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Lectin histochemistry study in the human vas deferens

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The oligosaccharide sequences of glycoconjugates in the normal human vas deferens and the nature of the saccharide linkage were studied by lectin histochemistry. The cytoplasm of all epithelial cell types (principal cells, basal cells, and mitochondria-rich cells) and luminal contents reacted positively with WGA, MAA, PNA, DSA, LTA, UEA-I, AAA, and ConA. The reaction was more intense in the stereocilia of principal cells. Cytoplasmic staining was diffuse except for PNA and DSA labeling which was limited to the apical cytoplasm and stereocilia of columnar cells. The cytoplasm of all cell types also reacted diffusely with HPA, although staining was weak and was not observed in the stereocilia. Positive reaction with SBA only was encountered in the stereocilia of principal cells. SNA, LTA, and DBA were unreactive. GNA-labeling showed a granular distribution in the supranuclear cytoplasm of columnar epithelial cells. Reactions with MAA, PNA, DSA, AAA, HPA and SBA disappeared after the β-elimination reaction. Reactions with WGA and UEA-I decreased after β-elimination or Endo-F digestion. Reactions with ConA and GNA were suppressed by Endo-F digestion. Reactions with PNA, HPA, and SBA increased after desialylation. Of all the lectins that label the luminal contents of the vas deferens, only UEA-I was not found in the luminal contents of seminiferous tubules and epididymis and, thus, this lectin would probably bind to glycoproteins secreted by the vas deferens. The chemical treatments used suggest that this secretion contains fucose residues located in both N- and O-linked oligosaccharides. The other lectins may label secreted proteins, but also structural proteins or proteins reabsorbed from the luminal fluid. The lectin- binding pattern of mitochondria-rich cells in the vas deferens differed from that found in the epididymis.

Keywords: **lectins, human vas deferens; oligosaccharides**

Abbreviations: **Endo-F: Endo-β-acetylglucosaminidase F/peptide N-glycosidase F; GlcNAc: N-acetylglucosamine; Neu5Ac: sialic acid; Gal: galactose; GalNAc: N-acetylgalactosamine.**

Introduction

The lumen of the human vas deferens is lined by an epithelium separated by a thick basal lamina from the underlying lamina propria. There are three concentric layers of smooth muscle cells covered by the tunica adventicia, which is located external to the lamina propria. The epithelium is composed of tall, pseudostratified, columnar cells with stereocilia protruding into the lumen, and stellate basal cells. Three types of columnar cells may be distinguished in the epithelium: principal cells, dark or pencil cells, and apical mitochondria-rich cells [1]. Whereas the mitochondria-rich cells seem to be a well-defined cell type, present also in the ductus epididymis, the dark cells are assumed to be a final degenerative stage of principal cells [2–3]. The vas deferens does not function simply as a conduit for the transport of spermatozoa, it plays some role in the absorption of fluid from the lumen, spermatozoon maturation, and spermiophagy [4–5].

The majority of proteins secreted by the excretory ducts of the reproductive system are glycosylated [6], and it has been demonstrated that glycans play an essential role in the maturation of spermatozoa [7–8]. Although most of these glycoproteins are secreted in the epididymis [9–10], it has also been reported that the vas deferens is involved in the secretion of some proteins [11].

Lectins are proteins or glycoproteins, each with an affinity for a specific sugar or sugar sequence [12–13]. They have been used to agglutinate several types of cells, including spermatozoa; or, when combined with an appropriate marker they have been employed as histochemical probes. The increasing use of lectins has provided information about sugars associated with glycoconjugates of spermatozoa and of testicular and epididymal epithelial cells, but

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there are no reports about the synthesis and secretion of glycoconjugates in the human vas deferens. In the present work, lectin cytochemistry, in combination with enzymatic and chemical treatments, was applied to map lectin binding sites on the human vas deferens.

Material and methods

Materials

Human vasa deferentia from 15 adult men (aged from 25 to 55 years) were obtained at autopsy. These men had died either in traffic accidents or from myocardial or cerebral infarction. The conditions for selection were the absence of reproductive, endocrine, or related diseases in the patient histories and the presence of complete spermatogenesis in their testes. The specimens were collected between 6 and 10 h after death. To evaluate postmortem changes in the autopsy specimens, three vasa deferentia obtained from testicular tumour surgery were immediately fixed and processed in the same way.

Lectin histochemistry

For light microscopy, the tissues were fixed in 10% (v/v) formaldehyde in phosphate buffered saline pH 7.4, for 24 h at room temperature. The samples were routinely processed, embedded in paraffin wax, and 5 µm sections were obtained. Sections were dewaxed in xylene and dehydrated in a graded ethanol series. Lectin binding patterns were established using horseradish peroxidase (HRP)- and digoxygenin (DIG)-conjugated lectins (Table 1).

For histochemical staining using HRP-conjugated lectins, previously endogenous peroxidase was blocked with 0.3% (v/v) hydrogen peroxide in Tris-buffered saline (TBS) and the sections were incubated for 90 min. at room temperature with the following HRP-conjugated lectins: PNA (25 µg/ml), ConA (20 µg/ml), LTA (25 µg/ml), WGA (10 µg/ml), SBA (18 µg/ml), UEA-I (20 µg/ml), HPA (6 µg/ml), and DBA (15 µg/ml). Peroxidase was detected with 0.05% (w/v) 3,3 diaminobenzidine and 0.015% (v/v) hydrogen peroxide in TBS.

For labeling with lectin-DIG conjugates, a two-step technique was applied. In brief, endogenous peroxidase activity was destroyed by a 30 min. treatment with 0.3% (v/v) H_2O_2 in TBS. Sections were rinsed in TBS, covered with 1% (w/v) BSA in TBS for 10 min., and incubated in the following lectin-DIG conjugates for 90 min. at room temperature: DSA (10 µg/ml), MAA (10 µg/ml), AAA (20 µg/ml), SNA (15 µg/ml), and GNA (60 µg/ml). After two rinses for 5 min. in TBS, sections were incubated with peroxidase-conjugated anti-DIG Fab' fragments (0.6 U/ml) in TBS supplemented with 1% (w/v) BSA for 60 min. at room temperature. The peroxidase activity was visualized with 0.05% (w/v) DAB and 0.015% (v/v) H_2O_2. Sections were counterstained with Harris's haematoxylin.

The staining intensity of the different lectins in the different cell types was subjectively evaluated as absent (−), weak (+), moderate (++), or intense (+++).

Enzymatic and chemical treatments

To expose carbohydrates that could be masked by sialic acid residues, acid hydrolysis was performed by immersing the sections in 0.1 M HCl for 2–3 h. at 82 °C.

Removal of sulpfate esters without modification of the oligosaccharide chains in the glycoproteins, was carried out by a sequential methylation-saponification process. Paraffin sections were treated with 0.15 N HCl in absolute methanol (5 h at 60 °C). For saponification, the sections were treated with 1.8 % (w/v) $Ba(OH)_2$ in aqueous solution (1 h at 0–4 °C).

To eliminate O-linked oligosaccharides (β-elimination reaction), the sections were incubated with 0.5 N NaOH in 70 % (v/v) ethanol at 4 °C, for 5 days [14]. After this treatment; staining of the incubated sections with HPA (a specific marker for O-linked oligosaccharides) was negative, whereas staining with GNA (a specific marker for N-linked oligosaccharides) was positive; and, thus, 5 days of treatment was the time required to remove O-linked oligosaccharides without modification of the N-linked oligosaccharides. To corroborate the effectiveness of this reaction, sections of human large intestine (which contain abundant O-linked oligosaccharides) were processed together with the vas deferens sections and used as positive controls.

Hydrolysis of N-linked oligosaccharides was carried out by enzymatic treatment with Endo-β-acetylglucosaminidase F/peptide N-glycosidase F (Endo-F). After incubation in 0.1 M Tris, 150 mM NaCl, 2.5 mM EDTA (pH 9) buffer containing 1% (w/v) BSA for 10 min, and a brief washing in the buffer without BSA, the sections were incubated with the enzyme, at a dilution of 6 U/ml, for 3 days.

Elimination of terminal glucose residues was carried out by the enzyme glucose oxidase from *Aspergillus niger.* After incubation in 0.2 M sodium acetate buffer (pH 5) for 5 min, the sections were incubated with the enzyme, which oxidizes glucose to glucuronic acid and H_2O_2, at a dilution of 50 U/ml, for 12 h at 37 °C.

Histochemical controls

Three types of controls were used: (1) substitution of conjugated and unconjugated molecules by the corresponding buffer; (2) preincubation of the lectins with the corresponding hapten-sugar inhibitor, used at a range of concentration from 0.025 to 1 M. The inhibitors were: D-Gal for PNA; methyl-α-mannose for both ConA and GNA; D-N-acetylgalactosamine for HPA and SBA; D-N-acetylglucosamine for WGA; N-acetyllactosamine for DSA; L-fucose for AAA, LTA and UEA-I; and Neu5Ac for SNA. The inhibitor used for MAA was 0.1M Neu5Ac (α 2,3) lactose. An

Table 1. Lectin binding pattern in human vas deferens.

Lectin	*Carbohydrate binding specificity*	*Principal cells*	*Mitochondria-rich cells*	*Basal cells*	*Lumen content*	*Observations*
WGA (Wheat germ agglutinin)	(GlcNAc)	+++	+++	+++	+++	Staining decreases with Endo-F and β-elimination in the three cell types
SNA (Sambucus nigra aggl.)	Neu5Acα2,6Gal; Neu5Acα2,6GalNAc	–	–	–	–	
MAA (Maackia amarensis aggl.)	Neu5Acα2,3Galβ1,4GlcNAc	++	++	+	++	No staining with β-elimination in the three cell types
PNA (Arachis hypogaea aggl.)	Galβ1,3GalNAc>Gal	++	++	++	++	Staining increases with acid hydrolysis and disappears with β-elimination in the three cell types
DSA (Datura stramonium aggl.)	Galβ1,4GlcNAc	++	++	++	++	Staining increases with acid hydrolysis and disappears with β-elimination in the three cell types
LTA (Lotus tetragonolobus aggl.)	L-fucose	–	–	–	–	
UEA-I (Ulex europaeus aggl.)	L-Fucα1,2Galβ1,4GlcNAcβ1,6	++	+++	++	++	Staining decreases with Endo-F in principal cells and basal cells and disappears with β-elimination in the three cell types
AAA (Aleuria aurantia aggl.)	αL-fucose	++	++	+	++	No staining with β-elimination in the three cell types
ConA (Canavalia ensiformis aggl.)	αMannose> αGlucose	+++	+++	+++	+++	No staining with Endo-F in the three cell types
GNA (Galanthus nivalis aggl.)	$(Mannose)_{1-3}$	+++	+++	+	–	No staining with Endo-F in the three cell types
DBA (Dolichos biflorus aggl.)	GalNAcα1,3	–	–	–	–	
HPA (Helix pomatia aggl.)	GalNAcα1,3GalNAc	+	+	+	+	Staining increases with desulfation and desialylation and disappears with β-elimination in the three cell types
GNA (Glycine max aggl.)	GalNAc-Ser/Thr; GalNAcβ1,3Gal; GalNAcβ1,4Gal	+	–	–	–	Staining increases with desulfation and desialylation and disappears with β-elimination in principal cells

GlcNAc: N-acetylglucosamine. Fuc: fucose. Neu5Ac: sialic acid. Gal: galactose. GalNAc: N-acelylgalactosamine. – no staining; + weak staining; ++ moderate staining; +++ intense staining.

additional control for SNA and MAA was pretreatment of the sections with 1 U/ml of Vibrio cholerae sialidase, diluted in acetate buffer (pH 5), for 3 h at 37 °C, to remove sialic acid residues from tissue sialoglycoconjugates.

Results

Comparison of the vasa deferentia obtained at surgery with the autopsy specimens showed neither histological nor histochemical changes. The lectin-binding pattern was similar in all specimens. The results are summarized in Table 1.

Reactions with WGA was intense in all epithelial cell types, and was particularly strong in the stereocilia. The luminal content, as well as the lamina propria and the adjacent connective tissue were also stained (Fig. 1). Reaction with WGA was not modified when the sections were subjected to acid hydrolysis, and decreased after the β-elimination reaction or Endo-F digestion (Fig. 2).

The vas deferens epithelium and luminal content were unreactive with SNA. A moderate reaction with MAA was visualized in the basal cells, basal cytoplasm of columnar cells, and luminal contents. Staining was more intense in the apical cytoplasm and stereocilia of columnar cells (Fig. 3). Reaction with MAA was abolished when the sections were subjected to the β-elimination reaction (Fig. 4).

Reactions with both PNA and DSA were similar. Staining was observed in all cell types and was more intense in the adluminal surface and luminal contents. The reactivity with these lectins was not modified by desulfation or Endo-F digestion, but no staining was observed after the β-elimination reaction. Staining with PNA (but not DSA) increased with acid hydrolysis.

LTA was unreactive in the vas deferens. UEA-I labeling was visualized in all epithelial cells, mainly in mitochondria-rich cells (Fig. 5). The luminal content also stained with this lectin. When the sections were subjected to enzymatic digestion, staining decreased in the principal cells, but remained in the mitochondria-rich cells (Fig. 6). After β-elimination, staining was abolished in all cell types. A diffuse reaction with AAA was found in the cytoplasm of all epithelial cells and luminal contents. The stereocilia were more intensely stained. AAA binding was not observed after NaOH treatment.

An intense reaction with ConA lectin was observed in the luminal contents and throughout the whole cytoplasm and stereocilia of all epithelial cell types. The reaction with this lectin was not modified after glucose oxidase digestion and was suppressed with Endo-F pretreatment. GNA-labeling was concentrated in the supranuclear cytoplasm of columnar epithelial cells, and showed a granular distribution. No staining was observed in the luminal content. Reaction with this lectin was abolished by Endo-F digestion.

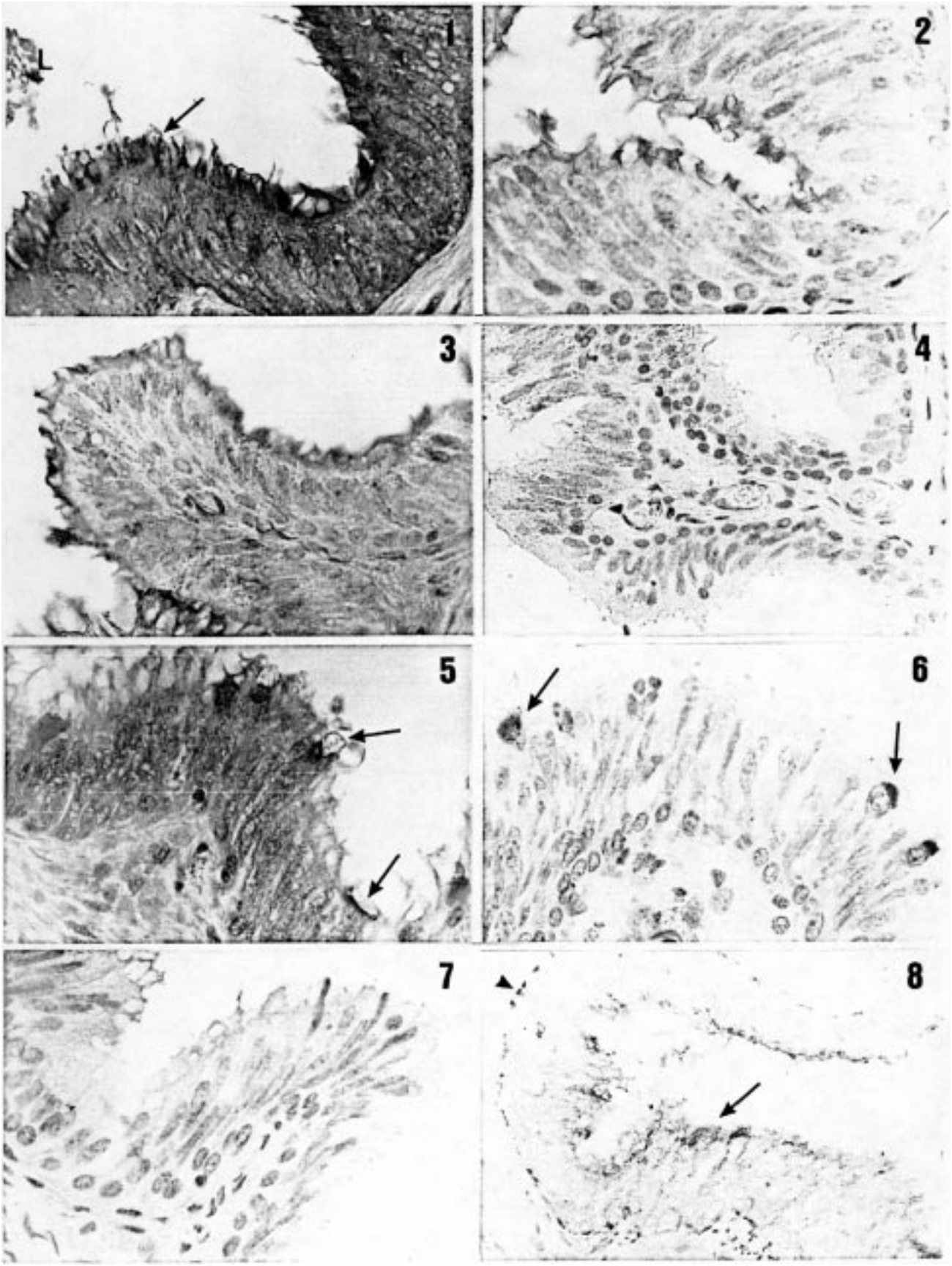
DBA showed a negative reaction in the vas deferens epithelium and luminal content. A slight reaction with HPA was observed throughout the cytoplasm of all epithelial cells and luminal content (Fig. 7). Positive reaction with SBA was only encountered in the adluminal surface of epithelial cells. The reactions with HPA and SBA were not modified after Endo-F digestion, but, after removal of O-linked oligosaccharides, the stainings were abolished. When the sections were subjected to desialylation or desulfation, staining with these lectins was more prominent (Fig. 8).

Discussion

The vas deferens should not be considered as a simple, passive duct for sperm transport [2]. The ultrastructural features of principal cells (stereocilia, apical vesicles, and lysosomes) suggest that these cells have a reabsorptive function. Based on the presence of abundant endoplasmic reticulum, Golgi apparatus, and a prominent nucleolus, some authors [15] have hypothesized that the epithelium of vas deferens synthesizes and secretes glycoproteins into the tubular lumen. Mitochondria-rich cells have only been reported in the vas deferens of humans [2] and rats [16], and their number increases along the length of the vas deferens while that of principal cells decreases. The high number of mitochondria in the mitochondria-rich cells could reflect calcium pumping or the generation of keto-acids for aminoacid synthesis, and suggests a high rate of utilization of acetyl coenzyme A. These cells express high levels of a vacuole-type proton-pumping H^+ ATPase on its luminal plasma membrane as well as in intracellular vesicles [17], and contain large amounts of carbonic anhydrase [18]. It has been suggested that this energy is required for intensification of fluid reabsorption, which mainly occurs at the prostatic end of the vas deferens [19].

Lectin histochemical studies have provided information on the synthesis and secretion of glycoconjugates in the testis and epididymis, but no studies relating to sugar residues of glycoconjugates in the human vas deferens have

Figure 1-8. Light microscopy lectin labeling of the human vas deferens. Sections have been counterstained with haematoxylin. X600 **Figure 1.** WGA labeling. All epithelial cell types and the luminal content (L) are stained. Labeling is more intense in stereocilia (arrow). **Figure 2.** WGA staining decreases in all cell types after Endo-F digestion. **Figure 3.** MAA labeling. All epithelial cells are moderately stained. Staining is more intense in the apical cytoplasm and stereocilia of columnar cells. **Figure 4.** MAA reaction disappears after the β-elimination reaction. **Figure 5.** UEA-I labeling is observed in all epithelial cells, mainly in mitochondria-rich cells (arrows). **Figure 6.** After Endo-F digestion, the staining intensity with UEA-I is maintained in mitochondria-rich-cells (arrows) while it decreases in the other cell types. **Figure 7.** Weak reaction with HPA in the most epithelial cells. **Figure 8.** After acid hydrolysis, reaction with HPA increases, overall in stereocilia (arrow) and basal cells (arrowhead).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been reported. Presents results show abundant glycoproteins in the vas deferens epithelium and ductal lumen. These glycoproteins might be classified into three groups: (a) proteins that are synthesized by the epithelial cells and are secreted into the lumen; (b) structural proteins; and (c) proteins that are reabsorbed by the epithelium. Although the chemical pretreatments used here to investigate the nature of the saccharide linkage are aggressive and might cause protein degradation, the use of positive and negative controls in this study validated the results and suggested certain conclusions.

WGA, MAA, PNA, DSA, UEA-I, AAA, ConA, and HPA positively stained the vas deferens epithelium and also the luminal contents and, thus, it might appear that these lectins were labeling glycoproteins secreted by the vas deferens. Except for UEA-I, however these lectins have also been detected in the lumen of the epididymal duct [20], therefore, the stained luminal material of the vas deferens could have originated in the epididymis, although it is possible that glycoproteins similar to those secreted by the epididymis may also be secreted by the epithelium of the vas deferens. The characteristics and possible function of these glycoproteins—which are involved in spermatozoon maturation [7–8]—have been considered in previously reported studies on the lectin histochemistry of human epididymis [20–21]. Additionaly, the stained glycoproteins within the epithelial cells of the vas deferens might correspond either to structural proteins or to proteins reabsorbed from the lumen and contained in endosomes and endolysosomes [4–5]. Endosomes have been shown to be involved in the sorting and recycling of receptors and ligands via small tubular extensions [22].

In contrast, the luminal content of the seminiferous tubules and that of the epididymal duct did not react with UEA-I lectin. Therefore, it is very likely that UEA-I-positive proteins are secreted by the vas deferens epithelium. This lectin identifies a number of terminal fucosyl residues. In the principal cells, staining decreased after Endo-F digestion and also after β-elimination. This suggests that these residues are located in both N- and O-linked oligosaccharides. This protein might correspond to a 34.5 kDa glycoprotein, named MVDP (mouse vas deferens protein) [23], which is released into the lumen of the vas deferens by an apocrine-like mechanism [11].

Among the structural proteins, the stereocilia of vas deferens principal cells reacted intensely with WGA, MAA, PNA, DSA, AAA and ConA and, less markedly, with SBA. The glycocalyx is particularly rich in carbohydrate moieties, above all sialylglycoconjugates; and some authors have suggested that terminal sialic acid residues play a role in several functions, including protection of cells from dehydration, transport of metabolites and ions across the plasmalemma, and hormone-binding [24]. The decrease in WGA reaction after β-elimination or Endo-F digestion suggests that the GlcNAc residues labeled by this lectin belong to both N- and O-linked oligosaccharides. After acid hydrolysis, the reaction with WGA was not modified, suggesting that sialic acid was not linked to these GlcNAc residues. MAA labeling showed the presence of Neu5Acα(2,3)Galβ(1,4)GlcNAc sequences [25] in O-linked oligosaccharides (reactivity was abolished by the β-elimination reaction). PNA labeling did not alter with desulfation treatment, but increased with acid hydrolysis, suggesting that two types of sequences belonging to glycoproteins with O-glycosidic linkage are labeled: Neu5AcGalβ(1,3)GalNAc, and Galβ(1,3)GalNAc. The DSA reaction suggested that the presence of the Galβ(1,4)GlcNAc sequence. AAA labeling was lost after the β-elimination reaction, suggesting that fucose residues are present in O-glycosylated proteins. ConA binds to mannose residues and this reaction was eliminated with Endo-F digestion, indicating that mannose residues belong to N-linked glycoproteins. The reaction with SBA indicated the presence of β-GalNAc in terminal residues. The use of this lectin combined with the desulfation technique suggested the presence of terminal residues with Neu5acGalNAc, and the results of the desulfation technique suggested the existence of the terminal GalNAc sulfated residues. Since staining disappeared after β-elimination, the above-mentioned residues are probably in O-linked oligosaccharides. Sulfation is a common post-transcriptional modification of both membrane and secreted glycoproteins in the trans-Golgi net [26], and there is considerable evidence to suggest that sulfation is important for intercellular recognition mediated by the carbohydrates of glycoproteins [27].

GNA labeling might be related to glycoproteins reabsorbed from the luminal fluid. This protein also belongs to the group of mannose-binding lectins selective for N-linked oligosaccharides [28–29]. The different staining pattern for each of the two mannose-binding lectins found in our study might explained on the basis that GNA binds to terminal Man in high mannose N-linked oligosaccharides [29], while ConA labels trimannosyl cores of oligomannoses, bianntenary hybrids, and complex type carbohydrates [28]. The granular appearance of GNA reaction could be related to lysosomes, which are known to contain oligomannose residues in the N-linked oligosaccharides of their enzymes [30].

The lectin-binding pattern of mitochondria-rich cells in the vas deferens differed from that found in the epididymis. In both ducts staining was positive for WGA, MAA, PNA, DSA, UEA-I, AAA, ConA, and HPA, and negative for LTA. However, the absence of reaction to SNA, DBA and SBA in the mitochondria-rich cells of the vas deferens contrasts with the intense reaction with these three lectins in the epididymal mitochondria-rich cells [21]. This suggests that the terminal residues of GalNAc, Neu5AcGalNAc, and SO_4GalNac present in O-linked oligosaccharides in the mitochondria-rich cells of the epididymis [21] are lacking in those of the vas deferens.

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