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Histochemical analysis of glycoconjugates in the domestic cat testis

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Summary. The localization and characterization of oligosaccharide sequences in the cat testis was investigated using 12 lectins in combination with the ß-elimination reaction, N-Glycosidase F and sialidase digestion. Leydig cells expressed O-linked glycans with terminal αGalNAc (HPA reactivity) and N-glycans with terminal/internal αMan (Con A affinity). The basement membrane showed terminal Neu5Acα2,6Gal/GalNAc, Galß1,3GalNAc, α/ßGalNAc, and GlcNAc (SNA, PNA, HPA, SBA, GSA II reactivity) in O-linked oligosaccharides, terminal Galß1,4GlcNAc (RCA_{120} staining) and αMan in N-linked oligosaccharides; in addition, terminal Neu5Acα2,3Galß1,4GlcNac, Forssman pentasaccharide, αGal, αL-Fuc and internal GlcNAc (MAL II, DBA, GSA I-B_4, UEA I, KOH-sialidase-WGA affinity) formed both O- and N-linked oligosaccharides. The Sertoli cells cytoplasm contained terminal Neu5Ac-Galß1,4GlcNAc, Neu5Ac-ßGalNAc as well as internal GlcNAc in O-linked glycans, αMan in N-linked glycoproteins and terminal Neu5Acα2,6Gal/GalNAc in both O- and N-linked oligosaccharides. Spermatogonia exhibited cytoplasmic N-linked glycoproteins with αMan residues. The spermatocytes cytoplasm expressed terminal Neu5Acα2,3Galß1,4 GlcNAc and Galß1,3GalNAc in O-linked oligosaccharides, terminal Galß1,4GlcNAc and α/ßGalNAc in N-linked glycoconjugates. The Golgi region showed terminal Neu5acα2,3Galß1,4GlcNac, Galß1,4GlcNAc, Forssman pentasaccharide, and αGalNAc in O-linked oligosaccharides, αMan and terminal ßGal in N-linked oligosaccharides. The acrosomes of Golgi-phase spermatids expressed terminal Galß1,3GalNAc, Galß1,4GlcNAc, Forssmann pentasaccharide, α/ßGalNAc, αGal and internal GlcNAc in O-linked oligosaccharides, terminal α/ßGalNAc, αGal and terminal/internal αMan in N-linked glycoproteins. The acrosomes of cap-phase spermatids lacked internal Forssman pentasaccharide and αGal, while having increased α/ßGalNAc. The acrosomes of elongated spermatids did not show terminal Galß1,3GalNAc, displayed terminal Galß1,4GlcNAc and α/ßGalNAc in N-glycans and Neu5Ac-Galß1,3GalNAc in O-linked oligosaccharides.

Key words: Testes, Glycoconjugates, Lectin histochemistry, Domestic cat

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Introduction

Male germ cells undergo many morphological and molecular changes during spermatogenesis. Such changes also regard the glycoprotein oligosaccharide chains which play important roles during spermatogenesis (Anakwe and Gerton, 1990; Martinéz-Menárguez et al., 1992; Akama et al., 2002) and fertilization (de Cerezo et al., 1996; Navaneetham et al., 1996)

Lectins have a specific binding affinity for the sugar residues of glycoconjugates, therefore they are a useful tool for investigating glycoconjugate distribution as well as cell differentiation and maturation (Spicer and Schulte, 1992). Among the carbohydrates that constitute the oligosaccharide chains in glycoproteins, sialic acids are known to be a large family of nine-carbon carboxylated sugars that usually occupy the terminal position of the oligosaccharide chains in a variety of glycoconjugates (Schauer, 1982). Sialic acids are also known to act as ligands in recognition phenomena (Varki, 1997), in *in vitro* sperm capacitation (Banerjee and Chowdhury, 1994; Focarelli et al., 1995) and in sperm-egg interaction (Geng et al., 1997). The oligosaccharide chains of glycoproteins have been classified into two families: N- and O-linked oligosaccharides. The first group is characterized by a

reducing terminal N-acetylglucosamine (GlcNAc) N-glycosidically bound to asparagine. The O-linked (mucin-type) oligosaccharides contain classically a reducing terminal N-acetylgalactosamine (GalNAc) linked O-glycosidically to serine or threonine. These aminoacidic residues can also link fucose (Fuc), glucose (Glc), mannose (Man), and N-acetylglucosamine (GlcNAc) to form other classes of O-linked glycans (reviewed in Lowe and Marth, 2003).

Lectin histochemistry has been successfully used to demonstrate the intracellular localization of the sugar residues as well as to evaluate the composition of the oligosaccharides in the testes of many mammals such as rodents (Arya and Vanha-Perttula 1984; Lee and Damjanov, 1984; Burket et al., 1987; Jones et al., 1992a,b, 1993; Kurohmaru et al., 1995, 1996; Pastor et al., 2003), domestic animals (Töpfer-Petersen et al., 1984; Arya and Vanha-Perttula, 1985; Kurohmaru et al., 1991; Calvo et al., 2000, Verini-Supplizi et al., 2000; Pinart et al., 2001), humans (Malmi et al., 1987, Wollina et al., 1989; Arenas et al., 1998) and other tetrapod vertebrates (Ballesta et al., 1991; Labate and Desantis, 1995; Sáez et al., 2004). In addition, the distribution of N- and O-linked oligosaccharides in germ cells has been identified in mammalian (Jones et al., 1992a,b; 1993; Martínez-Menárguez et al., 1992) and in amphibian testes (Sáez et al., 2004).

The studies on cat testis by histological means have regarded the testis structure, spermatogenic process and testis morphometry (Elock and Schoning, 1984; Sánchez et al., 1993a,b; Blanco-Rodriguez, 2002; França and Goninho, 2003; Tsutsui et al., 2004), while the *in situ* distribution of glycoconjugates had not been investigated. Therefore, the aim of the present study was to identify and localize the oligosaccharide sequences of glycoconjugates in domestic cat testis by means of the lectins most frequently used in glycohistochemistry, in combination with enzymatic and chemical treatments.

Materials and methods

Tissue preparation

The testes from four postpubertal (15-24 months old) privately owned domestic cats, submitted for routine surgical castration, were fixed in Bouin's fluid for 12 h at room temperature (RT). Following fixation, the tissues were washed and dehydrated in an ethanol series, cleared in xylene, and embedded in paraffin wax. 4-µm thick sections were cut and, after de-waxing with xylene and hydration in an ethanol series of descending concentrations, were stained by means of the following histochemical methods according to Desantis et al. (2003).

Lectin histochemistry

The lectins used are listed in Table 1. The lectins PNA, RCA_{120}, DBA, SBA, HPA, Con A, WGA, GSA-II, and UEA-I were HRP-conjugated. They were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). MAL II, SNA, and GSA I-B_4 were biotinylated lectins and were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

De-waxed and re-hydrated tissue sections were immersed in 3% (v/v) solution of H_2O_2 in methanol for 10 min to suppress the endogenous peroxidase activity, rinsed in 0.05 M Tris-HCl buffered saline (TBS) pH 7.4 and incubated in lectin solution at appropriate dilutions (Table 1) for 1 h at room temperature (RT). After 3 rinsings in TBS, the peroxidase activity of HRP-conjugated lectins was visualized by incubation in a solution containing 0.05% 3,3'-diaminobenzidine (DAB) and 0.003% H_2O_2 in 0.05 M TBS (pH 7.6) for 10 min at RT before dehydration and mounting. Tissue sections incubated in biotinylated lectins (MAL II, SNA and GSA I-B_4) were rinsed 3 times with 0.05 M phosphate-

Table 1. Lectins used, their sugar specificities and inhibitory sugars used in control experiments.

LECTIN ABBREVIATION	SOURCE OF LECTIN	CONCENTRATION (µg/ml)	SUGAR SPECIFICITY	INHIBITORY SUGAR	REFERENCE
MAL II	*Maackia amurensis*	15	Neu5acα2,3Galß1,4GlcNAc	Neu5NAc	Sata et al. 1990
SNA	*Sambucus nigra*	15	Neu5Acα2,6Gal/GalNAc	Neu5NAc	Shibuya et al. 1987
PNA	*Arachis hypogea*	20	Terminal Galß1,3GalNAc	Gal	Lotan et al. 1975
RCA_{120}	*Ricinus communis*	25	Terminal Galß1,4GlcNAc	Gal	Baenziger and Fiete 1979
SBA	*Glycine max*	15	Terminal α/ßGalNAc	GalNAc	Hammarström et al. 1977
DBA	*Dolichos biflorus*	20	Terminal FP>GalNAcα1,3GalNAc	GalNAc	Hammarström et al. 1977
HPA	*Helix pomatia*	20	Terminal αGalNAc	GalNAc	Roth 1984
Con A	*Canavalia ensiformis*	20	Terminal and internal αMan>αGlc	Mannose	Goldstein & Hayes 1978
WGA	*Triticum vulgaris*	20	Terminal and internal ßGlcNAc>>NeuNAc	GlcNAc	Debray et al. 1981
GSA I-B4	*Bandeiraea simplicifolia*	25	Terminal αGal	Galactose	Hayes & Goldstein 1974
GSA II	*Bandeiraea simplicifolia*	20	Terminal D-GlcNAc	GlcNAc	Shanker Iyer et al. 1976
UEAI	*Ulex europaeus*	25	Terminal L-Fucα1,2Galß1,4GlcNAcß	Fuc	Pereira et al. 1978

Fuc, Fucose; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; FP, Forssman pentasaccharide GalNAcα1,3GalNAcα1,3Galß1,4Galß1,4GlcNAc; Man, mannose; NeuNAc, N-acetyl neuraminic (sialic) acid.

buffered saline (PBS) and were incubated in streptavidin/peroxidase complex (Vector Lab. Inc.) for 30 min at RT. After washing in PBS, peroxidase was developed in a DAB-H_2O_2 solution as above.

Controls for lectin staining included: (1) substitution of the substrate medium with buffer without lectin; (2) incubation with each lectin in the presence of its hapten sugar (0.2-0.5 M in Tris buffer).

Enzymatic and chemical treatments

Before staining with MAL II, SNA, PNA, RCA_{120}, DBA, SBA, WGA some sections were incubated at 37°C for 16 h in 0.86 U/mg protein of sialidase (Type V, from *Clostridium perfringens*) (Sigma Chemicals Co., St. Louis, MO, USA) dissolved in 0.1 M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. Prior to the neuraminidase treatment, a saponification technique was performed to render the enzyme digestion effective, with 0.5% KOH in 70% ethanol for 15 min at RT (Reid et al., 1978).

Hydrolysis of N-linked oligosaccharides was carried out by enzymatic treatment with 10 U/ml of N-Glycosidase F (Roche, Mannheim, Germany) diluted in 20 mM sodium phosphate buffer, pH 7.2, containing 10 mM EDTA and 0.5% Triton X-100, overnight at 37°C. After a brief wash, the sections were incubated with each of the lectins used in this study.

As controls of the enzyme (sialidase and N-glycosidase F) digestion procedures, sections were incubated in the specific enzyme-free buffer solutions under the same experimental conditions.

To eliminate O-linked oligosaccharides (ß-elimination reaction), sections were incubated with 0.5 N NaOH in 70% (v/v) ethanol at 4°C for 5 days (Ono et al., 1983) and then incubated with each of the lectins.

Results

The results of lectin staining of the cat testes are summarized in Table 2.

MAL II gave a moderate staining of the basement membrane, a faintly visible reaction of the cytoplasm of spermatocytes which showed a moderate affinity for a cytoplasm granule, presumed Golgi zone (Fig. 1a). Removal of O-linked glycans by the ß-elimination reaction decreased the staining intensity in the basement membrane, and abolished the reactivity of the spermatocytes (Fig. 1b). Removal of N-linked oligosaccharides by N-glycosidase F pre-treatment did not modify the MAL II binding pattern. Saponification, followed by neuraminic acid cleavage (KOH-sialidase), abolished MAL II reactivity.

SNA showed a moderate staining of the basement membrane and a weak reaction with the Sertoli cell cytoplasm, which had a reticular appearance around the germ cells (Fig. 2a). ß-elimination abolished the basement membrane affinity, whereas it decreased the staining of the Sertoli cells (Fig. 2b). N-glycosidase F pre-treatment did not modify the SNA binding pattern. After KOH-sialidase treatment no positive reaction was observed.

PNA gave a moderate reaction for the basement membrane, a faintly visible staining of the cytoplasm of spermatocytes, and strongly reacted with the acrosome of round spermatids (Golgi- and cap-phase spermatids), whereas the elongated spermatids were unreactive (Fig. 3a). ß-elimination annulled the reactivity, whereas N-glycosidase F incubation did not modify the PNA staining pattern. After KOH-sialidase treatment the lectin PNA revealed cryptic binding sites in the acrosome of elongated spermatids (Fig. 3b). ß-elimination abolished the affinity, whereas N-glycosidase F incubation did not modify the KOH-sialidase-PNA staining pattern.

RCA_{120} displayed a faintly visible reaction for the basement membrane and the cytoplasm of spermatocytes which contained a moderately reactive Golgi zone. The acrosome of round spermatids showed a moderate staining, whereas the acrosomes of elongated spermatids were unreactive (Fig. 4a). ß-elimination abolished the affinity for the Golgi zone of spermatocytes and the acrosome of round spermatids, revealed binding sites in the acrosome of elongated spermatids as well as increasing the staining of the basement membrane and the cytoplasm of spermatocytes (Fig. 4b). N-glycosidase F treatments did not change the binding pattern. KOH-sialidase incubation did not reveal cryptic RCA_{120} binding sites. ß-elimination and N-glycosidase F treatments prior to KOH-sialidase RCA_{120} staining gave the same binding pattern as the RCA_{120} procedure.

SBA showed a weak staining of the basement membrane and of the acrosome of spermatids in the Golgi phase, and a strong reaction of the acrosome in cap-phase spermatids (Fig. 5a). ß-elimination annulled the basement membrane reactivity, decreased the staining of round spermatids and revealed binding sites in the cytoplasm of spermatocytes as well as in the acrosome of elongated spermatids (Fig. 5b). The removal of N-linked oligosaccharides did not change the SBA binding pattern. KOH-sialidase incubation revealed cryptic binding sites in the cytoplasm of Sertoli cells (Fig. 5c). This staining was annulled by ß-elimination treatment, whereas N-glycosidase F incubation was ineffective.

DBA showed a moderate staining of the basememt membrane and a weak affinity of the Golgi zone of spermatocytes and the acrosome of Golgi-phase spermatids (Fig. 6). After ß-elimination treatment the basement membrane showed a faintly visible reaction whereas the other structures were negative. N-glycosidase F incubation did not modify the DBA binding pattern. KOH-sialidase incubation did not reveal cryptic binding sites to DBA and the staining did not change after either ß-elimination or N-glycosidase F treatment.

HPA displayed a moderate reaction with the basement membrane, a weak staining of the Leydig

Table 2. Lectin staining pattern in domestic cat testes.

LECTIN	LEYDIG CELLS	BASEMENT MEMBRANE	SERTOLLI CELLS	SPERMATOGONIA	SPERMATOCYTES	SPERMATIDS		
						ROUND		ELONGATED
						Golgi	Cap	
MAL II	-	++	-	-	±/++G	-	-	-
ß-elimination	-	+	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	±/+G	-	-	-
SNA	-	++	+	-	-	-	-	-
ß-elimination	-	-	±	-	-	-	-	-
N-Glycosidase F	-	++	+	-	-	-	-	-
PNA	-	++	-	-	±	+++	+++	-
ß-elimination	-	-	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	±	+++	+++	-
KOH-si-PNA	-	++	-	-	±	+++	+++	+++
ß-elimination	-	-	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	±	+++	+++	+++
RCA120	-	±	-	-	±/++G	++	++	-
ß-elimination	-	+	-	-	+	-	-	++
N-Glycosidase F	-	±	-	-	±/++G	++	++	-
KOH-si-RCA120	-	±	±	-	±/++G	++	++	-
ß-elimination	-	+	-	-	+	-	-	++
N-Glycosidase F	-	±	±	-	±/++G	++	++	-
SBA	-	+	-	-	-	+	+++	-
ß-elimination	-	-	-	-	+	±	++	+
N-Glycosidase F	-	+	-	-	-	+	+++	-
KOH-si-SBA	-	+	+	-	-	+	+++	-
ß-elimination	-	-	-	-	+	±	++	+
N-Glycosidase F	-	+	+	-	-	+	+++	-
DBA	-	++	-	-	+G	+	-	-
ß-elimination	-	±	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	+G	+	-	-
KOH-si-DBA	-	++	-	-	+G	+	-	-
ß-elimination	-	±	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	+G	+	-	-
HPA	+	++	-	-	+G	+	+	+
ß-elimination	-	-	-	-	-	-	-	-
N-Glycosidase F	+	++	-	-	+G	+	+	+
Con A	+	+	++/+++a	±	±/+s/+G	±	±	±
ß-elimination	+	+	++/+++a	±	±/+s/+G	±	±	±
N-Glycosidase F	-	-	-	-	-	-	-	-
KOH-si-WGA	-	+	±	-	-	++	++	++
ß-elimination	-	±	±a	-	-	-	-	-
N-Glycosidase F	-	+	±	-	-	++	++	++
GSA I-B_4	-	++	-	-	++G	++	-	-
ß-elimination	-	++	-	-	++G	++	-	-
N-Glycosidase F	-	+	-	-	-	++	-	-
GSA II	-	+	-	-	-	-	-	++*
ß-elimination	-	-	-	-		-	-	-
N-Glycosidase F	-	+	-	-	-	-	-	++*
UEA I	-	++	-	-	-	-	-	-
ß-elimination	-	+	-	-	-	-	-	-
N-Glycosidase F	-	++	-	-	-	-	-	-

a, apical zone; G, Golgi zone; s, cell surface; si, sialidase; *, post-acrosomal region; -, negative reaction; ±, faintly visible reaction; +, ++, +++: weak, moderate, strong reactions. When not specified, the reactions concern the cytoplasm, except for spermatids where the labelling concerns the acrosome.

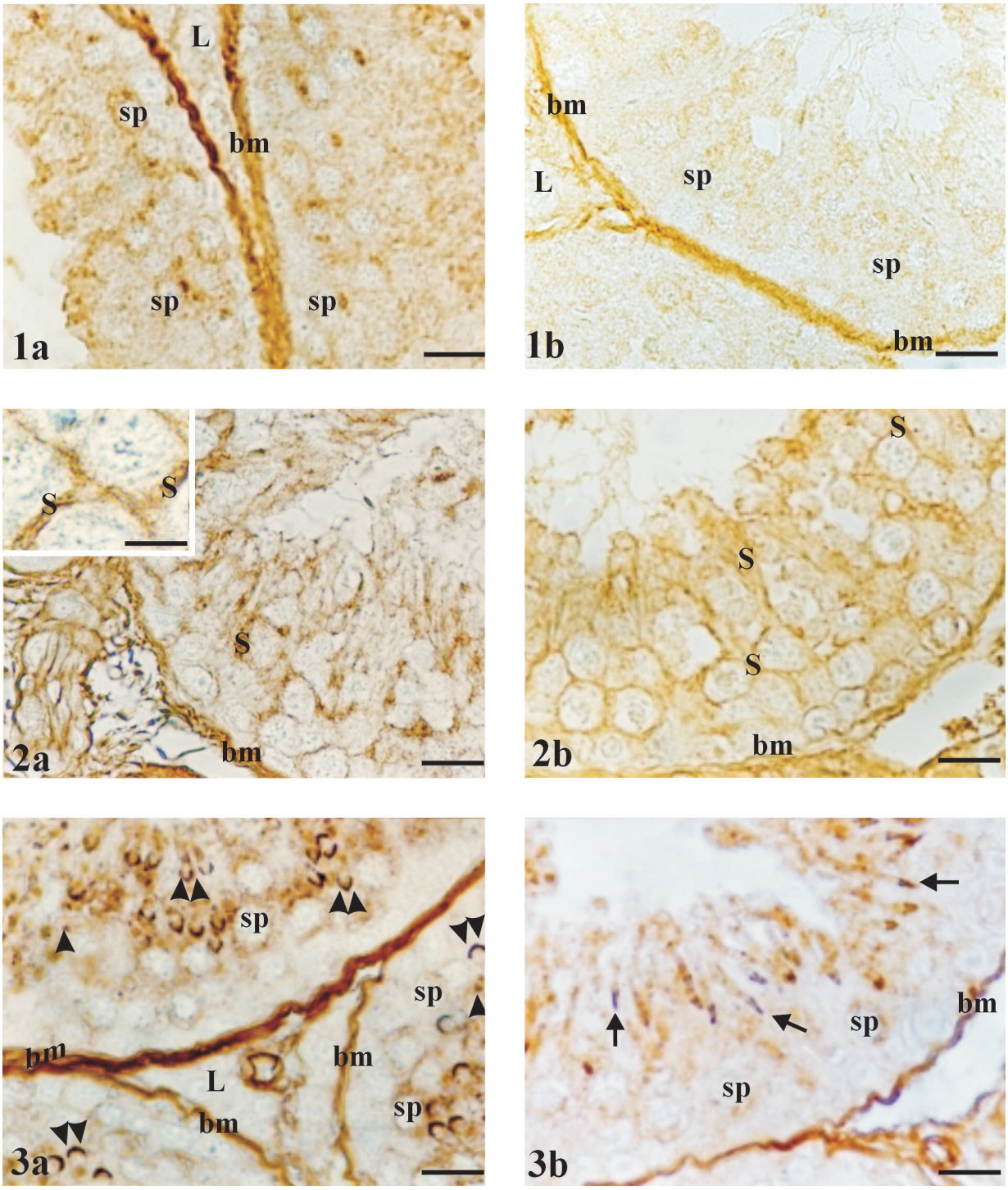


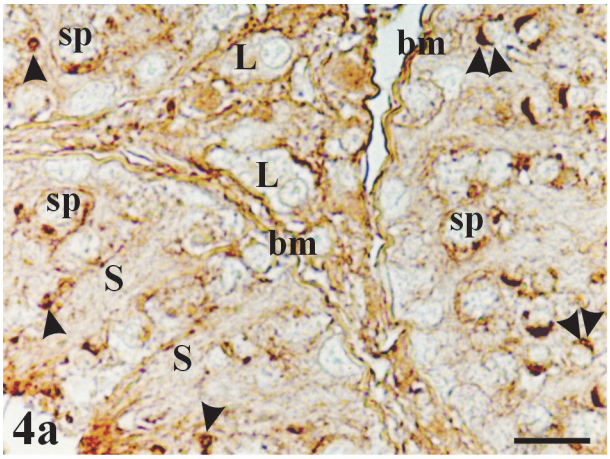
Fig. 1. MAL II reactivity in the cat testis. **a.** MAL II moderately stained the basement membrane, the Golgi zone of spermatocytes and very faintly the cytoplasm of spermatocytes. **b.** ß-elimination procedure: removal of O-linked glycans decreased the intensisty of MAL II staining in the basement membrane and abolished the reactivity in the spermatocytes. bm, basement membrane, L, Leydig cells; sp, spermatocytes. Scale bars: 18 µm.

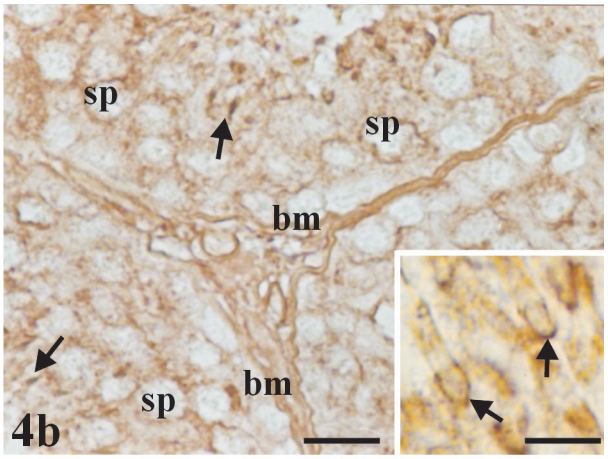
Fig. 2. SNA binding pattern in the cat testis. **a.** SNA reactivity present in the basement membrane and in the Sertoli cells. **b.** After ß-elimination procedure only a faintly visible reaction was found in the Sertoli cells. bm, basement membrane, S, Sertoli cells. Scale bars in a,b: 18 µm. Scale bar in inset: 45 µm.

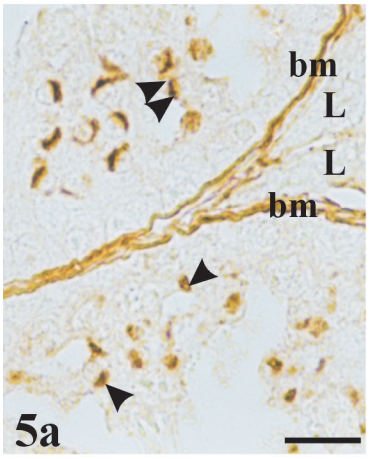
Fig. 3. PNA **(a)** and KOH-sialidase-PNA **(b)** staining in the cat testis. **a.** PNA: the lectin moderately stained the basement membrane, very weakly the cytoplasm of spermatocytes, strongly the acrosome of round spermatids. **b.** KOH-sialidase/PNA: the sialic acid cleavage revealed cryptic binding sites to PNA in the acrosome of elongated spermatids. bm, basement membrane, L, Leydig cells; sp, spermatocytes; arrow, elongated spermatids; arrowhead, Golgi-phase spermatids; double arrowhead, cap-phase spermatids. Scale bars: 18 µm.

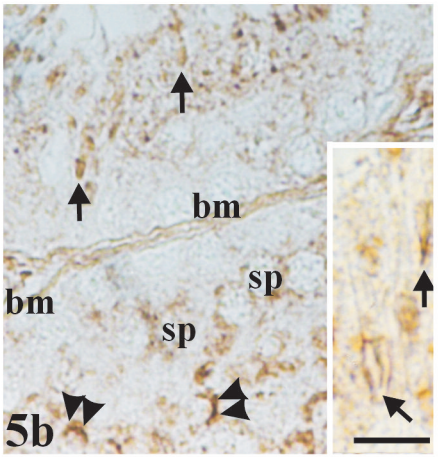
cells, the Golgi zone of spermatocytes and of the acrosome in round and elongated spermatids (Fig. 7). ß-elimination eliminated these binding sites, whereas glycosidase F incubation did not modify the staining pattern.

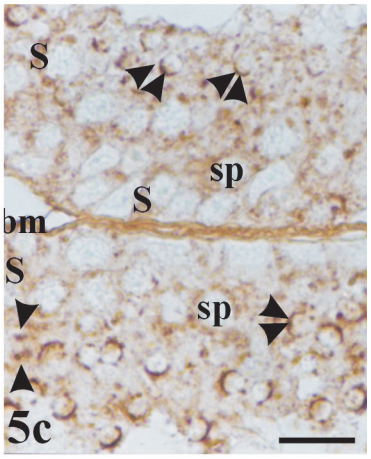
Con A showed a weak staining of the Leydig cell cytoplasm, the basement membrane and the Sertoli cell cytoplasm, which displayed a more intense staining in the apical zone (Fig. 8). The lectin also gave a faintly visible reaction with the cytoplasm of spermatogonia

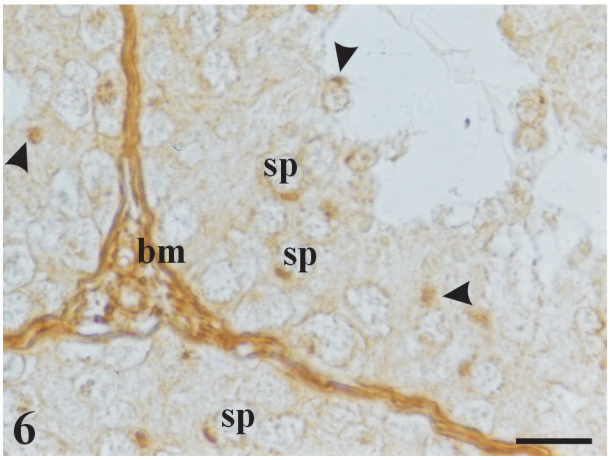












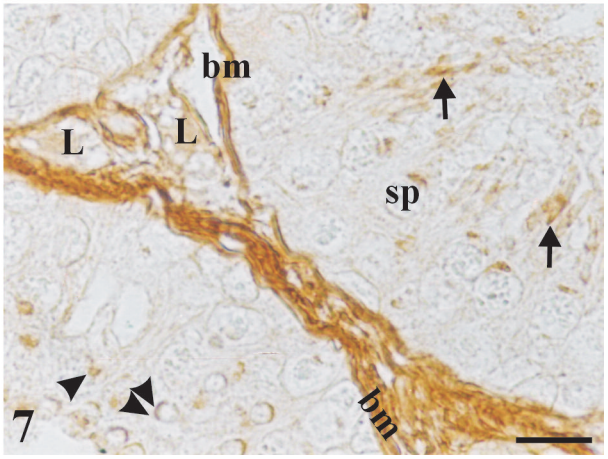


Fig. 4. RCA_{120} labelling of the cat testis. **a.** RCA_{120} very weakly stained the basement membrane, the cytoplasm of spermatocytes, and reacted moderately with the Golgi zone of spermatocytes as well as with the acrosomes of round spermatids. **b.** ß-elimination/RCA_{120} procedure increased staining of the basement membrane and cytoplasm of spermatocytes, as well as binding sites in acrosomes of elongated spermatids, while abolishing the reactivity of the Golgi zone of spermatocytes and in the acrosomes of round spermatids. bm, basement membrane, L, Leydig cells; S, Sertoli cells; sp, spermatocytes; arrow, elongated spermatids; arrowhead, Golgi-phase spermatids; double arrowhead, cap-phase spermatids. Scale bars in a,b: 18 µm. Scale bar in inset: 45 µm.

Fig. 5. SBA binding pattern in the cat testis. **a.** SBA weakly stained the basement membrane and the acrosome of spermatids in Golgi phase, and strongly the acrosome of cap-phase spermatids. **b.** ß-elimination/SBA: ß-elimination abolished the binding sites in the basement membrane and decreased staining in round spermatids, while showing binding sites in the cytoplasm of spermatocytes and in the acrosome of elongated spermatids. (Inset: elongated spermatids). **c.** KOH-sialidase/SBA: the KOH-sialidase treatment induced the appearance of binding sites in the Sertoli cells. bm, basement membrane, L, Leydig cells; S, Sertoli cells; sp, spermatocytes; arrow, elongated spermatids; arrowhead, Golgi-phase spermatids; double arrowhead, cap-phase spermatids. Scale bars in a,b,c: 18 µm. Scale bar in inset: 45 µm.

Fig. 6. DBA staining of the cat testis. DBA reactivity present in the basement membrane, Golgi zone of spermatocytes and acrosome of Golgi-phase spermatids. bm, basement membrane, sp, spermatocytes; arrowhead, Golgi-phase spermatids. Scale bar: 18 µm.

Fig. 7. HPA staining of cat testis. HPA binding sites are located at the level of Leydig cells, basement membrane, Golgi zone of spermatocytes and acrosome of both round and elongated spermatids. bm, basement membrane, L, Leydig cells; sp, spermatocytes; arrow, elongated spermatids; arrowhead, Golgi-phase spermatids; double arrowhead, cap-phase spermatids. Scale bar: 18 µm.

and spermatocytes as well as with the developing acrosomes. The spermatocytes revealed a weak staining of the Golgi zone and the cell surface (Fig. 8). ß-elimination did not modify this binding pattern, whereas N-glycosidase F abolished staining.

KOH-sialidase-WGA treatment (performed to highlight ßGlcNAc, but not sialic acid) gave a faintly visible reaction with the cytoplasm of Sertoli cells, weakly stained the basement membrane and moderately the acrosome in both round and elongated spermatids (Fig. 9a). ß-elimination reduced basement membrane staining and annulled the binding sites of the Sertoli cells (except to the apical zone), and spermatids (Fig. 9b). N-glycosidase F incubation did not change the binding pattern.

GSA $I\text{-}B_4$ showed a moderate reactivity of the basement membrane, the Golgi zone of spermatocytes and the acrosome of Golgi-phase spermatids (Fig. 10a). ß-elimination did not modify the staining pattern, whereas N-glycosidase F incubation decreased basement membrane staining and abolished the spermatocytic reactivity (Fig. 10b).

GSA II displayed a weak staining of the basement membrane and a moderate affinity in the post-acrosomal region of elongated spermatids (Fig. 11). ß-elimination annulled the staining. N-glycosidase F treatment did not modify the GSA II binding pattern.

UEA-I showed a moderate affinity with the basement membrane (Fig. 12a). ß-elimination decreased the basement membrane reactivity (Fig. 12b). N-glycosidase F treatment did not change the binding pattern.

Discussion

The present lectin histochemistry study on the domestic cat testis allowed us to identify the two groups of glycoconjugates, O- and N -linked types, as well as to

Fig. 8. Con A binding pattern in the cat testis. Con A reactivity present in the Leydig cells, basement membrane and in the each cell type constituting the seminiferous epithelium with the strongest staining in the apical zone of Sertoli cells. bm, basement membrane; L, Leydig cells; S, Sertoli cells; sg, spermatogonia; sp, spermatocytes; arrow, elongated spermatids. Scale bar: 18 µm. Scale bar in inset: 45 µm.

Fig. 9. KOH-sialidase/WGA staining of cat testis. **a.** WGA binding sites present in the basement membrane, Sertoli cells and acrosome of both round and elongated spermatids. **b.** ß-elimination/KOH-sialidase/WGA: ß-elimination procedure abolished the staining of Sertoli cells (except in the apical zone) and decreased the basement membrane reactivity. bm, basement membrane; L, Leydig cells; S, Sertoli cells; arrow, elongated spermatids; double arrowhead, cap-phase spermatids. Scale bars: 18 µm.

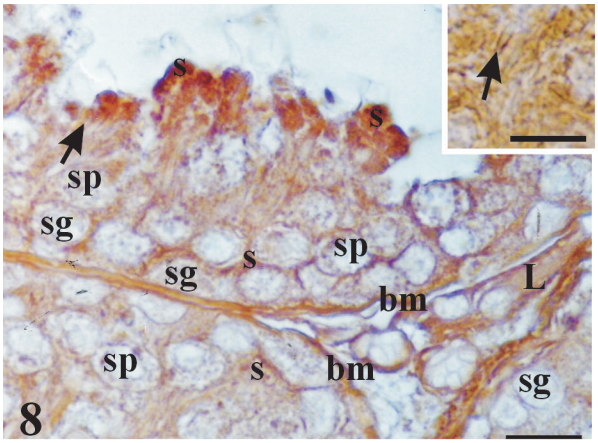
Fig. 10. GSA $I\text{-}B_4$ labelling of the cat testis. **a.** Staining present in the basement membrane, Golgi zone of spermatocytes and acrosomes of spermatids in Golgi phase. **b.** N-glicosidase F/ GSA $I\text{-}B_4$: removal of N-linked oligosaccharides decreased the affinity of basement membrane and abolished the binding sites in the Golgi zone of spermatocytes. Inset: Golgi-phase spermatids. bm, basement membrane; S, Sertoli cells; sp, spermatocytes; arrowhead, Golgi-phase spermatids. Scale bars in a and in b: 18 µm. Scale bar in inset: 45 µm.

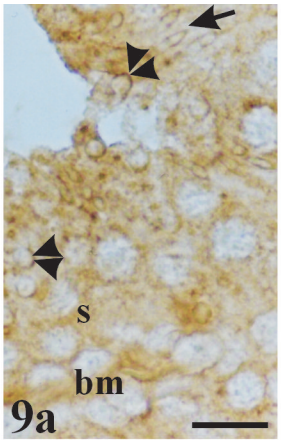
Fig. 11. GSA II binding pattern of the cat testis. GSA II stained the basement membrane and the post-acrosomal region of elongated spermatids. Inset: elongated spermatids. bm, basement membrane; arrow, elongated spermatids. Scale bar: 8 µm. Scale bar in inset: 45 µm.

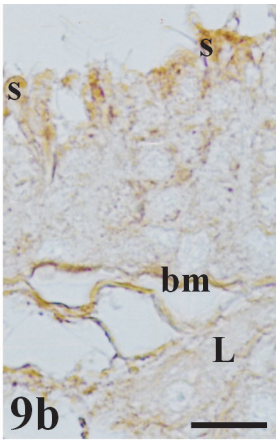
Fig. 12. UEA I binding pattern in the cat testis. **a.** UEA I binding sites present in the basement membrane. **b.** ß-elimination reduced the reactivity in the basement membrane. bm, basement membrane; L, Leydig cells. Scale bars: 18 µm.

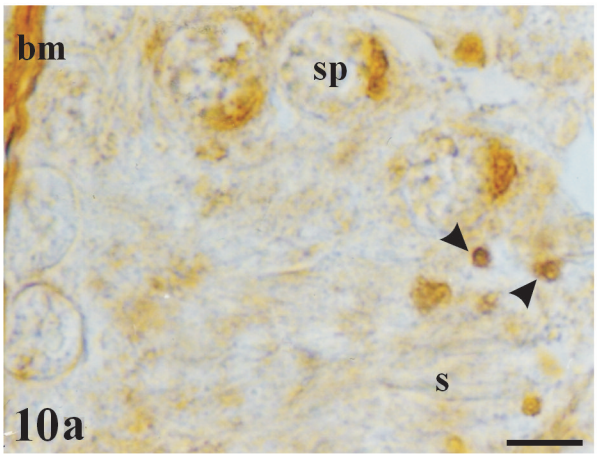
characterize the oligosaccharide sequences in the Leydig cells, the basement membrane, and the seminiferous epithelial cells. In addition, the results provide information about the changes of glycoconjugate pattern taking place in the developing acrosomes during cat spermiogenesis.

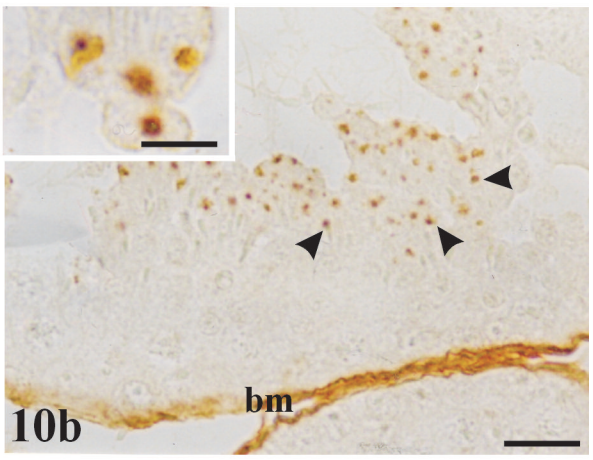
Leydig cells expressed cytoplasm binding sites for lectin HPA and Con A indicating the presence of oligosaccharides with terminal αGalNAc and terminal/internal αMan. The HPA and Con A stainings were abolished after ß-elimination and N-glycosidase F, respectively. Affinity for HPA identifies the many O-

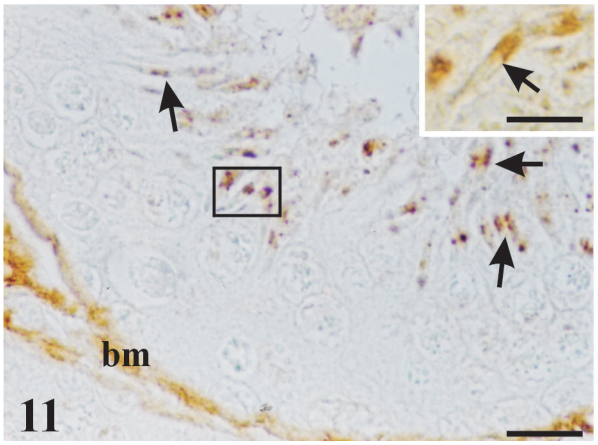


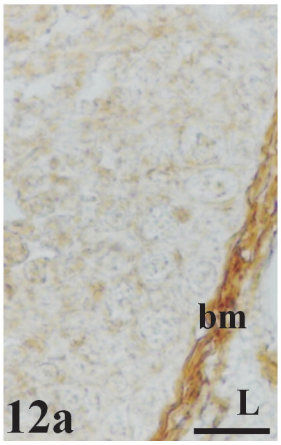


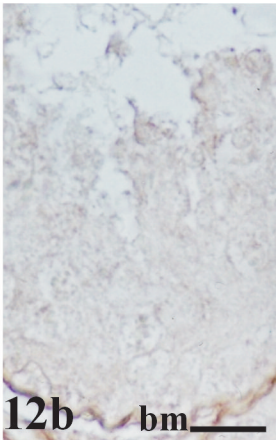












linked oligosaccharides containing GalNAc (Spicer and Schulte, 1992). This suggests that αGalNAc and αMan are located in O-linked and N-linked oligosaccharides, respectively. It is well known that Con A binds to a range of N-linked glycans from high-Man, through intermediate/hybrid, to small bi-antennary complex type, irrespective of bisection (Goldstein and Hayes, 1978; Debray et al., 1981). The possible involvement of the Leydig cells oligosaccharides as structural glycoproteins, in transport functions, endocrine and cell regulation (Pinart et al., 2002), in luteinizing hormone receptor expression (Zhang et al., 1995; Arenas et al., 1998; Pastor et al., 2003) has been considered on lectin histochemistry studies in the testis of other mammalian species.

The basement membrane reacted with all the utilized lectins. The abolition of the SNA, PNA, HPA, SBA and GSA II reactions after ß-elimination suggests that the terminal Neu5Acα2,6Gal/GalNAc, Galß1,3GalNAc, α/ßGalNAc, and GlcNAc residues labelled by those lectins belong to O-linked oligosaccharides. The increase in the RCA_{120} reaction after ß-elimination suggests the presence of terminal Galß1,4GlcNAc in N-linked oligosaccharides crypted by O-linked oligosaccharides. The decrease in MAL II, DBA, UEA I, and KOH-sialidase-WGA affinity after ß-elimination indicates that basement membrane contains both O- and N-linked oligosaccharides with terminal Neu5Acα2,3Galß1,4GlcNac, Forssman pentasaccharide, Fucα1,2Galß1,4GlcNAc and internal GlcNAc. The abolition of the Con A reaction with N-glycosidase F treatment shows the presence of high-Man N-linked oligosaccharides. In addition, terminal αGal is present in both O- and N-linked glycans as revealed by the decreased reactivity of GSA I-B_4 after N-glycosidase F treatment. This complex lectin-binding pattern could be related to the presence of a meshwork of several large glycoconjugate components such as laminins, collagen IV, perlecan, and nidogen (Timpl, 1993; Malinda and Kleinman, 1996; Timpl and Brown, 1996) which are responible for many of the biological functions attributed to basement membranes. The present findings show that the basement membrane of the cat seminiferous epithelium contains a more complex oligosaccharide pattern than the other mammalian species studied (Arya and Vanha-Perttula, 1984, 1986; Jones et al., 1992b, 1993; Arenas et al., 1998; Pastor et al., 2003).

The cytoplasm of Sertoli cells from the base to the apical region expressed binding sites to SNA, KOH-sialidase-RCA_{120}, KOH-sialidase-SBA, KOH-sialidase-WGA, and Con A. The binding pattern of these lectins after ß-elimination and N-glycosidase F reaction suggests that terminal Neu5Acα2,6GalßGalNAc (labelled by SNA) belongs to both O- and N-linked oligosaccharides, Neu5AcGαlß1,4GlcNAc and Neu5AcßGalNAc (shown with KOH-sialidase-RCA_{120} and KOH-sialidase-SBA, respectively) and internal GlcNAc (revealed by KOH-sialidase-WGA) constitute O-linked glycans, and Man residues (Con A affinity) are present in N-linked glycoproteins. Lectin labelling of Sertoli cells has been related to structural and secreted glycoproteins. Sertoli cells secrete various paracrine factors involved in the control of germ cells, peritubular cells and Leydig cells (Skinner, 1993). As regards sialoglycoconjugates, their presence has been found in rat (Jones et al., 1992b, 1993), human (Arenas et al., 1998), bull (Calvo, 2000), and hamster Sertoli cells (Pastor et al., 2003). Although the role of sialoglycoproteins is unknown, it has been reported that in the rat Sertoli cells secreted sulphated glycoprotein (SGP-2) binds SNA (Sensibar et al., 1993). The apical extension of Sertoli cells, with respect to the remaining cytoplasm, showed a high presence of N-linked glycoproteins containing terminal/internal Man and internal GlcNAc residues, evidenced by the abolition of Con A staining after the N-glycosidase F reaction and by the appearance of staining with KOH-sialidase-WGA after ß-elimination, respectively. The Con A and WGA reaction observed in the apical extensions of Sertoli cells has been associated with the presence of lysosomes containing residual bodies from degradation of the residual spermatid cytoplasm in bull (Arya and Vanha-Perttula, 1985), rat (Arya and Vanha-Perttula, 1985), gerbil, guinea pig, mouse and nutria (Arya and Vanha-Perttula,1986), horse (Verini-Supplizi et al., 2000), hamster (Ballesta et al., 1991; Pastor et al., 2003) and human (Malmi et al., 1990; Arenas et al., 1998).

Spermatogonia exhibited cytoplasmic binding to Con A and this reaction was abolished after N-glycosidase treatment. The presence of high-Man N-linked binding sites has been detected in mouse (Lee and Damjanov, 1984; Burkett et al., 1987), rat (Arya and Vanha-Perttula, 1984; Jones et al., 1992a), boar (Calvo et al, 2000), hamster (Pastor et al., 2003), and human spermatogonia (Arenas et al., 1998). Since other lectins also label spermatogonia of these species , it is possible to suppose that domestic cat spermatogonia contain a very simple glycoconjugate pattern which will become more complex during the transition to spermatocytes.

The cytoplasm of spermatocytes reacted with MAL II, PNA, RCA_{120} and Con A. ß-elimination abolished MAL II and PNA reactivity, whereas it increased RCA_{120} staining and revealed SBA binding sites. This suggests that terminal Neu5acα2,3Galß1,4GlcNAc and Galß1,3GalNAc belong to O-linked oligosaccharides, whereas terminal Galß1,4GlcNAc and α/ßGalNAc are present in a subset of N-linked glycoconjugates masked by O-linked oligosaccharides. N-linked glycans containing high-Man residues were also expressed in the surface of spermatocytes. The cytoplasmic staining of spermatocytes could indicate synthesis and processing of glycoproteins in the endoplasmic reticulum. Some of these glycoproteins are transported to the cell surface where they might be involved in the transport of ions (Spicer and Schulte, 1992; Parillo et al., 1998) and/or the interaction with Sertoli cells (Blackmore and Eisoldt, 1999). Recently, an N-glycan has been found to regulate

spermatocyte-Sertoli cell adhesion and to permit germ cell survival through spermatogenesis in mouse (Akama et al., 2002). The Golgi complex in spermatocytes, shown as a cytoplasmic positive granule, was positive for MAL II, RCA_{120}, DBA, HPA, Con A, and GSA I-B_4. The absence of reaction for MAL II, RCA_{120}, DBA, and HPA after ß-elimination and for Con A and GSA I-B_4 after N-glycosidase F digestion indicates the presence of both O- and N-linked oligosaccharides. O-linked oligosaccharides terminate with Neu5acα2,3Galß1,4 GlcNac, Galß1,4GlcNAc, Forssman pentasaccharide, and αGalNAc, while N-linked oligosaccharides express terminal/internal αMan and terminal αGal. The Golgi zone reactivity could represent the precursor of acrosome glycoproteins. Acrosomes are Golgi-derived secretory granules whose biogenesis begins in the spermatocytes (Anakwe and Gerton, 1990). O- and N-linked glycoproteins have been previously found in the Golgi zone of spermatocytes of mouse (Burkett et al., 1987) and human testis (Arenas et al., 1998).

The lectin pattern of the acrosomal glycoconjugates changed in relation to the acrosomal developing state. The acrosomal region of Golgi-phase spermatids bound PNA, RCA_{120}, DBA, SBA, HPA, Con A, KOH-sialidase-WGA, and GSA I-B_4. ß-elimination eliminated the staining with PNA, RCA_{120}, DBA, HPA, and KOH-sialidase-WGA and decreased the reaction of SBA. N-glycosidase F incubation abolished the reaction with Con A. GSA I-B_4 labelling was not modified with ß-elimination or N-glycosidase F treatment. These findings suggest that the acrosomes of Golgi-phase spermatids express terminal Galß1,3GalNAc, Galß1,4GlcNAc, Forssmann pentasaccharide, α/ßGalNAc, and internal GlcNAc in O-linked oligosaccharides, terminal α/ßGalNAc and Gal in O-and N-linked glycans, and high-mannose in N-linked glycoproteins. The fact that the removal of O- and N-linked oligosaccharides did not change the GSA I-B_4 reaction could depend on the smasking of new N- and O-linked oligosaccharides terminating in αGal after ß-elimination and N-glycosidase F treatment, respectively. During the cap-phase, the acrosomes lacked the reaction with DBA and GSA I-B_4, whereas they showed an increase in SBA staining. This indicates that in this developing phase the acrosome lacks oligosaccharides terminating with Forssman pentasaccharide and αGal, whereas there is an increase in terminal α/ßGalNAc residues. In the next developmental stage, the acrosomes of elongated spermatids did not express O-linked oligosaccharides terminating with Galß1,3GalNAc, Galß1,4GlcNAc, and α/ßGalNAc, as revealed by the absence of staining with PNA, RCA_{120}, and SBA, respectively. After KOH-sialidase, PNA showed a strong reaction, which was eliminated by ß-elimination, suggesting the presence of sialic acid linked to Galß1,3GalNAc in O-linked oligosaccharides. Since RCA_{120} and SBA labelling appeared after ß-elimination, it is possible to suppose that cryptic N-linked oligosaccharides terminating with Galß1,4GlcNAc and α/ßGalNAc were unmasked. A characteristic feature of elongated spermatids is represented by the staining of the post-acrosome region with GSA II, suggesting the presence of GlcNAc in O-linked oligosaccharides (reactivity was abolished by ß-elimination). Among the vertebrates investigated, the post-acrosome of spermatids has only been found in the mouse testis but with other lectins (Burkett et al., 1987). The presence of glycoconjugates in the acrosomal region of developing spermatids mirrors the complex nature of acrosomal glycoproteins related to the presence of carbohydrate-rich enzymes which are crucial to fertilization (Malmi et al., 1987, 1990). It is well known that the acrosome contains several enzymes, such as acid phosphatase, acrosin, and hyaluronidase, which are glycoproteins or closely associated with complex saccharide moieties and that are essential for fertilization (Gould and Bernstein, 1977). The changes in carbohydrate pattern shown during acrosome development could be related to the addition of new residues to the carbohydrate chains, or to compression of acrosomal components that mask some sugar residues, as well as to conformational modifications and redistribution of glycoprotein, rather than to a loss in glycan content (Kurohmaru et al., 1995; Martínez-Menárguez et al., 1999). O- and N-linked glycans have been found in rat (Jones et al., 1992b, 1993; Martínez-Menárguez et al., 1992); human (Arenas et al., 1998), boar (Calvo et al., 2000), horse (Verini-Supplizi et al., 2002), and hamster spermatids (Pastor et al., 2003). Modifications of acrosomal glycoconjugate have been reported during spermiogenesis of these mammalian as well as in non-mammalian species (Ballesta et al., 1991; Labate and Desantis, 1995; Sáez et al., 2004) but it is not possible to show a common pattern of glycosylation during acrosome development. As regards sialic acid residues, the presence of sialoglycoproteins has been demonstrated in round and elongated mouse (Burkett et al., 1987), rat (Jones et al., 1992b) and human spermatids (Arenas et al., 1998). Sialic acids are considered to be involved in the inhibition of intermolecular and intercellular interactions by means of their negative charge (Varki, 1997), in an increase in solubility, and can affect the conformation of glycoproteins and cell adhesive properties (Hilkens et al., 1992; Arenas et al., 1998).

In conclusion, the present histochemical analysis provides information on the oligosaccharide sequences of the Leydig cells and the seminiferous epithelium of cat testis, as well as reporting the specific changes occurring in the glycans of developing acrosomes during spermatogenesis.

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