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Immunohistochemical and lectin histochemical analysis of the alpaca efferent ducts

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Summary. An immunohistochemical and lectin histochemical study of the efferent ducts was performed in the alpaca. Two types of epithelium, consisting of principal and ciliated cells, were detected on the basis of the different cytokeratins expression and lectin binding pattern. AE1/AE3 and 13 cytokeratin antibodies intensely immunostained the entire cytoplasm of type I PCs, whereas AE1/AE3, but not anti cytokeratin 13, immunoreacted in type II principal cells along the apical, lateral and basal plasma-membrane.

The histochemical characterization of the epithelial cells was carried out using a battery of different lectins: Con-A, UEA-I, LTA, WGA, GSA-II, GSA-IB4, SBA, PNA, ECA, DBA, MAL-II and SNA. Sialidase digestion and deglycosilation pre-treatments were also employed.

In type I principal cells, the staining of the Golgi zone was interpreted giving evidence for the synthesis and secretion of O- and N-linked oligosaccharides. In particular, α-Man/α-Glc, GlcNAc, ß-Gal-(1-4)-GlcNAc, Neu5Acα2,3Gal and Neu5Acα2,6Gal/GalNAc residues were included in both O- and N-linked glycans, whereas α-Fuc, ß-GalNAc and α-Gal were only found in O-linked oligosaccharides; α-GalNac and ß-Gal-(1-3)-D-GalNAc were found subterminal to sialic acid moieties and they were included in O- and N-glycans. In type II principal cells, the lectin staining was observed in the apical cytoplasmic granules and in vacuoles that were interpreted as components of an elaborate endocytotic apparatus specialized for the uptake of particulate material and fluid from the lumen. These results suggest the existence of two structurally different epithelial segments along the ductuli efferentes of the alpaca, with a high degree of compartmentalization of the secretory and absorptive activities.

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Introduction

The ductuli efferentes are portions of the excurrent duct system which connect the rete testis to the epididymis. They are lined by columnar ciliated cells and principal non ciliated cells that create an optimal microenvironment in which spermatozoa undergoes many morphological and physiological changes, essential for acquiring fertilizing ability and motility through the absorption and secretion of different macromolecules that contact spermatozoa (Queiròz et al., 2006; Kujala et al., 2007).

The ciliated cells (CCs) have very similar morphological features in a great variety of species studied (Arrighi et al., 1993, 1994; Ilio and Hess, 1994; Stoffel and Friess, 1994; Wakui et al., 1996) whereas the principal cells (PCs) show more inter-species variation. Indeed, previous histochemical and morphological investigations have described unique types of PCs in many laboratory rodents (Arya and Vanha Perttula et al., 1986; Burkett et al., 1987), lizards (Labate et al., 1997) and horses (Arrighi et al. 1993, 1994; Parillo et al., 1998). Conversely, PCs have been divided into three types in man (Ilio and Hess, 1994), bull (Goyal and Hrudka, 1981), goat (Goyal et al., 1992) and dog (Wakui et al., 1996) on the basis of the variation in the presence or absence of granules and vacuoles that may contain endocytotic material or secretory components of the cells.

The use of lectins as histochemical probes allows the "in situ" identification of the glycomolecules present in tissues and cells, because it is possible to precisely localize the different sugar residues by the specific carbohydrate binding of the lectins (Spicer and Schulte, 1992; Madrid et al., 2000).

The objective of the present report was to identify and localize the oligosaccharide sequences of glycoconjugates and the nature of their linkages in the epithelial cells lining the ductuli efferentes of the alpaca, using a broad battery of lectins in conjunction with enzymatic and deglycosilative pre-treatments. Additionally, an immunohistochemical study using different anti-cytokeratin antibodies was performed, in order to more clearly elucidate the structural and functional differences of these epithelial cells.

It is inspected that this information with the data obtained in our recent analogous investigation carried out in the alpaca epididymis (Parillo et al., 2008) may improve the knowledge in the reproductive biology of this camelid species.

Materials and methods

For this study ductuli efferentes were taken following castration from 4 to 10 year-old alpaca (n=5) without reproductive diseases and showing complete spermatogenesis. The specimens were immediately fixed in Carnoy's fluid for 24 hours and post-fixed in a solution of 2% calcium acetate and 4% paraformaldehyde (1:1 v/v) for 3 hours at room temperature (Menghi, 1984). They were then routinely dehydrated in graded series of alcohols, cleared in xylene and subsequently embedded in paraffin.

Serial sections 5µm thick were mounted on Superfrost Plus slides (Bio-Optica, Milano, I) and subjected to the following lectin histochemical and immunohistochemical procedures.

Lectin histochemistry

The specimens were processed for lectin histochemistry according to the procedures that were described previously by Parillo et al. (1997, 1998).

The sections were dipped in 0.3% H_2O_2/methanol for 30 minutes to inhibit endogenous peroxidase activity and, after washing with PBS, were incubated in a moist chamber for 1 hour at room temperature with a solution of horseradish peroxidase (HRP) conjugated lectins in 0.1M PBS pH 7.2 containing 0.1mM $CaCl_2$, $MgCl2_2$ and $MnCl_2$. The sections were gently rinsed with PBS and the peroxidase activity sites were visualized with a 3-3' diaminobenzidine (DAB kit, D.B.A. Italia S.R.L., Milano, I) for 5 minutes. Finally, all the sections were rinsed in distilled water, dehydrated and mounted in Eukitt.

Biotinylated lectins specific for sialic acid

The direct visualization of sialic acid residues was carried out with SNA and MAL-II biotinylated lectins. Sections were dipped in 0.3% H_2O_2/methanol for 30 minutes to inactivate endogenous peroxidase. Endogenous avidin-binding activity was blocked using the avidin-biotin blocking kit (D.B.A. Italia S.R.L., Milano, I). Subsequently, the sections were processed as above with the exception that, after lectin incubation, the slides were exposed to avidin-biotin complex (ABC kit, Vector Laboratories) for 30 minutes.

All the lectins used in this research, as well as their carbohydrate specificies, inhibitory sugars, and optimal concentrations, are reported in Table 1.

The lectins used in this research were purchased from Sigma Chemicals (Milano, Italy) with the exception of GSA-II, LTA, ECA, MAL-II and SNA that were obtained from Società Italiana Chimici (Roma, I).

Controls

Negative controls for the lectin labelling, except MAL-II and SNA, were run either by substitution of lectin-conjugates with the respective unconjugated lectins or by preincubation of lectins with the addition of 0.2/0.4M corresponding hapten sugars (Sigma, Milano - Italy). Preincubation of sections with neuraminidase eliminated staining with MAL-II and SNA.

Table 1. Lectins used and their carbohydrate specificities.

Source of lectin	Acronym	Carbohydrate specificity[a]	Inhibitory sugars[b]	Lectin concentration
Canavalia ensiformis	Con-A	α-D-Man>α-D-Glc	α-D-Methylman	20 µg/ml
Triticum vulgaris	WGA	GlcNAc>sialic acid	D-GlcNAc	10 µg/ml
Griffonia simplicifolia	GSA-II	α and ß GlcNAc	D-GlcNAc	50 µg/ml
Glycine max	SBA	α-D-GalNAc>ß-D-GalNAc	D-GalNAc	10 µg/ml
Arachis hypogaea	PNA	ß-D-Gal-(1→3)-D-GalNAc	D-Gal	40 µg/ml
Griffonia simplicifolia IB4	GSA-IB4	α-D-Gal	D-Gal	20 µg/ml
Ricinus communis	ECA	ß-D-Gal-(1→4)-D-GlcNAc	D-Gal	50 µg/ml
Dolichos biflorus	DBA	α-D-GalNAc	D-GalNAc	10 µg/ml
Lotus tetragonolobus	LTA	α-L-Fuc	L-Fuc	20 µg/ml
Ulex europaeus	UEA-I	α-L-Fuc	L-Fuc	20 µg/ml
Sambucus nigra	SNA	NeuAc(α2,6)Gal/GalNAc	NeuAc	100 µg/ml
Maackia amurensis	MAL-II	NeuAc(α2,3)Gal	NeuAc	100 µg/ml

[a]: ß-D-Gal, ß-D-galactose; α-D-Gal, α-D-galactose; D-GalNAc, D-N-acetylgalactosamine; ß-D-GalNAc, ß-D-N-acetylgalactosamine; α-D-GalNAc, α-D-N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; α-D-Man, α-D-mannose; α-D-Glc, α-D-glucose; NeuAc, N-acetylneuraminic acid. [b]: α-D-Methylman, α–D-methylmannose.

An additional control was performed by dipping the sections in a DAB system without lectins in order to demonstrate endogenous peroxidase activity in the tissue samples.

Enzymatic digestion

GSA-II-, SBA-, DBA-, PNA-, GSA IB4- and ECA-lectin staining procedures were preceded by neuraminidase (sialidase type V from *Clostridium perfringens*, Sigma Chemical Co., Milano - Italy) digestion, which was carried out for 18h at 37°C in a solution of 0.1M acetate buffer, pH 5.5 and 10 mM $CaCl_2$ containing the enzyme at a concentration of 0.86 U/ml.

Controls for the enzymatic digestion were provided by sections exposed to the buffer in which the enzyme was dissolved.

Sialic acid residues with O-acyl groups at C4 resisted *C. perfringens* neuraminidase, but were cleaved after removal of the acetyl groups by saponification. Saponification was performed by immersing the sections in 1% potassium hydroxide solution in 70% ethanol for 15 minutes prior to sialidase degradation.

Deglycosylation pre-treatments

Two chemical pre-treatments were performed: a) hydrolysis of N-linked oligosaccharides was done with endo-ß-acetylglucosaminidase F/peptide N-glycosidase F (PNGase F - Roche, Mannheim, Germany) as previously described (Lucocq et al., 1987; Madrid et al., 2000); b) ß-elimination reaction, to eliminate O-linked oligosaccharides was carried out as previously reported (Ono et al., 1983; Madrid et al., 2000).

Immunohistochemistry

The epididymal sections were incubated with 3% H_2O_2 (20 minutes) to block the endogenous peroxidase activity. After rinsing with PBS containing 0.2% Triton X-100 and 0.1% bovine serum albumin (BSA), the sections were incubated with citrate buffer solution (pH 6.0) and heated in a microwave oven at 700 watts for 10 minutes to enhance their antigenicity. Slides were then allowed to cool at room temperature for at least 20 minutes. Background labelling was prevented by incubating the sections with normal goat serum (NGS), diluted 1:10, for 30 minutes at RT. The slides were then incubated overnight in a moist chamber at 4°C with monoclonal antibodies against cytokeratins (CK) using the following working dilutions: anti-CK AE1/AE3 (DakoCytomation, Carpinteria, CA, 1:50), anti-CK 5 (Novus Biologicals, 1:10), anti CK 13-16 clone K 8.12 (Sigma, 1:10) and anti CK 18 clone CY-90 (Sigma, 1:10). The next day, the slides were washed with PBS and incubated with biotin goat anti-mouse IgG conjugate (Zymed 81-6140) diluted 1:200 for 30 min. After PBS washes, the slides were exposed to avidin-biotin complex (ABC kit) for 30 min. The sections were rinsed gently with PBS and the peroxidase activity sites were visualized with DAB kit and processed as lectin histochemistry.

Slides, on which the primary antibody was omitted, were used for the negative control for unspecific staining.

Results

Histologically and histochemically, we categorized the epithelium (columnar pseudostratified) lining the ductuli efferentes of the alpaca into two types: an initial segment lined by type I epithelium, and a distal segment, near the epididymis, lined by type II epithelium, consisting both of CCs and PCs (Fig. 1a,b). CCs were morphologically similar in both epithelia showing a cytoplasm containing a finely granular material. In type I epithelium, the PCs were characterized by a homogenous cytoplasmic matrix and numerous granules filled in the apical cytoplasm just beneath the plasma-membrane (Fig. 1c). These cells exhibited lectin reactive sites in the supranuclear cytoplasm (Golgi area), apical granules and glycocalyx. In type II epithelium, the PCs appeared vacuolated and contained apical cytoplasmic granules (Fig. 1d). Lectin histochemistry revealed reactive sites in the glycocalyx, vacuoles and granules of these cells.

The lectin binding profiles of epithelial cells lining the ductuli efferentes in the alpaca are reported in Table 2.

Lectin histochemistry of type I epithelium

Con-A was initially negative. Binding sites for Con-A appeared in PCs (glycocalyx, apical granules Golgi area and basal cytoplasm) and CCs after both deglycosylation procedures (Fig. 2a). UEA-I (Fig. 3a) and LTA lectins stained PCs (all levels) and CCs only after PNGase-F pre-digestion. WGA binding sites were observed in PCs (all levels) after both deglycosylation procedures (Fig. 4a) and in the glycocalyx of CCs but only after PNGase-F pre-digestion. GSA-II reacted in the glycoclayx of CCs and in the Golgi zone of PCs. ß-elimination reaction decreased the staining of the Golgi area and it positively stained the glycocalyx and the apical granules of PCs (Fig. 5a), whereas PNGase-F abolished the reactivity in CCs and PCs (Fig. 5b). Sialidase digestion, preceded or not by saponification with KOH, did not modify WGA and GSA-II staining. SBA labelling sites were found in a subgroup of CCs and in PCs (all levels) (Fig. 6a) and they disappeared after ß-elimination; PNGase-F pre-digestion, sialidase and KOH/sialidase did not modify SBA results. DBA and PNA (Fig. 7a) reacted only after the removal of sialyl residues in PCs (all levels) and CCs. ECA signals were observed at all levels of PCs and CCs. ß-elimination caused the total disappearance of ECA labelling, whereas PNGase-F decreased the CCs and PCs (Golgi area) positive staining. GSA-IB4 stained both CCs and PCs at all levels (Fig. 9a). ß-elimination

abolished the above staining, except the glycocalyx of a subgroup of CCs (Fig. 9b), whereas PNGase did not modify GSA-IB4 binding pattern. MAL-II and SNA (Fig. 10a) reacted only after both deglycosylation procedures in CCs and PCs.

Lectin histochemistry of type II epithelium

Con-A showed a reaction after both deglycosylation pre-treatments in PCs (glycocalyx and apical granules; the majority of vacuoles showed a moderate positivity but some of them remained unstained) (Fig. 2b) and in CCs. UEA-I labelling appeared after ß-elimination in PCs (glycocalyx and apical granules) (Fig. 3b) and after PNGase-F in PCs (glycocalyx, vacuoles and apical granules) (Fig. 3c) and in CCs. LTA was always negative. WGA binding sites were detected after ß-elimination in PCs (at all levels) and in CCs and after PNGase-F pre-digestion in the glycocalyx of PCs and CCs. GSA-II reacted in PCs (glycocalyx and apical granules); this positivity was not modified by ß-elimination, whereas it was abolished by PNGase-F pre-

Table 2. Cytologic binding of lectins to the ductuli efferentes epithelial cells in the alpaca.

Lectins and treatments	Type I epithelium					Type II epithelium				
	Ciliated cells		*Principal cells*			*Ciliated cells*		*Principal cells*		
	GLY[a]	CY[b]	GLY[a]	GOLGI	ACG[c]	GLY[a]	CY[b]	GLY[a]	CV[d]	ACG
Con-A	-	-	-	-	-	-	-	-	-	-
ß-elimination-Con-A	++	++	+++	++[e]	+++	+++	++	+++	-/++[g]	+++
PNGase F-Con-A	++	+	++	++[e]	+++	+++	++	+++	-/++[g]	+++
UEA-I	-	-	-	-	-	-	-	-	-	-
ß-elimination-UEA-I	-	-	-	-	-	-	-	++	-	++
PNGase F-UEA-I	+	+	+	+++	++	+	+	++	++	++
LTA	-	-	-	-	-	-	-	-	-	-
ß-elimination-LTA	-	-	-	-	-	-	-	-	-	-
PNGase F-LTA	+	+	+	+++	++	-	-	-	-	-
WGA, Sialidase/WGA	-	-	-	-	-	-	-	-	-	-
ß-elimination-WGA	-	-	+++	++	+++	+++	++	+++	+	+++
PNGase F-WGA	+	-	+++	++	+++	++	-	++	-	-
GSA-II, Sialidase/GSA-II	++	-	-	++	-	-	-	++	-	++
ß-elimination-GSA-II	++	-	+++	+	+++	-	-	++	-	++
PNGase F-GSA-II	-	-	-	-	-	-	-	-	-	-
SBA, Sialidase/SBA	-/+++[f]	++	++	++	++	-/+++[f]	-	-	-	-
ß-elimination-SBA	-	-	-	-	-	-	-	-	-	-
PNGase F-SBA	-/+++[f]	-	++	++	++	-/+++[f]	-	+	-/+[g]	+
DBA	-	-	-	-	-	-	-	-	-	-
ß-elimination-DBA	-	-	-	-	-	-	-	-	-	-
PNGase F-DBA	-	-	-	-	-	-	-	-	-	-
Sialidase-DBA	+++	+	+++	+++	+++	+++	+	+++	+	+++
PNA	-	-	-	-	-	-	-	-	-	-
ß-elimination-PNA	-	-	-	-	-	-	-	-	-	-
PNGase F-PNA	-	-	-	-	-	-	-	-	-	-
Sialidase/PNA	++	+	+++	+++	+++	+++	+	+++	-/+[g]	+++
ECA, Sialidase/ECA	+++	++	+	+++	+	+++	+	+++	++	+++
ß-elimination-ECA	-	-	-	-	-	-	-	-	-	-
PNGase F-ECA	+	+	+	++	+	+++	+	+++	++	+++
GSA-IB4, sialidase/GSA-IB4	++	++	++	++	++	+++	-	++	-	++
ß-elimination-GSA-IB4	-/+++[f]	-	-	-	-	-/+++[f]	-	-	-	-
PNGase F-GSA-IB4	++	++	++	++	++	-	-	+	-	+
MAL-II	-	-	-	-	-	-	-	-	-	-
ß-elimination-MAL-II	+	+	+++	+	+++	+++	+	+++	++	+++
PNGase F-MAL-II	+	+	+++	+	+++	+++	+	+++	++	+++
SNA	-	-	-	-	-	-	-	-	-	-
ß-elimination-SNA	++	+	+++	++	+++	++	+	+++	++	++
PNGase F-SNA	+++	+	+++	++	++	++	+	++	++	++

(+) and (-) indicate staining intensity on a subjective scale that attributes (-) to negative reaction and (+++) to strong reaction. [a]GLY = glycocalyx; [b]CY = cytoplasm; [c]ACG = apical cytoplasmic granules; [d]CV = cytoplasmic vacuoles. [e]Binding sites appeared in the supranuclear and basal cytoplasm. [f]Only a subgroup of CCs were strongly reactive. [g]Vacuoles resulted positive or negative.

digestion. Sialidase degradation, also preceded by deacetylation with KOH, did not affect WGA and GSA-II results. SBA signals were demonstrated only in the glycocalyx of a subgroup of CC, and ß-elimination abolished this staining; PNGase-F treatment did not modify the reactivity of CCs and it determined SBA positivity in the glycocalyx, apical granules and numerous vacuoles of PCs (Fig. 6b). Sialidase and KOH/sialidase failed to influence SBA results. DBA and PNA (Fig. 7b) binding sites were discovered after sialidase digestion in both PCs (at all levels) and in CCs; saponification with KOH did not change the staining

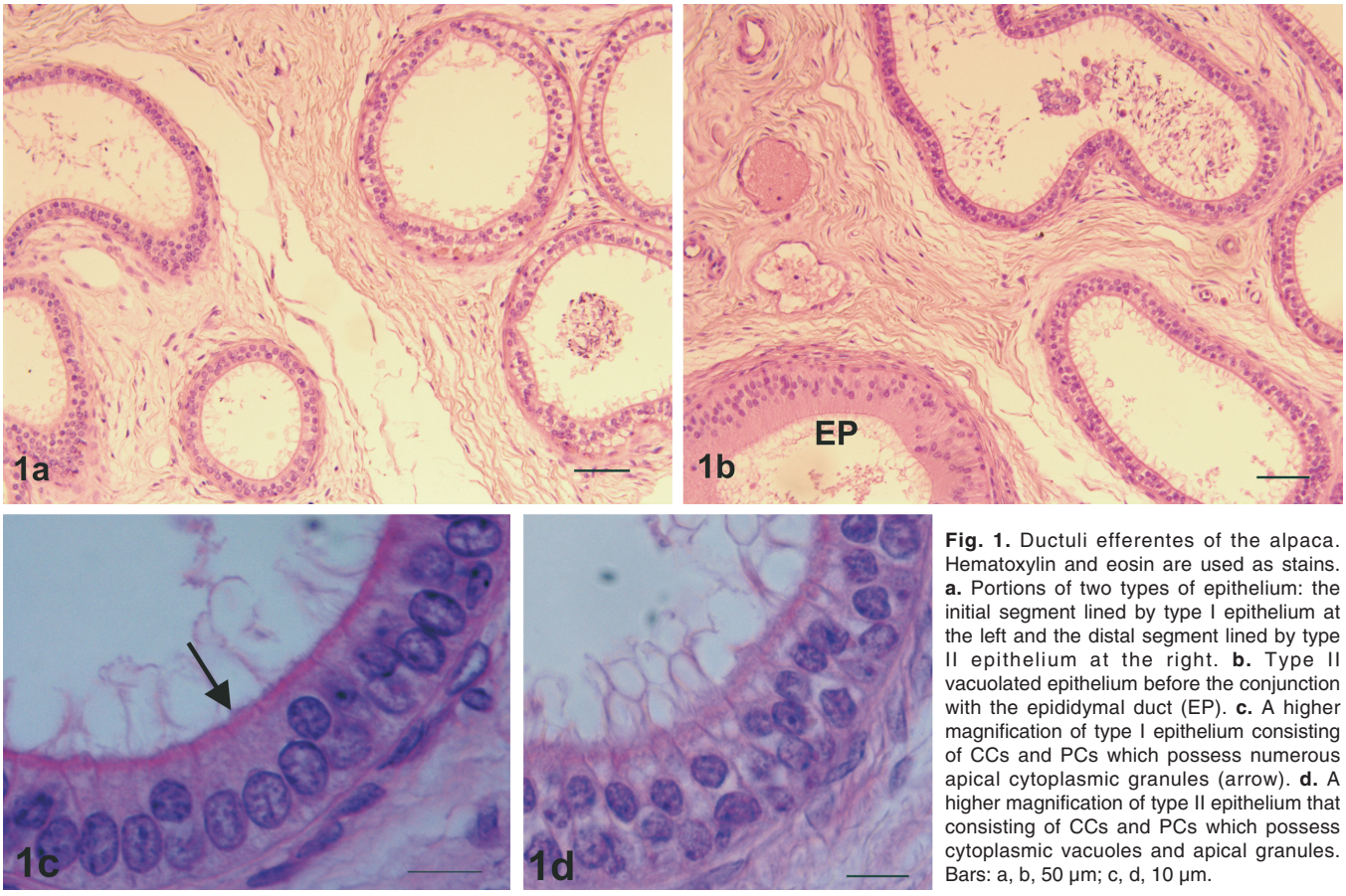

Fig. 1. Ductuli efferentes of the alpaca. Hematoxylin and eosin are used as stains. **a.** Portions of two types of epithelium: the initial segment lined by type I epithelium at the left and the distal segment lined by type II epithelium at the right. **b.** Type II vacuolated epithelium before the conjunction with the epididymal duct (EP). **c.** A higher magnification of type I epithelium consisting of CCs and PCs which possess numerous apical cytoplasmic granules (arrow). **d.** A higher magnification of type II epithelium that consisting of CCs and PCs which possess cytoplasmic vacuoles and apical granules. Bars: a, b, 50 µm; c, d, 10 µm.

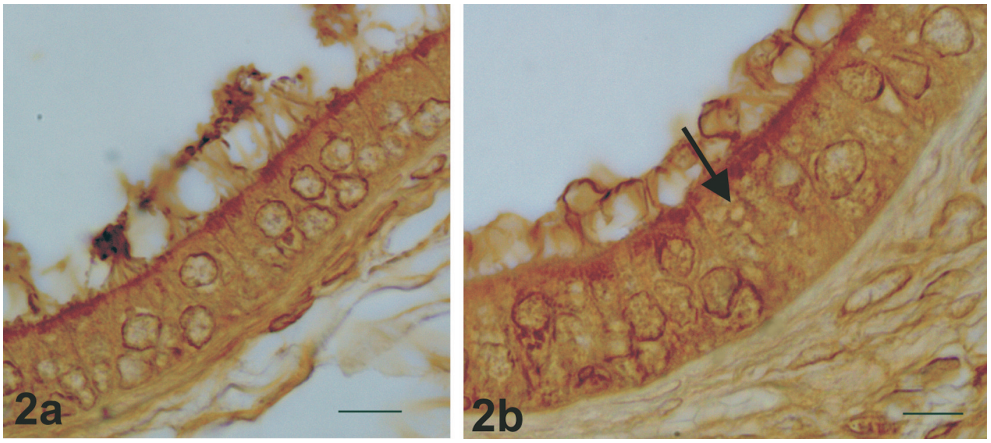

Fig. 2. a. Type I epithelium, ß-elimination/Con-A. The binding sites are localized in PCs (glycocalyx, apical granules, supranuclear and basal cytoplasm) and in CCs. **b.** Type II epithelium, ß-elimination/Con-A. In PCs the glycocalyx and apical granules strongly react, whereas the vacuoles are moderately stained. Note the negativity of some vacuoles (arrow). Bars: 10 µm.

obtained with sialidase/DBA-PNA sequences. ECA affinity was observed in PCs (at all levels) and in CCs (Fig. 8c); ß-elimination abolished the above reactivity, whereas PNGase-F pre-digestion did not affect ECA labelling. GSA-IB4 reactivity was localized in PCs (glycocalyx and apical granules but not in vacuoles) and in the glycocalyx of CCs (Fig. 9c); ß-elimination determined the abolition of the above staining, except the glycocalyx of a subgroup of CCs, whereas PNGase-F pre-digestion decreased GSA-IB4 affinity in PCs (glycocalyx and apical granules). Sialidase digestion, preceded or not by saponification, did not affect ECA and GSA-IB4 stainings. MAL-II and SNA were initially negative; both deglycosylation procedures produced reactivity in PCs (Fig. 10b) and in CCs.

In this type of epithelium, lectin histochemistry

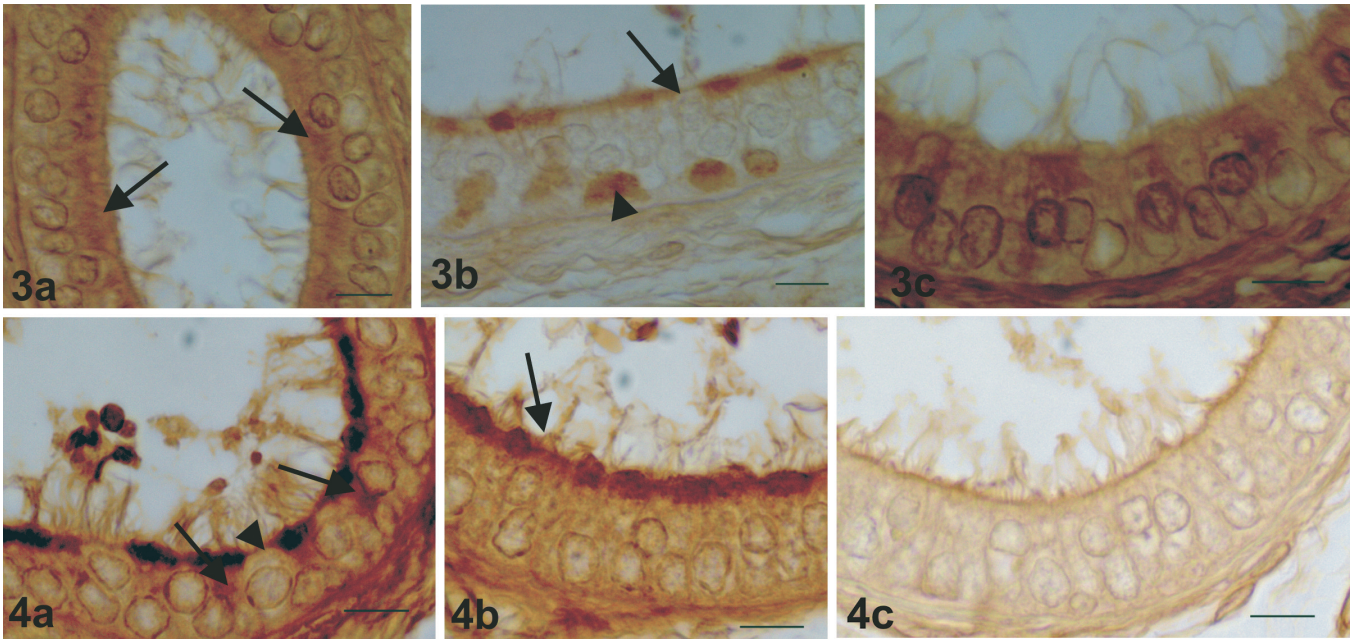

Fig. 3. a. Type I epithelium, PNGase F/UEA-I. PCs display a positive reaction at the glycocalyx, apical granules and Golgi area levels (arrows). **b.** Type II epithelium, ß-elimination/UEA-I. Labelling sites are localized in the glycocalyx and apical granules of PCs, whereas the vacuoles are unreactive. CCs are unstained (arrow); the IEMs display a moderate positivity (arrowhead). **c.** Type II epithelium, PNGase F/UEA-I. After PNGase-F digestion PCs show reactive sites in the glycocalyx, vacuoles and granules. Bars: 10 µm.

Fig. 4. a. Type I epithelium, ß-elimination/WGA. PCs showing a strong reaction in the glycocalyx and apical granules and a moderate reaction in the Golgi zone (arrows). CCs are negative (arrowhead). **b.** Type II epithelium, ß-elimination/WGA. Reactive sites are localized strongly in the glycocalyx and apical granules and weakly in the vacuoles; CCs also react (arrow). **c.** Type II epithelium, PNGase F/WGA. PCs show a moderate staining only in the glycocalyx, whereas vacuoles and apical granules remain unstained. Bars: 10 µm.

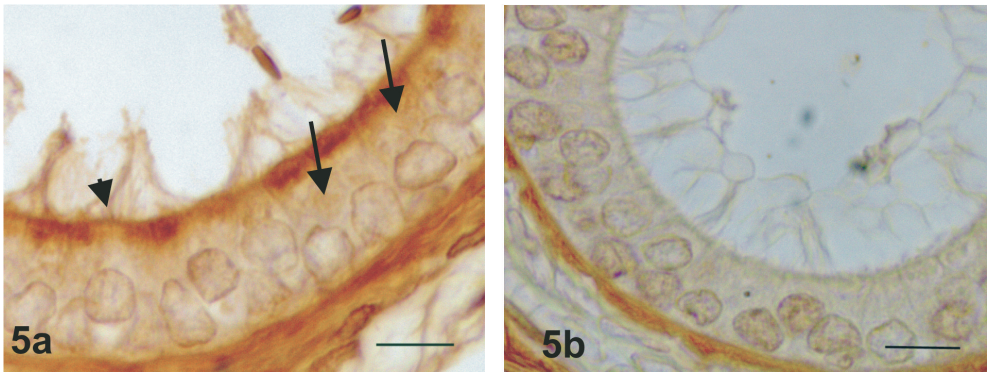

Fig. 5. a. Type I epithelium, ß-elimination/GSA-II. CCs display moderate staining in the glycocalyx (arrowhead) whereas PCs show a reaction which is strong in the glycocalyx and apical granules and weak in the Golgi area (arrows). **b.** Type I epithelium, PNGase F/GSA-II. The staining is abolished in both CCs and PCs. Bars: 10µm.

revealed the appearance of large and irregular intra-epithelial masses (IEMs) occupying the basal zone of the epithelium. In particular, they stained positive with ß-elimination/UEA-I (Fig. 3b), -/SBA, -/ECA sequences, sialidase/PNA (Fig. 7b), GSA-IB4 (Fig. 9c), and with Con-A, MAL-II and SNA (Fig. 10b) after both deglycosylation pre-treatments.

Immunohistochemistry

The distribution pattern of the intermediate filaments revealed topographic differences between type I and II epithelium. In type I epithelium, the entire cytoplasm of PCs were immunolabelled strongly with AE1/AE3 and moderately with 13-16 CK antibodies. In particular, the reaction product was very strongly localized in the apical and baso-lateral regions of the cells (Fig. 11a,b). In type II epithelium, immunostaining using AE1/AE3 antibodies was moderate in PCs along the plasma-membrane (apical, lateral and basal) and at times a strong reactivity was observed in the upper half of the lateral plasma membrane; their cytoplasm was negative or very weakly stained (Fig. 11c). CK 13-16 antisera did not react in type II PCs (Fig. 11d) whereas CK 5 and 18 antibodies gave negative results in both types of PCs. CCs weakly stained with CK AE1/AE3 antibodies in both types of epithelia and with CK 13-16 in type I epithelium (Fig. 11a-d).

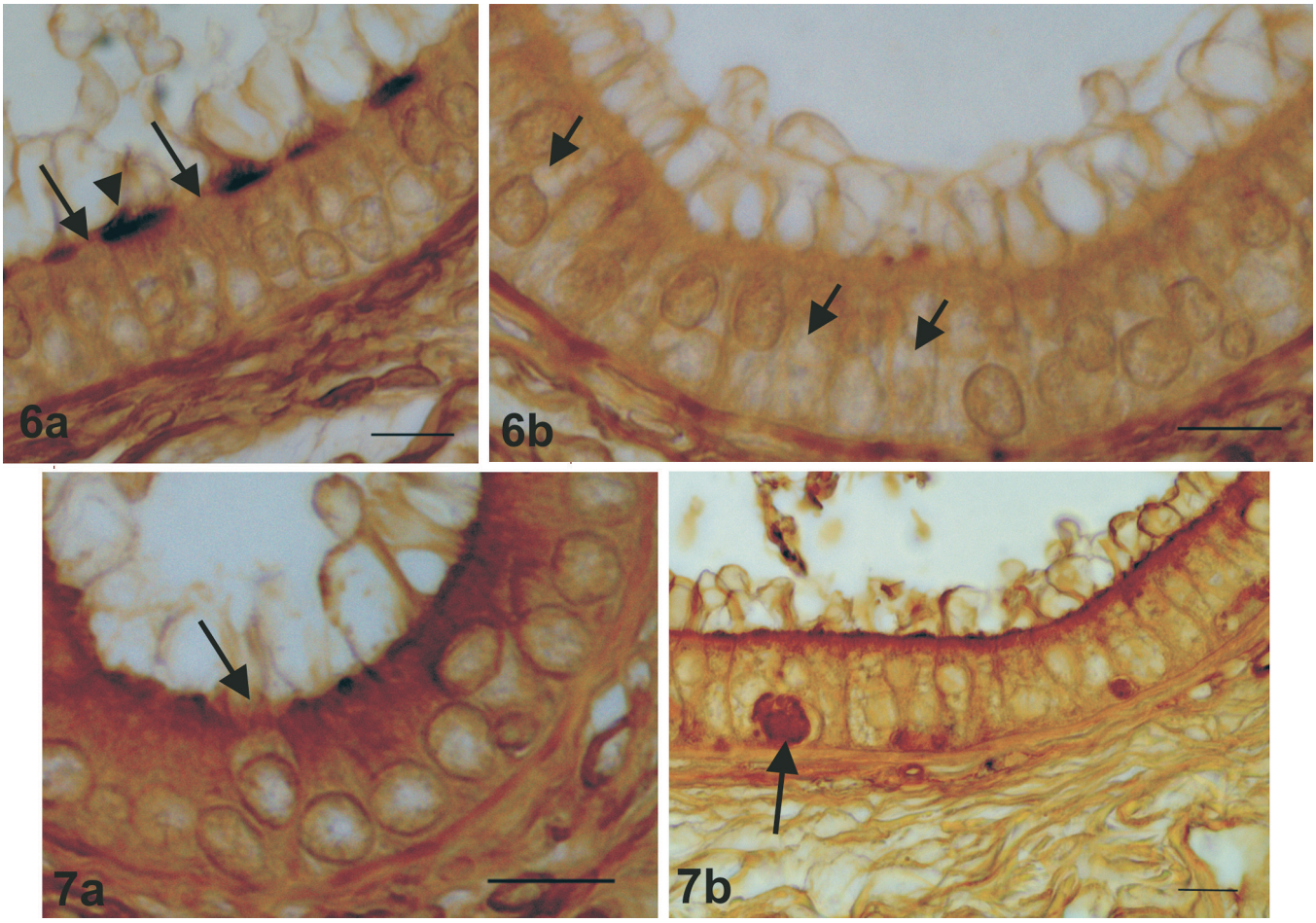

Fig. 6. a. Type I epithelium, SBA staining. The reactive sites are strongly localized in the glycocalyx and moderately in the cytoplasm of a subgroup of CCs (arrowhead); PCs show a moderate reaction in the glycocalyx, apical granules and supranuclear cytoplasm (arrows). **b.** Type II epithelium, PNGase F/SBA. The treatment results in SBA positivity in the glycocalyx, apical granules and numerous vacuoles of PCs; note that some vacuoles (arrows) remain negative. Bars: 10 µm

Fig. 7. a. Type I epithelium, sialidase/PNA sequence. PCs show a strong staining in the glycocalyx, Golgi area and apical granules; CCs display moderate staining in the glycocalyx and weak in the cytoplasm (arrow). **b.** Type II epithelium, sialidase/PNA sequence. PCs exhibit a strong reaction in the glycocalyx and apical granules, whereas the vacuoles are negative or weakly stained. The IEMs react strongly (arrow). Bars: 10 µm.

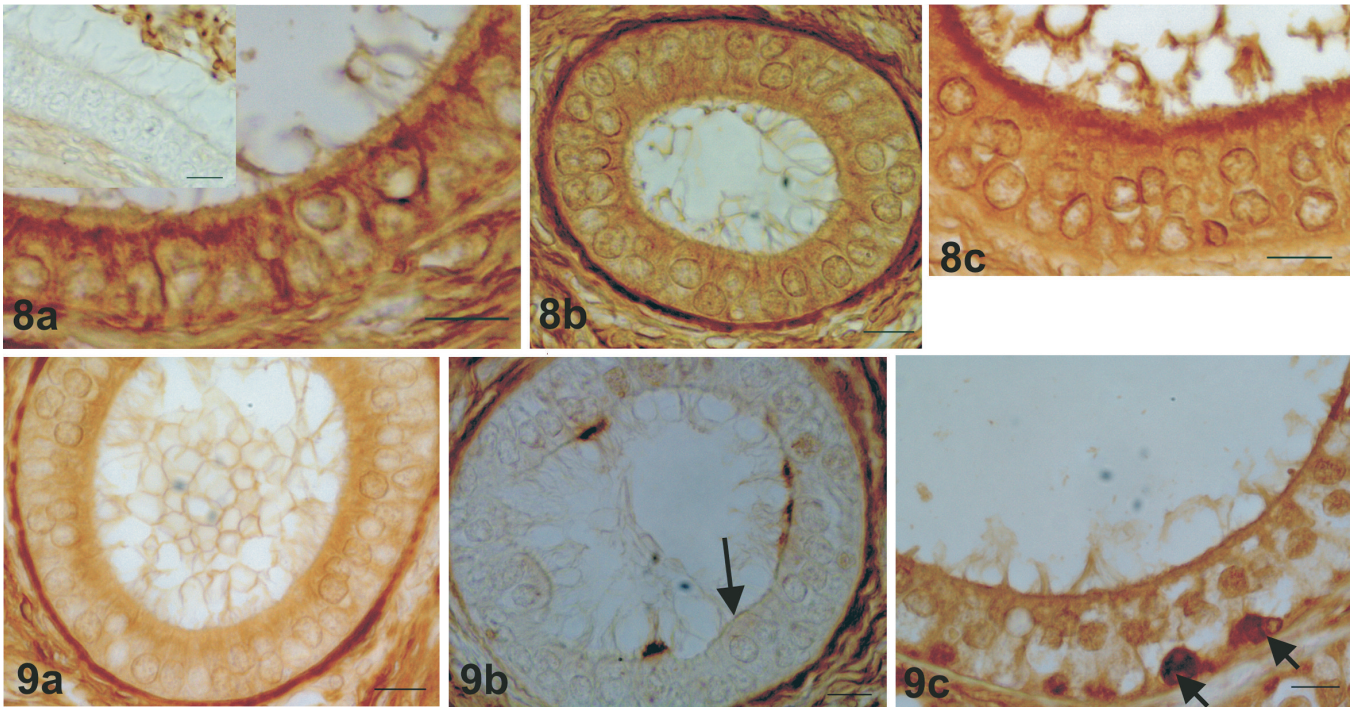

Fig. 8. a. Type I epithelium, ECA staining. PCs show a positivity which is weak in the glycocalyx and apical granules and strong in the Golgi area; the staining is abolished after ß-elimination (insert). **b.** Type I epithelium, PNGase F/ECA. The treatment causes a decrease of the reactivity in the Golgi area of PCs. **c.** Type II epithelium, ECA staining. Binding sites are localized strongly within the glycocalyx and apical granules and less so within the vacuoles of PCs. Bars: 10 µm.

Fig. 9. a. Type I epithelium, GSA-IB4 staining. PCs exhibit a moderate reaction within the glycocalyx, apical granules and Golgi area. **b.** Type I epithelium, ß-elimination/GSA-IB4 staining. A subgroup of CCs display a strong reaction in the glycocalyx, whereas other CCs are negative (arrow). **c.** Type II epithelium, GSA-IB4 staining. PCs show a moderate reaction of the glycocalyx and apical granules whereas vacuoles remain negative. Note the strong staining of the IEMs (arrows). Bars: 10µm.

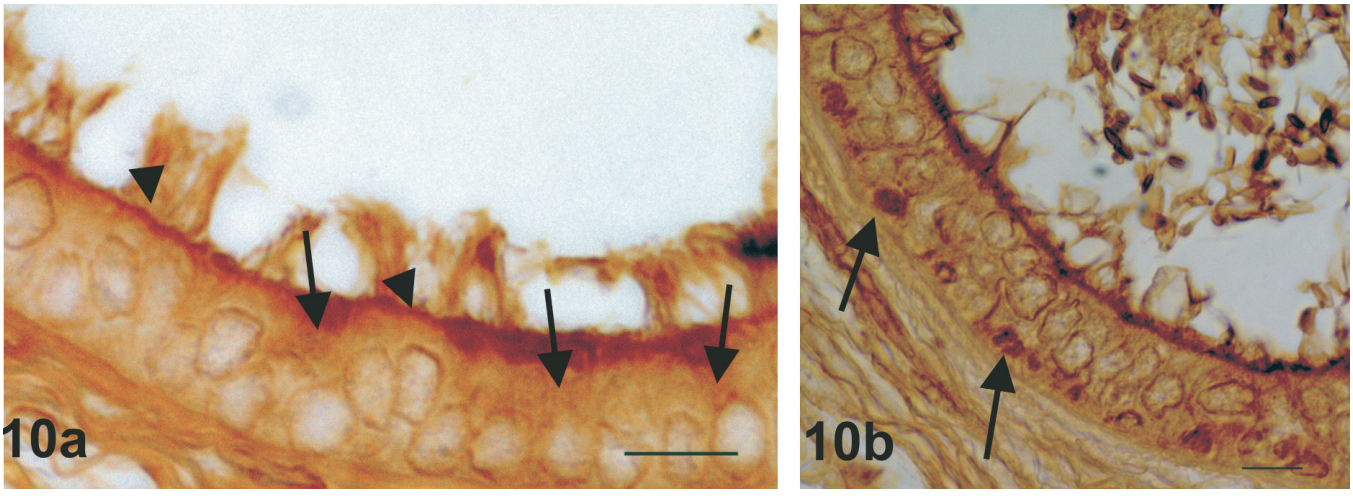

Fig. 10. a. Type I epithelium, ß-elimination/SNA. PCs display a strong reactivity within the glycocalyx and apical granules and moderate reactivity in the supranuclear cytoplasm (Golgi area, arrows); CCs show moderate staining of the glycocalyx and weak staining of the cytoplasm (arrowheads). **b.** Type II epithelium, ß-elimination/SNA. PCs show a strong positivity within the glycocalyx and moderate positivity of the apical granules and cytoplasmic vacuoles. The IEMs are strongly stained (arrows). Bars: 10 µm.

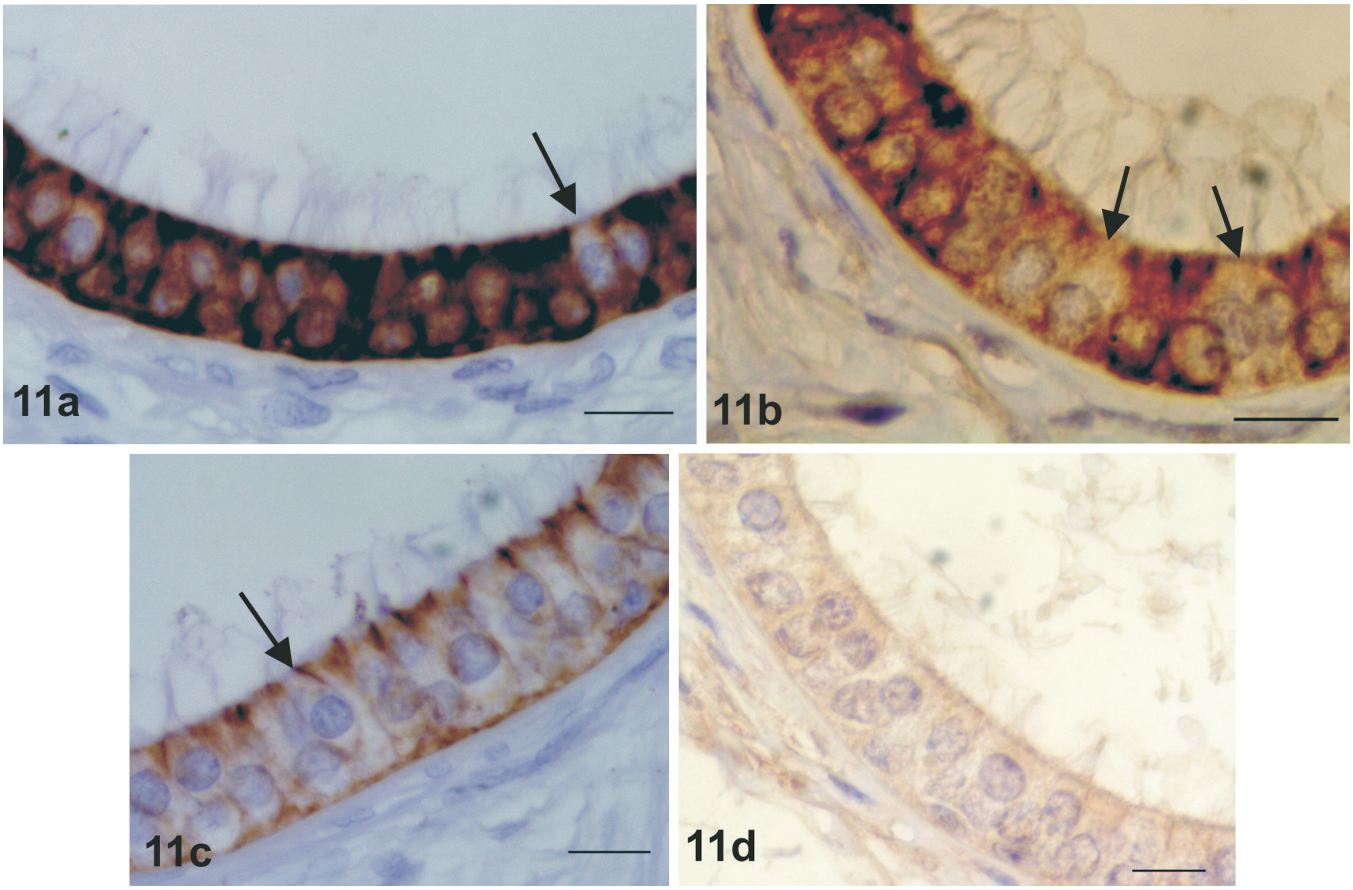

Fig. 11. Type I epithelium, immunohistochemical demonstration of AE1/AE3 **(a)** and 13-16 cytokeratins **(b)**. Immunoreaction in PCs is strong and moderate, respectively, throughout the cytoplasm and very strong in the apical, basal and lateral cytoplasm. CCs are weakly immunostained (arrows). Type II epithelium, immunohistochemical demonstration of AE1/AE3 **c)** and 13-16 cytokeratins **d)**. **c.** PCs immunoreact along the plasma-membrane (apical, lateral and basal) and some of them show a strong reactivity in the upper half of the lateral plasma membrane; their cytoplasm was negative or very weakly stained. CCs are weakly stained (arrows). **d.** No immunoreaction is observed in the epithelial cells. Bars: 10 µm.

Staining control

All the control staining procedures failed to disclose appreciable reactivity at any of the above sites.

Discussion

From the pattern of expression of lectins and intermediate filaments, we have been able to discern two types of PCs in the ductuli efferentes of the alpaca.

In the type I PCs, the main structure that was reactive with all lectins used in this study was the supranuclear region, which corresponded to the Golgi area, suggesting that these cells were actively involved in the synthesis and secretion of glycoconjugates. Deglycosylation methods which removed O- and N-linked oligosaccharides exposed masked carbohydrates and helped us in the detailed elucidation of oligosaccharidic sequences of these glycomolecules. In particular, since the removal of either O-linked or N-linked oligosaccharides unmasked Con-A labelling, α-D-Man/α-D-Glc residues could be included in both types of glycans. Although the presence of the above sugar in O-linked oligosaccharides is considered unusual, it has been previously demonstrated in other tissues (Madrid et al., 2000). In addition, the occurrence of Con-A positive sites in the basal cytoplasm probably corresponded to the binding at the cisternae of the rough endoplasmic reticulum (RER). This finding agreed with the ultrastructural labelling of this cytoplasmic site by Con-A lectin (Chan and Wong, 1992). In fact, it has been reported that mannose moieties present in the RER may be associated with the initial assembly of the N-linked oligosaccharides of glycoproteins, which is completed in the Golgi stack. The Fuc moieties, recognized by UEA-I and LTA, were included in O-linked oligosaccharides; the above sugar residues were masked by N-linked glycans and, thus, they were labelled to the lectins after their removal. The result obtained with LTA disagreed with the well known specificity of this lectin for α-L-

Fuc (Menghi et al., 1989). Indeed, these authors reported that LTA preferentially binds to fucose α 1,4 linked to GlcNAc. However, the presence of fucose in O-linked oligosaccharides recognized by LTA was also reported in other studies (Leis et al., 1997). The fact that WGA was negative, but that the cleavage of both N- or O-linked oligosaccharides rendered the Golgi area positive, indicated the synthesis of internal GlcNAc in N- and O-linked glycans. The same carbohydrate also occurred in terminal position included in N- and O-linked oligosaccharides, as demonstrated by the decrease of GSA-II reactivity after ß-elimination and the abolition of the staining after PNGase-F pre-digestion. SBA and GSA-IB4 reactivity were indicative of the occurrence of terminal α-GalNAc and α–Gal moieties, respectively, in the Golgi zone; ß-elimination abolished their labelling, thus suggesting the presence of the above carbohydrates in O-linked oligosaccharides. The result obtained with ECA lectin indicated the occurrence of terminal ß-D-Gal-(1-4)-D-GlcNAc in both O- and N- linked oligosaccharides. The fact that the Golgi area rendered positive to sialic acid labelling lectins only after removal of N- and O-linked oligosaccharides suggests that these chains block the access of MAL-II and SNA to Neu5Acα2,3Gal and Neu5Acα2,6Gal/GalNAc sugar sequences, respectively, and that these sequences belong to both N- and O-linked glycans. The acceptor sugars for sialic acid were α-GalNAc and the disaccharide ß-D-Gal-(1-3)-D-GalNAc as demonstrated by DBA and PNA positivity after sialidase degradation.

The lectin affinity of the apical cytoplasmic granules observed in type I PCs might represent the staining of the above secretory glycoconjugates preceding their extrusion. The correspondence of the lectin binding pattern between the apical granules and the Golgi area of these cells supports our hypothesis. In ultrastructural studies carried out in the rat ductuli efferentes, it has been argued that although the PCs are probably secretory, the Golgi apparatus does not exhibit typical secretory granules as observed in glandular tissues (Rambourg et al., 1987). Additionally, in the alpaca ductuli efferentes we did not note apical protrusions in PCs and secretory blebs into the lumen that are indicative of an apocrine mode of secretion as evidenced in different male accessory sex glands (Chan and Ho, 1999; Parillo and Verini Supplizi, 2008). However, the appearance of apocrine-like secretion in PCs of the ductuli efferentes has been reported in different mammals and birds (Ilio and Hess, 1994).

In type II PCs the Golgi area appeared to show no evidence of lectin labelling, contrary to what has been observed in vacuoles and in the apical cytoplasmic granules. In particular, apical granules expressed Glu/Man and Fuc moieties, recognized by Con-A and UEA-I respectively, in both N- and O-linked oligosaccharides. Internal GlcNAc residues identified by WGA were included in N-linked glycans crypted by O-linked chains, whereas terminal GlcNAc visualized by GSA-II belonged to N-linked oligosaccharides. Terminal ß-GalNAc and ß-D-Gal-(1-4)-D-GlcNAc were included in O-linked oligosaccharides, as shown by SBA and ECA positivities respectively, whereas ß-Gal moieties, detected by GSA-IB4, occurred in both O- and N-linked glycans. Additionally, sialic acid linked either via α2,3-6 to the penultimate ß-Gal or via α2,6 to subterminal α-GalNAc was detected in the apical granules, as demonstrated by the results obtained with MAL-II-, SNA-, DBA- and PNA-lectins. The cytoplasmic vacuoles contained a very similar sugar sequence to that described above in the cytoplasmic granules. In our opinion the lectin-stained material which we observed in the vacuoles and apical granules of type II PCs can be interpreted as representing the staining of glycoprotein material taken up from the lumen. The granules may correspond to the numerous pinocytotic vesicles that are components, as well as vacuoles, of an elaborate endocytotic apparatus specialized for the uptake of particulate material and fluid from the lumen, as previously reported in rat, (Robaire and Hermo, 1988), boar (Stoffel and Friess, 1994) and horse (Arrighi et al., 1994). Conversely, in bull, goat and dog the granules and vacuoles, presumably of Golgi origin, are thought to contain secretory products of the cells (Goyal and Williams, 1988; Ilio and Hess, 1994; Wakui et al., 1996).

The marked differences in the cellular localization of keratin filaments between two types of PCs further suggests the possibility of different specific functions along the efferent ductules. The expression of AE1/AE3 keratins mainly in the upper lateral half and in basal plasmalemma of type II PCs could be related to the conspicuous presence at this level of cytoskeletal structures typical of cells subjected to mechanical forces caused by the intense adsorptive process, as reported in the PCs of the human ductuli efferentes (Regadera et al., 1993).

The intra-epithelial masses containing lectin labelled material that we observed only in the basal portion of the type II epithelium could correspond to the presence of lipofuscinic-like material, further testifying to the absorptive function of type II PCs in the alpaca. This finding is in agreement with ultrastructural observations performed in equine ductuli efferentes (Arrighi et al., 1994). These authors showed epithelial cells with cytoplasm filled with lipofuscinic-like material, which sometimes contained fragments of spermatozoa, suggesting that PCs were also involved in spermatophagic processes.

Conversely, in type I PCs, the intense expression of AE1/AE3 and 13-16 CKs throughout their entire cytoplasm may be interpreted as a cytoskeletal adaptation in response to secretory processes. Indeed, it is well known that CK 13-16 is expressed as a major component of non-keratinised epithelia, including those with secretory activity. CKs 5 and 18 were undetectable in the alpaca efferent ductules but they were demonstrated in man (Achtstatter et al., 1985), whereas

in dog the above CKs were not detected in PCs but CK 18 was expressed in CCs (Wakui et al., 1994). Taken together, this data indicates that the epithelia of efferent ducts differ in their cytoskeletal feature between different species.

In our opinion, the intraepithelial differences we observed in PCs should not represent different phases of the cycle because the two types of PCs are not present throughout the length of the efferent ductules similarly to what has been reported in goat (Goyal et al., 1992). Furthermore, the present study demonstrated that the two types of PCs have a peculiar cytoskeletal and lectin labelling pattern supporting the abovementioned hypothesis that these cells are independent cells in agreement with the results obtained in dogs (Wakui et al., 1996).

In the alpaca, the cytoplasm of CCs reveal a weak immunoreaction to cytokeratins antisera, and also displayed a lectin binding pattern. Some lectins, such as Con-A, UEA-I and ECA similarly stained CCs in both types of epithelium, whereas other lectins reacted differently. Indeed, LTA and GSA-IB4 positive sites were localized in CCs of type I epithelium, whereas SBA, sialidase/DBA and sialidase/PNA reactivity was demonstrated in those of type II epithelium. As the CCs are not known to secrete macromolecules, it is possible that the lectin staining material detected in their cytoplasm could correspond to synthesized structural glycoproteins (Arenas et al., 1996). Moreover, the finely granular material which reacted with different lectins could also be attributed to the vesicular elements, considering that these cells are involved in both fluid phase and adsorptive endocytosis (Ilio and Hess, 1994).

The reactivity to different classes of lectins indicates the complexity and diversity of the glycocalyx in the two types of epithelium of ductuli efferentes. The conspicuous presence of oligosaccharidic chains at this level could be ascribed to the expression of several glycoproteins acting as anion exchangers with different specificity, mainly localized in the apical border of PCs in the human efferent ducts (Kujala et al., 2007). Additionally, the authors argued that some of these anion transporters, such as NHERF-1, were also detected in the apical edge of a subgroup of CCs. In this regard, it is interesting to note that in the alpaca, a subpopulation of CCs in both types of epithelia reacted strongly with SBA and ß-elimination/GSA-IB4 sequence.

The above anion exchangers, regulating fluid and electrolyte absorption, provide an accurate control of intraluminal pH and HCO_3^- concentration, essential for maturation of sperm and, thereby, for male fertility (Kujala et al., 2007). We do not exclude the possible role of estrogen in modulating and regulating fluid and ion resorption as previously demonstrated in the rat (Ilio and Hess, 1994).

In summary, we provide evidence of the existence of two epithelial segments with different cytokeratin and lectin histochemical composition along the ductuli efferentes of the alpaca, suggesting a high degree of compartmentalization of the secretory and absorptive activities.

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