent of agglutination of spermatozoa: 0 = no agglutination; 4 = maximum agglutination with no free spermatozoa. Three replicates were carried out.

Fig. 4. Western blot analysis of plasma membrane extracts of brushtail possum (*Trichosurus vulpecula*) (a) caput and (b) cauda epididymal sperm extracts stained with ConA. Note caput epididymal spermatozoa have five bands that were not present in blood plasma (105, 86, 53, 47 and 39 kDa) and cauda epididymal spermatozoa have two unique bands (> 200 and 77 kDa) stained with lectin. (c) Caput epididymal sperm extracts stained with SBA showing four bands that were not present in blood plasma (116, 103, 82 and 63 kDa). No unique bands were present in cauda epididymal sperm extracts stained with SBA.

of sialic acid by the epididymal epithelium that attaches to the sperm surface and masks the terminal sugar residues; and (iii) loss of saccharides at the time of shedding of the cytoplasmic droplet (Nicolson *et al.*, 1977; Holt, 1980; Margargee *et al.*, 1988). Such changes in the distribution and abundance of surface saccharides may relate, at least in part, to the preparation of the spermatozoon for molecular interactions with the female reproductive tract, capacitation changes and binding of the spermatozoon to the zona pellucida that surrounds the egg.

The present study, which extends the findings of Temple-Smith and Bedford (1976) on fixed spermatozoa and the preliminary observations of Cooper *et al.* (1998), used three approaches to characterize the saccharide content of the possum sperm surface and the changes that take place during epididymal transit. Firstly, caput epididymal spermatozoa stain positively with a variety of fluorescently labelled lectins and show regionalization of staining of some of the lectins. The positive staining on the sperm surface with FITC-WGA and FITC-GSLII indicates *N*-acetyl-D-glucos-

samine is present, RCA₁₂₀, RCA₆₀ and MPL (together with the effect of the blocking sugars) indicates D-galactose is present; ConA and PSA indicate mannose and α -D-glucose are present, whereas DBA, HPA and SBA indicate N-acetyl-galactosamine is present. The absence of staining with FITC-UEA1 indicates that fucose may not be present on the surface of these spermatozoa. In the fluorescent-labelling studies of unfixed spermatozoa, propidium iodide was used to control for sperm viability (Harrison and Vickers, 1990); thus the staining with FITC-lectins was used to expose saccharide residues on, or over, the plasma membrane of live spermatozoa. The increased intensity of staining that occurred with some of the lectins after fixation was probably due, at least in part, to the lectin-binding of intracellular components as fixation permeabilizes cells.

In the present investigation, when unfixed spermatozoa from the cauda epididymides were stained with the fluorescent-bound lectins, in many cases different results were obtained compared with those obtained with spermatozoa from the caput epididymides. For instance, cauda epididymal spermatozoa unlike caput epididymal spermatozoa did not stain with RCA₁₂₀, RCA₆₀, MPL, DBA or SBA, indicating that, similar to the situation in eutherian spermatozoa (Nicolson *et al.*, 1977, Margaree *et al.*, 1988), either loss or masking of various saccharides occurs during epididymal transit. Spermatozoa from the cauda epididymides were also incubated with neuraminidase before the lectin staining to determine which of the two possibilities had resulted in decreased staining. In the case of FITC-RCA₁₂₀, -RCA₆₀, -MPL and -DBA, but not -SBA, positive staining with the lectin was observed, even though spermatozoa that had not been incubated with the enzyme were negative. This finding indicates that the absence of fluorescence of the cauda epididymal spermatozoa is likely to be due to the secretion of sialic acid and its resultant binding to the sperm surface, and thus the masking of the galactose and possibly some of the N-acetyl-galactosamine residues.

In general, the findings of the agglutination studies support the conclusions from the FITC-labelled lectin observations. Specifically, after incubation with ConA, agglutination was less marked in cauda epididymal spermatozoa than in caput epididymal spermatozoa, indicating that, similar to observations in laboratory rats (Olson and Danzo, 1981), but unlike those observations in rabbits (Nicolson *et al.*, 1977) and rams (Hammerstedt *et al.*, 1982), there were fewer glycoproteins with α -D-glucose and α -D-mannose on the surface of cauda epididymal spermatozoa. The fact that both head-head and head-tail agglutination occurred strongly supports the results of the FITC-lectin studies, that these sugars are present on the cell membrane of both these regions. Observations from the western blot analysis indicate that, similar to eutherian mammals (Srivastava and Olson, 1991), there are several glycosylated proteins containing glucose or mannose present on the sperm surface of caput epididymal spermatozoa, but by the time the spermatozoa reach the cauda

epididymidis there are fewer glycoproteins that stain with ConA.

Results from the western blot analysis with SBA were more marked than those with ConA, as four SBA-positive glycoproteins appeared in caput epididymal sperm extracts, but by the time the spermatozoa reached the cauda epididymides, these differences were no longer apparent. This finding was supported, not only by FITC-labelling studies, but also by the agglutination results indicating that the N-acetyl-galactosamine units to which the SBA binds become reduced during epididymal transit. In relation to these observations, it is possible that exposure of possum spermatozoa to glycosidases, and also to galactosyl transferases, occurs during epididymal transit and that these enzymes cleave some of the glucose and galactosamine residues from the sperm surfaces, as has been suggested in various eutherian mammals (for examples, see Srivastava and Olson, 1991; Srivastava, 2000).

What is the broader significance of the present observations? Even though marsupials diverged from eutherian mammals over 100 million years ago and the morphology of the spermatozoa differ significantly (Bedford, 1991, 1998), it is apparent that the caput epididymal sperm surface has a complex series of surface saccharides similar to that seen in eutherian spermatozoa and that, during epididymal migration, there are modifications in the saccharides on the sperm surface. In eutherian mammals, Bedford (1979) suggested that these changes prepare spermatozoa for capacitation and binding to the zona pellucida. However, in possums, culturing cauda epididymal spermatozoa with oocytes does not result in sperm-zona binding, and recent observations indicate that secretions from the oviduct may be necessary to facilitate binding (Mate *et al.*, 2000). It is possible, therefore, that some of the surface carbohydrates on possum spermatozoa facilitate interaction with the epithelial lining of the female reproductive tract (Suarez, 1998; Topfer-Petersen, 1999) or are involved in protecting the sperm surface molecules during sperm migration (see Schroter *et al.*, 1999). Although possums do not have a distinct series of crypts in the isthmus of the oviduct for sperm storage, unlike those of dasyurid marsupials (Breed *et al.*, 1989; Bedford and Breed, 1994), the spermatozoa may interact with, and may even become bound to, the surface epithelium for a period between mating and ovulation. Thus, this process may involve molecular interaction between saccharides on the sperm surface and those of cells lining the female reproductive tract.

The present study has demonstrated that when the possum spermatozoa enter the epididymis a variety of sugars is already present on their surface and that during epididymal transit a complex series of changes takes place in the saccharide composition. Some of the changes observed in lectin staining may be due to deposition of sialic acid, whereas other changes are probably brought about by the removal of the terminal saccharides by glycosidases and glycosyl transferases in the epididymal

fluid. The reasons for this complex series of molecular changes in the sperm surface remain to be determined but, in general, the changes appear to be similar to those that take place in spermatozoa of eutherian mammals. In eutherians it has been suggested that the complex series of changes may relate to changes that spermatozoa undergo in the female reproductive tract in preparation for fertilization and, in particular, for those associated with capacitation and the development of sperm–zona binding ability (Bedford, 1979). However, in possums, sperm capacitation is poorly understood and cauda epididymal spermatozoa do not appear to be able to bind to the zona pellucida without secretions from the female reproductive tract. Thus, these changes in carbohydrates on the sperm surface may relate to extratesticular sperm maturation processes other than the development of sperm–zona binding. Alternatively, changes may relate, at least in part, to sperm–female tract interactions that take place between insemination and ovulation.

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