Glycoconjugate Profiles of Insect Spermatogenesis: Lectin Cytochemical Analysis of the Cricket, Gryllus bimaculatus

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Glycoconjugate Profiles of Insect Spermatogenesis: Lectin Cytochemical Analysis of the Cricket, *Gryllus bimaculatus*

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ABSTRACT—The distribution of glycoconjugate in testicular germ cells of the cricket, *Gryllus bimaculatus*, was cytochemically investigated using a panel of lectins and several kinds of glycosidases. Observations were focused on the early process of spermatogenesis. The binding pattern with *N*-acetylgalactosamine-binding lectins, especially with *Dolichos biflorus* agglutinin, and their susceptibility to peptide-*N*-glycosidase F (PNGase F) suggested the stage-specific expression of *N*-linked glycoproteins with terminal α-*N*-acetylgalactosamine in the early meiotic prophase. In the primary spermatocyte, the transitory expression of PNGase F-resistant glycoproteins was detected by several mannose-binding and fucose-binding lectins such as *Lens culinaris* agglutinin and *Anguilla anguilla* agglutinin. Hyaluronidase-sensitive glycoconjugates also distributed widely in cricket testes, exemplified by perinuclear granular structures recognized by *Helix pomatia* agglutinin and nuclear staining with *Phaseolus lunatus* agglutinin. These lectin-binding affinities in meiotic prophase were discussed in reference to many informations about carbohydrate-lectin binding specificities.

INTRODUCTION

Evidence for glycosylation and its developmental changes in male germ cells has been accumulated through several biochemical approaches. We know about, for instance, the glycosylation of particular proteins in mouse pachytene spermatocytes and spermatids (Millette and Scott, 1984; Maylie-Pfenninger, 1994), its elevated dolichol synthesis, which might be associated with glycosylation (Potter *et al.*, 1981), the fucosylation of glycoproteins in rat spermatocytes and spermatids (Grootegoed *et al.*, 1982), and the sialylation of surface carbohydrates of mouse prepachytene spermatocytes (Fenderson *et al.*, 1984) and that of rat spermatozoa at the maturation stage (Yamamoto *et al.*, 1991). These fragmentary studies should be integrated to create a comprehensive understanding of the distribution of glycoconjugates in spermatogenesis.

A large number of lectin-histochemical analyses have been conducted on mammalian testicular tissues such as rabbit (Nicolson *et al.*, 1977), mouse (Watanabe *et al.*, 1981; Kanai *et al.*, 1989), rat (Arya and Vanha-Perttula, 1984; Malmi *et al.*, 1990; Jones *et al.*, 1993; Martínez-Menárguez *et al.*, 1992) and bull (Ertl and Wrobel, 1992). There are still very few descriptions from non-mammal vertebrates (Ballesta *et al.*, 1991; Labate and Desantis, 1995) and from insects such as *Drosophila* (Perotti and Pasini, 1995) and three orthopteran species (Esponda and Guerra, 1991). The interests of most of these studies, however, focused mainly on glycoprotein distribution at the latest stages of spermatogenesis. Information about glycoconjugates in the early stages of spermatogenesis, particularly in invertebrates, remains to be elucidated.

Our previous study on male germ cells of the cricket, *Gryllus bimaculatus*, showed a dramatic change of the binding activity to soybean agglutinin (SBA) during the primary spermatocyte stage (Suzuki and Nishimura, 1995). In the present study, we further explored the glycoconjugates of cricket testis employing a panel of lectins and several glycosidases in order to characterize the nature of glycoconjugate changes during the development of spermatogonia to early spermatids.

MATERIALS AND METHODS

Tissue processing

Crickets, *Gryllus bimaculatus*, were reared on an artificial mouse diet (MF; Oriental Yeast Co. Ltd., Tokyo). Testes were dissected from ether-anesthetized animals, washed in 10 mM phosphate buffered saline (PBS), pH 7.4, and fixed with one of the following solutions: (1) Bouin's fluid, (2) 4% paraformaldehyde in PBS, (3) 3% formaldehyde/0.1% glutaraldehyde in PBS, and (4) 1% glutaraldehyde in PBS. After fixation, they were dehydrated in ethanol series, embedded in paraffin, and 5-μm sections were obtained. They were dewaxed and treated for lectin cytochemistry. To make a comparison of the effects between fixatives, 4 pieces of testis were obtained from a 7th-instar animal and each piece was fixed with one of the 4 fixatives. After fixation all pieces were processed at the same time and embedded all together.

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Lectins

The following lectins conjugated with biotin were used in this study: *Dolichos biflorus* agglutinin (DBA), soybean agglutinin (SBA) from *Glycine max*, *Sophora japonica* agglutinin (SJA), *Griffonia simplicifolia* lectin I-B_4 (GS-I-B_4), peanut agglutinin (PNA) from *Arachis hypogaea*, *Ricinus communis* agglutinin I (RCA-I), succinylated wheat germ agglutinin (S-WGA) from *Triticum vulgaris*, concanavalin A (Con A) from *Canavalia ensiformis*, *Lens culinaris* agglutinin (LCA), *Pisum sativum* agglutinin (PSA), *Ulex europaeus* agglutinin I (UEA-I), the above lectins were purchased from Vector Laboratories, Inc., Burlingame, CA; *Helix pomatia* agglutinin (HPA), *Phaseolus lunatus* agglutinin (LBA), *Anguilla anguilla* agglutinin (AAA), and *Limax flavus* agglutinin (LFA), the last 4 lectins from E-Y laboratories, Inc., San Mateo, CA.

Lectin cytochemistry

The dewaxed sections were soaked in 50 mM Tris-HCl buffered saline (TBS), pH 7.4, and the non-specific sites were blocked with TBS containing 1% bovine serum albumin (BSA-TBS) for 30 min at the room temperature. BSA-TBS was also used as a diluent. The sections were then incubated with biotinylated lectins (Table 1) diluted in BSA-TBS for 1 hr at the room temperature. After washing with TBS, they were incubated with the peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD; or Zymed Laboratories, Inc., San Francisco, CA; they showed same results) at 1:200 dilution for 30 min at the room temperature. Development was carried out after final washing with TBS using 0.02% 3-amino-9-ethylcarbasol in 50 mM acetate buffer, pH 5.0, containing 0.03% H_2O_2, and rinsed with water. Sections were counterstained with hematoxylin if necessary, and then mounted sequentially in Crystal/Mount (Biomeda Corp., Foster City, CA) and Eukitt (O. Kindler GmbH & Co., Freiburg, Germany).

In control experiments, biotinylated lectins were preincubated with 0.1 M of the specific inhibitory sugars (Table 1) for 30 min before cytochemistry.

Enzyme treatment of the paraffin sections

Some dewaxed sections were incubated with glycosidases before lectin cytochemistry. The enzymes used in the experiments were as follows: 100 mU/ml (400 Oxford GlycoSystems de-glycosylation Units/ml) of Endo-β-*N*-acetylglucosaminidase F/Peptide *N*-glycosidase F (EndoF/PNGaseF) or 50 mU/ml (50 Oxford GlycoSystems Units/ml) of PNGaseF from *Flavobacterium meningosepticum* (Oxford GlycoSystems Ltd, Oxon, UK) in 20mM sodium phosphate buffer, pH 7.5, containing 50 mM EDTA (24 hr, 37°C); 0.5% hyaluronidase from bovine testes (Sigma) in 0.1 M phosphate buffer, pH 6.0 (18 hr, 37°C); 0.5 U/ml of fucosidase from bovine kidney (Sigma) in 0.1 M citric acid-0.2 M Na_2HPO_4, pH 5.5 (24 hr, 37°C). Control sections were incubated with enzyme-free buffers.

RESULTS

Among the 6th- to 8th- (the last) instar testes subjected to the experiments, early 7th-instar testes were most suitable for the aim of the present investigation because of the high ratio of spermatocytes and the appearance of early spermatids. It was difficult to discriminate meiotic prophase stages by the chromosomal phase even on sections counter-stained with hematoxylin. Thus, in this study, we referred to primary spermatocyte stages using three terms according to their size: early-spermatocytes for those smaller than 10-11 μm in diameter, late-spermatocytes larger than 18 μm, and mid-spermatocytes for those in-between. Figure 1 shows a scheme of the early 7th-instar testis. A large number of follicles were bundled in a pair of testis sheaths.

Comparison of fixatives

Testis staining patterns with SBA, DBA and PNA were compared between specimens treated with four fixatives.

Table 1. Biotinylated lectins used in this study

Acronym	Sources	Concentration (μg/ml) tested range; optimum		Inhibitory sugar (0.1 M)	Major carbohydrate-binding specificity[c]	Ref.[c]
S-WGA	*Triticum vulgaris*	5 - 20	20	GlcNAc	GlcNAcβ1-4GlcNAc > GlcNAc	1, 4
DBA	*Dolichos biflorus*	5 - 20	10	GalNAc	GalNAcα1-3GalNAcβ1- > GalNAcα1-3(±Fucα1-2)Galβ1-	6
LBA	*Phaseolus lunatus*	1.25 - 20	5 - 10	GalNAc	GalNAcα1-3(Fucα1-2)Galβ1-	7
HPA	*Helix pomatia*	1.25 - 10	5 - 10	GalNAc	GalNAcα1-3(±Fucα1-2)Galβ1-, GalNAcα1- > GalNAcβ1-4Galβ1-	3, 6
SBA	*Glycine max*	1.25 - 20	1.25 - 5	GalNAc	αGalNAc > βGalNAc > Gal	1, 6
SJA	*Sophora japonica*	20 - 100	—[a]	GalNAc	Galβ1-3GalNAcα1- > Galβ1-3/4GlcNAcβ1- > GalNAc >> Gal	10
GS-I-B_4	*Griffonia simplicifolia*	20 - 100	—[a]	Gal	Galα1-3Galβ1-4GlcNAcβ1-	3, 9
RCA-I	*Ricinus communis*	10 - 200	10 - 20	Gal	Galβ1-4GlcNAcβ1- > β-Gal	1, 3
PNA	*Arachis hypogaea*	2.5 - 10	2.5 - 10	Gal	Galβ1-3GalNAcα1- > Galβ1-4GlcNAcβ1-	8, 10
Con A	*Canavalia ensiformis*	1 - 20	1	Man	Trimannosyl core of high monnose- or hybrid-type	1, 3
LCA	*Lens culinaris*	0.5 - 20	0.5 - 1	Man	Core region of complex-type with α1-6-linked fucose	1, 3
PSA	*Pisum sativum*	1 - 20	1	Man	Core region of complex-type with α1-6-linked fucose	1, 3
AAA	*Anguilla anguilla*	10 - 200	20[b]	Fuc	αL-Fuc	2
UEA-I	*Ulex europaeus*	10 - 20	20[b]	Fuc	Fucα1-2Galβ1-4GlcNAcβ1-	1, 5
LFA	*Limax flavus*	20 - 100	—[a]	Neu5Ac	Neu5Ac > Neu5Gc	11

GlcNAc, *N*-acetyl-D-glucosamine; GalNAc, *N*-acetyl-D-galactosamine; Gal, D-galactose; Man, D-mannose; Fuc, L-fucose; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycoloylneuraminic acid.

[a] No specific response was observed at the tested concentration.

[b] Weak or no response at this concentration without EndoF/PNGaseF treatment.

[c] Generally referred to Goldstein and Poretz (1986). Other references are numbered: 1, Debray *et al.* (1981); 2, Gercken and Renwrantz (1994); 3, Kobata and Yamashita (1993); 4, Monsigny *et al.* (1979); 5, Pereira *et al.* (1978); 6, Piller *et al.* (1990); 7, Sikder *et al.* (1986); 8, Sueyoshi *et al.* (1988); 9, Wood *et al.* (1979); 10, Wu (1984); 11, Zeng and Gabius (1992).

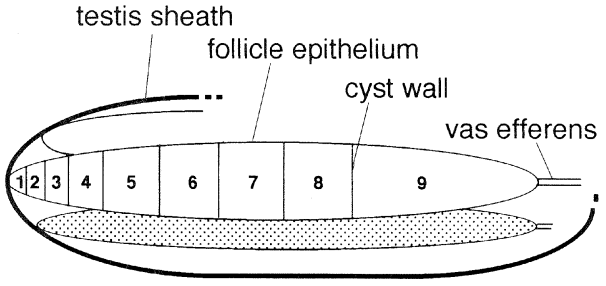

Fig. 1. Scheme of early 7th-instar testis. Several spermatocysts (9 in this figure) are arrayed in a testis lobe. Each cyst contains many germ cells synchronously developing in numerical order, e.g., spermatogonia (1), early-spermatocytes (2, 3), mid-spermatocytes (4), late-spermatocytes (5–7), secondary spermatocytes or round spermatids (8), and elongated spermatids (9). There may be some varieties in the number and the constitution of developmental stages.

Compared to the tissue fixed with Bouin's fluid showing specific staining patterns with these lectins as described in the following section, glutaraldehyde fixation deteriorated the staining activities with all three lectins. DBA binding thoroughly disappeared with 0.1% glutaraldehyde. Tissue fixed with 4% formaldehyde caused reduced staining with PNA. Therefore further experiments were carried out with specimens fixed with Bouin's fluid.

Lectin histochemistry on sections of testes fixed with Bouin's fluid

Staining patterns with 15 lectins were studied on the Bouin-fixed sections and the results are summarized in Table 2. Control sections that were incubated with 0.1 M of each corresponding inhibitory sugar (Table 1) showed no staining, except for the case with LFA as described later. The results of the staining by each lectin are reported below in detail. The lectins were categorized according to Goldstein and Poretz (1986).

N-ACETYLGLUCOSAMINE-BINDING LECTIN

S-WGA — As shown in Fig. 2a, several granular structures reacted with S-WGA in spermatogonia, early- and mid-spermatocytes. Cytoplasm in early-spermatocytes also showed diffuse staining. Staining with S-WGA disappeared entirely by PNGase F treatment. Hyaluronidase treatment showed no effect.

N-ACETYLGALACTOSAMINE/GALACTOSE-BINDING LECTINS

DBA — Granular structures were stained in spermatogonia, early- and mid-spermatocytes. The lectin binding activity was most intense in early-spermatocytes. Many granular structures and diffuse cytoplasm were strongly stained at this stage. The stain decreased in later stages, and was negative in late-spermatocytes (Figs. 2b, 3a). The reactivity was removed thoroughly by PNGase F treatment (Fig. 3b), but was not affected by hyaluronidase.

LBA — Various granular structures in spermatogonia, early- and mid-spermatocytes reacted with LBA. The granular bodies in mid-spermatocytes were remarkably large and only a few numbers were found in a single cell. Nuclei of spermatogonia and early-spermatocytes showed diffuse staining. Nuclei of the testis sheath cells, small granular structures in cyst cells, and tracheae were also stained with LBA (Fig. 4a, c). After Endo F/PNGase F treatment, all responses in granular structures disappeared, but the nuclei of germ cells and testis sheath cells, cytoplasm of the early- and mid-spermatocytes,

Table 2. Lectin staining pattern in cricket testicular germ cells

	Spermatogonia			Spermatocytes *early*			*mid*		*late*		Spermatids	
	N	G	C	N	G	C	G	C	G	C	G	C
S-WGA	–	$+^{f}$	–	–	$+^{f}$	$+^{f}$	$+^{f}$	$\pm^{f}$	–	–	–	–
DBA	–	$+^{f}$	–	–	$++^{f}$	$++^{f}$	$++^{f}$	$\pm^{f}$	–	–	–	–
LBA	$+^{h}$	$+^{f}$	–	$\pm^{h}$	$++^{f}$	$++^{f}$	$++^{f}$	$\pm^{f}$	–	–	–	–
HPA	–	+	$\pm^{f}$	–	++	$++^{f}$	+	$+^{f}$	$++^{h}$	$\pm$	++	+
SBA	–	+	$+^{f}$	–	++	$++^{f}$	+	$+^{f}$	$++^{h}$	$\pm$	++	$+^{h}$
RCA-I	–	$+^{f}$	$\pm^{f}$	–	$++^{f}$	$++^{f}$	+	$\pm^{f}$	+	$\pm$	$++^{h}$	$\pm$
PNA	–†	–	–	–†	–	–	–	–	–	–	–	–
ConA	–†	$\pm^{f}$	$\pm^{f}$	–†	$+^{f}$	$+^{f}$	$+^{f}$	$+^{h*}$	$++^{fh}$	$+^{*}$	–	+
LCA	–†	$+^{f}$	$+^{f}$	–†	$+^{f}$	$+^{f}$	$+^{f}$	$+^{*}$	$++^{f}$	$+^{*}$	$\pm$	+
PSA	–†	$+^{f}$	$+^{f}$	–†	$+^{f}$	$+^{f}$	$\pm^{f}$	$+^{*}$	$\pm^{f}$	$+^{*}$	–	+
AAA	–	–	–	–	–	–	$-^{*}$	$+^{h*}$	$-^{*}$	$+^{h*}$	–	$-^{*}$
UEA-I	–	–	–	–	–	–	$-^{*}$	$-^{*}$	$-^{*}$	$-^{*}$	–	$-^{*}$

N, nucleus; G, various granular strucures; C, cell surface and/or cytoplasm stained diffusely.
Intensity of staining: – negative, ± faint, + moderate, ++ strong. GS-IB_4, SJA and LFA showed no specific staining in germ cells.
[f] The staining activity sensitive to PNGaseF or EndoF/PNGaseF.
[h] The staining activity sensitive to hyaluronidase.
* Different binding sites appeared after EndoF/PNGaseF treatment.
† Faintly positive after hyaluronidase treatment.

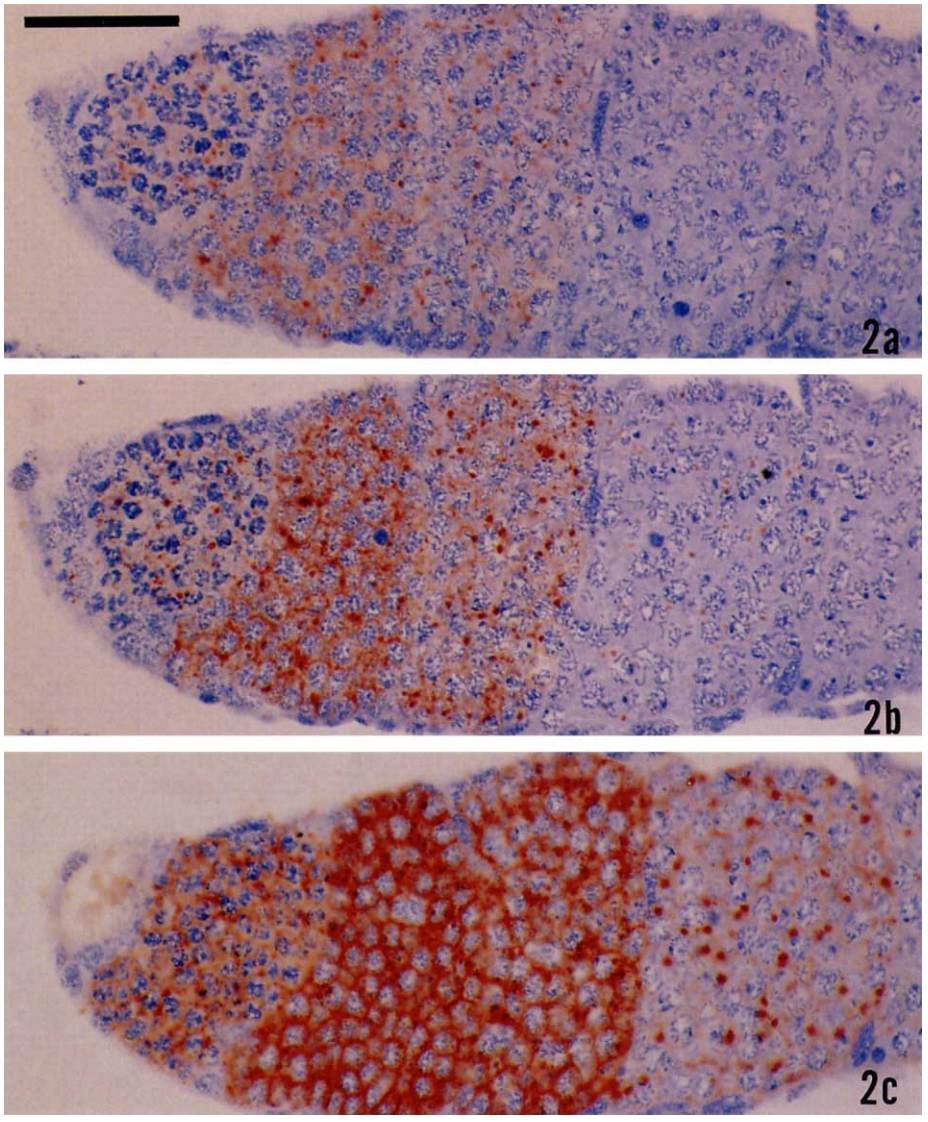

Fig. 2. Three adjacent sections of testis follicles stained with S-WGA (**a**), DBA (**b**) and SBA (**c**). Counterstained with hematoxylin. Scale bar, 50 μm.

and tracheae were still stained weakly (Fig. 4b). On the other hand, hyaluronidase treatment halted binding activities that were resistant to Endo F/PNGase F (Fig. 4d). Comparing these results, it revealed that nuclei of most testicular cells showed weak binding susceptible to hyaluronidase.

HPA — Various sizes of granular structures displayed staining from spermatogonia to spermatids. The cytoplasm and cell surface of early-spermatocytes were also strongly stained (Fig. 5a, c). Among the testis tissues other than germ cells, the testis sheath, testis follicle epithelia, cyst walls and tracheae showed affinity to this lectin. Endo F/PNGase F treatment halted intense staining in early-spermatocytes while retaining granular structures (Fig. 5b). Incubation with hyaluronidase caused reduced staining of the perinuclear granular structures in late-spermatocytes (Fig. 5d).

SBA — Details about SBA staining pattern were as described (Suzuki and Nishimura, 1995). As shown in Fig. 2, the strong response with SBA was observed at the same stage as the cases with S-WGA and DBA. Incubation with PNGase F caused reduced staining in early-spermatocytes but not in late-spermatocytes or spermatids. Incubation with hyaluronidase caused reduced staining in the late-spermatocytes (Suzuki and Nishimura, 1995).

SJA — Although SJA is also the lectin that recognizes *N*-acetylgalactosamine residue, it could not bind to any part of the testis.

GS-I-B_4 — This lectin specific to α-galactose residue could not bind to any part of the testis.

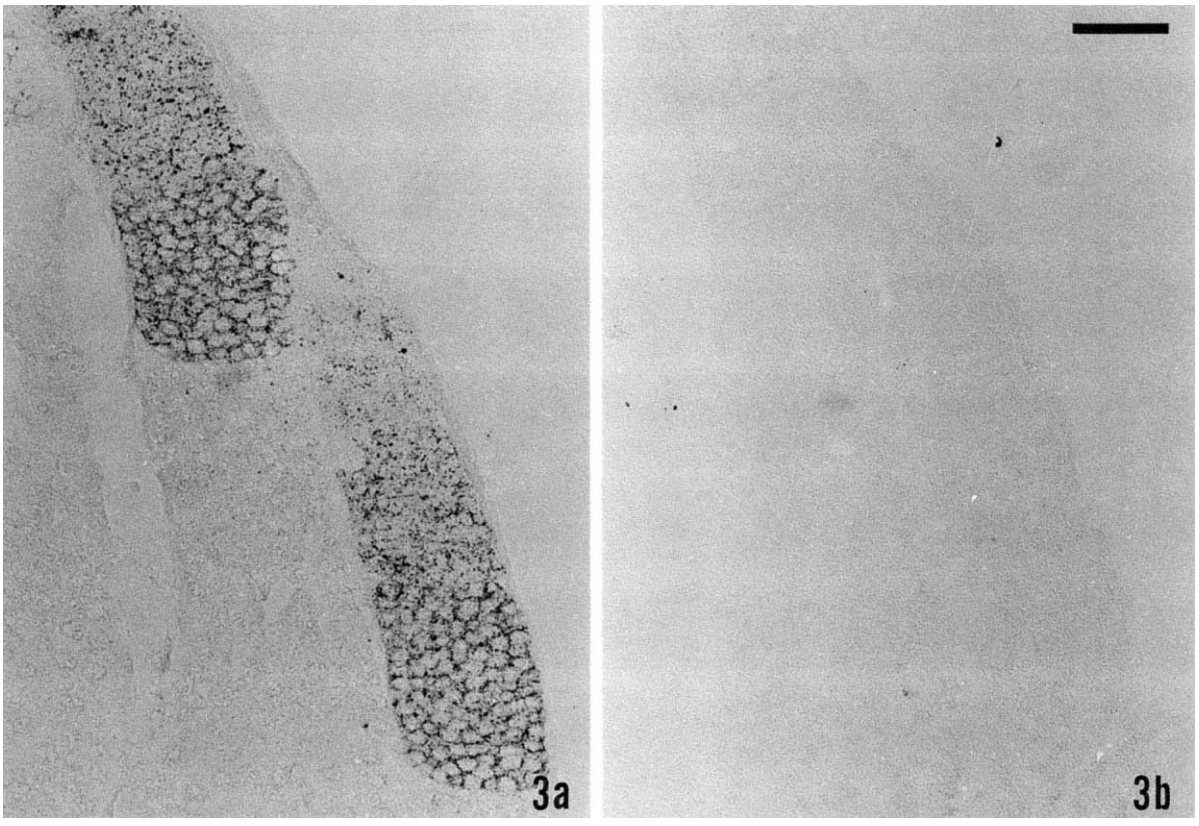

Fig. 3. DBA staining after treatment without (**a**) and with (**b**) PNGase F. Scale bar, 50 μm.

RCA-I — Various granules or vesicles are stained with RCA-I in various germ cell stages. In early-spermatocytes, cell surface and granular structures were strongly positive (Fig. 6a). After this stage the staining intensity decreased. In spermatids, granular structures, that might be acrosomes and/or Golgi bodies, reacted to this lectin (Fig. 6c). Cyst walls, testis follicle epithelia, and the testis sheath were also stained. Endo F/PNGase F treatment halted positive responses in early-spermatocytes and cyst cells (Fig. 6b), but had no effect on spermatids, testis sheath and follicle cells. Hyaluronidase treatment reduced the staining of granular structures in spermatids (Fig. 6d).

PNA — Testicular germ cells and cyst cells were all negative against PNA on sections without enzyme treatment. The testis sheath and testis follicle walls, however, showed strong affinity to this lectin. At the tip of a testis follicle, a weakly stained cell was observed with surrounding negative cells (Fig. 7). Endo F/PNGase F treatment had no effect. Hyaluronidase treatment produced a weak binding with PNA to the nuclei of spermatogonia and spermatocytes.

Mannose/glucose-binding lectins

Con A — This lectin bound to most areas, except nuclei, of the testis and showed non-stage specific staining patterns without enzyme treatment (Fig. 8a, c). After incubation with Endo F/PNGase F, Con A could not bind to spermatogonia and early-spermatocytes (Fig. 8b). Cell surfaces or extracellular matrices of mid-spermatocytes and later stages were positive after enzyme treatment. Hyaluronidase treatment caused reduced staining of the granular structures and revealed weak staining in the nuclei in germ cells (Fig. 8d).

LCA — Several granular structures in spermatogonia and early-spermatocytes were weakly positive. In mid-spermatocytes many granules and the cell surface showed strong affinity to LCA (Fig. 9a, c). The cell surface, extracellular matrices and granular structures in late-spermatocytes reacted weakly. The spermatid cell surface showed a weak response but no granular structure was observed in it. The testis sheath, testis follicle epithelium and cyst cells were also stained. Pretreatment with PNGase F diminished the staining in spermatogonia, early-spermatocytes, granular structures in late-spermatocytes, the testis sheath, and follicle epithelia (Fig. 9b). Hyaluronidase treatment revealed weak staining in spermatogonial and spermatocyte nuclei (Fig. 9d).

PSA — Although the reactivity of granular structures in late-spermatocytes to PSA was relatively weak, the staining pattern was very similar to that with LCA.

Fucose-binding lectins

AAA — This lectin bound weakly but specifically to mid- and late-spermatocytes at a concentration of 20 μg/ml (Fig. 10a).

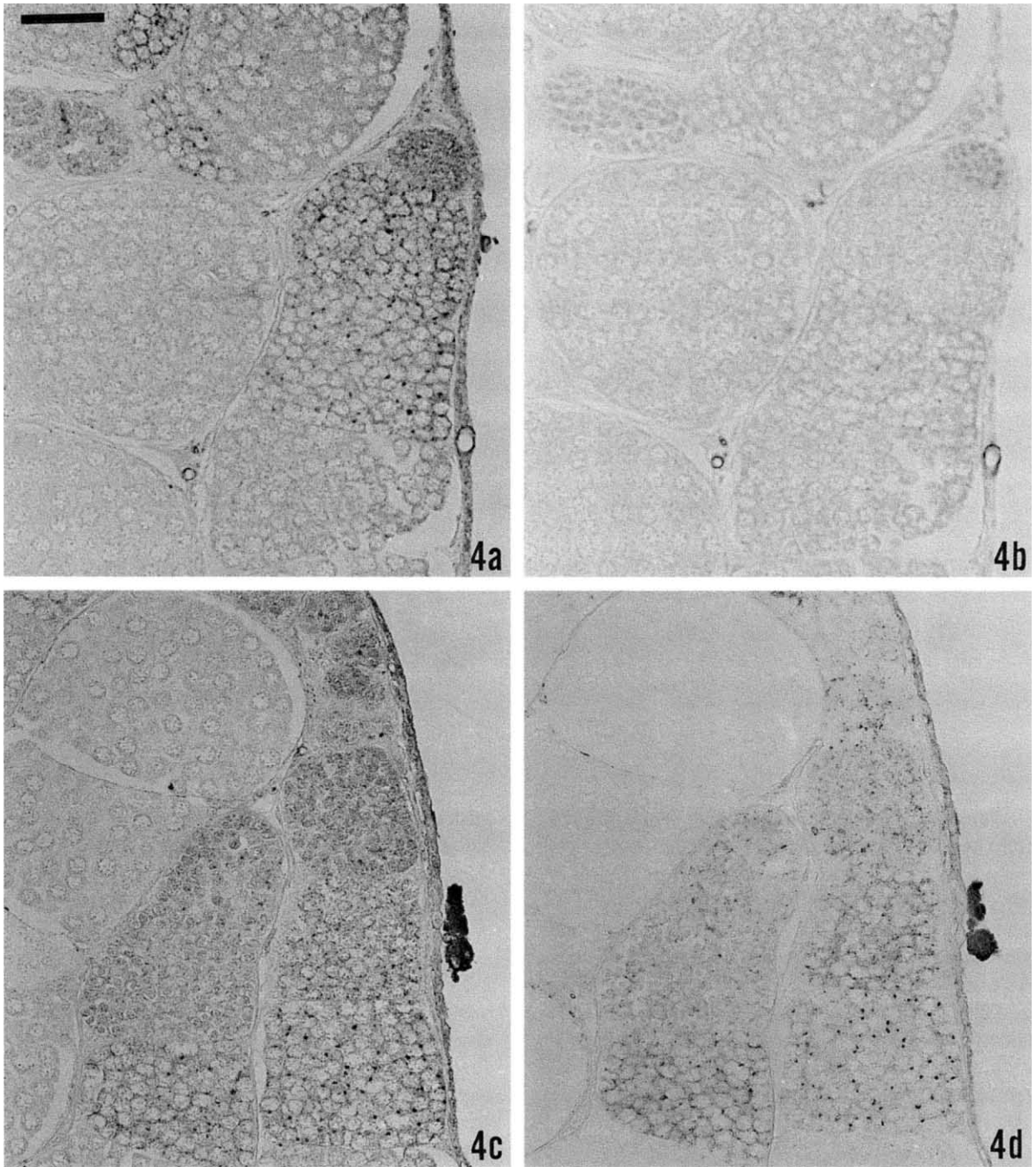

Fig. 4. Enzyme treatment and LBA staining. Endo F/PNGase F (**b**) and the control (**a**), hyaluronidase (**d**) and the control (**c**). Scale bar, 50 μm.

This binding was inhibited by adding 0.1 M fucose, or by hyaluronidase treatment, but not by α-fucosidase from bovine kidney (Table 3). After treatment with Endo F/PNGase F, the spermatocytes displayed strong staining with AAA (Fig. 10b). This staining pattern was similar to that with LCA and PSA after Endo F/PNGase F or PNGase F treatment. The response was inhibited by preincubation of AAA with 0.1 M fucose. Staining was not observed in sections incubated with α-fucosidase from bovine kidney after Endo F/PNGase F treatment (Table 3).

UEA-I — Although this lectin showed no staining in any region without enzyme treatment, it could stain spermatocytes treated with Endo F/PNGase F as the case with AAA. This binding was inhibited by adding fucose to the lectin solution (Table 3).

Sialic acid-binding lectin

LFA — Germ cells showed no reactivity to LFA. Testis sheath epithelium was weakly stained. This staining, however, seemed to be due to non-specific binding, because preincubation of LFA with 0.1 M of *N*-acetylneuraminic acid was not

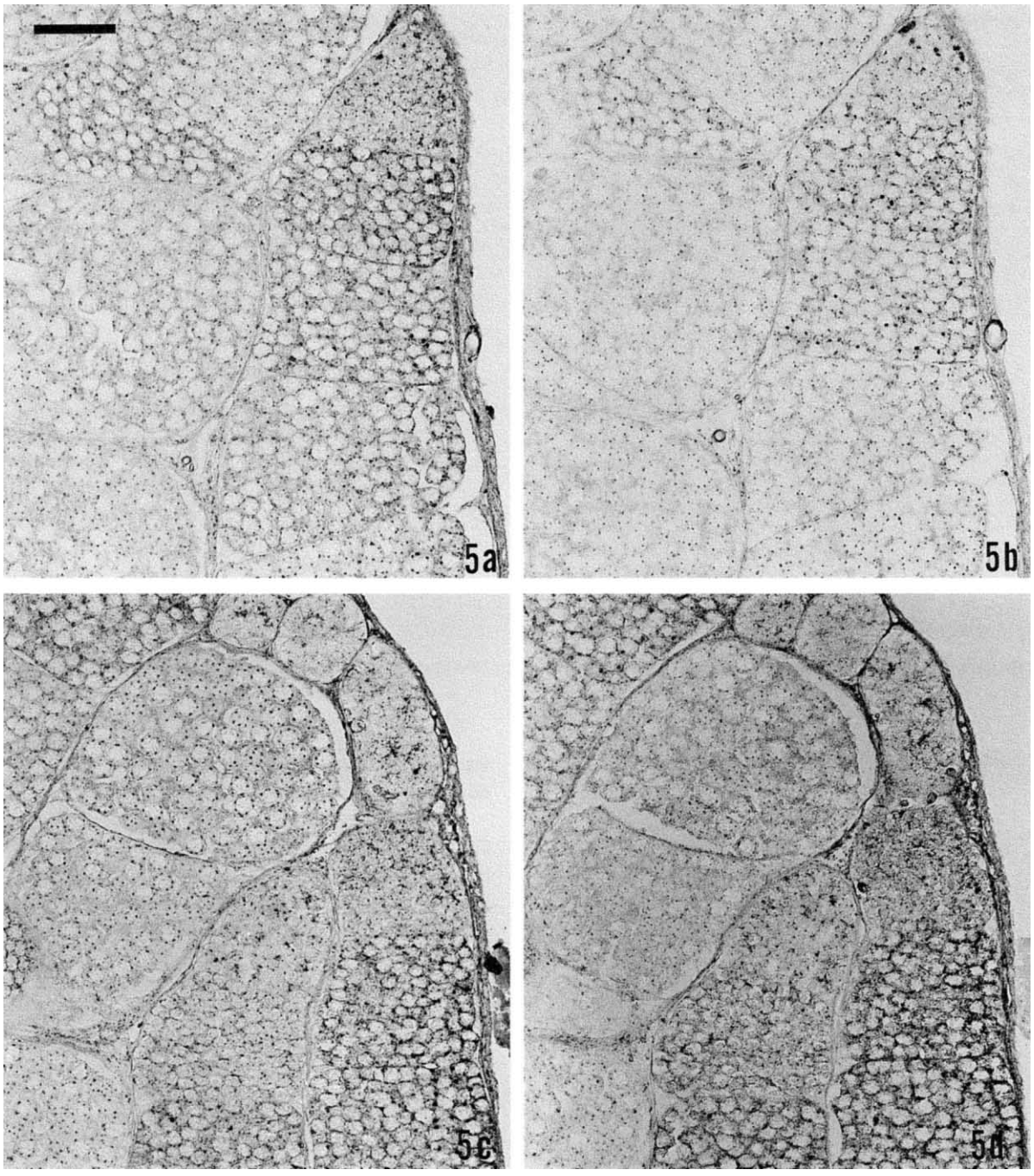

Fig. 5. Enzyme treatment and HPA staining. Endo F/PNGase F (**b**) and the control (**a**), hyaluronidase (**d**) and the control (**c**). Scale bar, 50 μm.

able to reduce the staining intensity.

DISCUSSION

A large number of germ cells in insect testes develop synchronously in each spermatocyst. In cricket testicular follicles of 6th- and early 7th-instar nymph, we can observe a tandem 'suite' containing several stages of primary spermatocytes. This provides a great advantage, in contrast with those in mammalian testes, in comparing developing spermatocyte stages. As for mammalian spermatogenesis, there are sparse descriptions about glycoconjugate changes during the long time span of the primary spermatocyte stage among the accumulation of lectin histochemical studies, although several works (Kanai *et al.*, 1989; Malmi *et al.*, 1990; Ertl and Wrobel, 1992; Jones *et al.*, 1993) referred to those during development from spermatogonia to spermatocytes. The fractional description in a study on lizard spermatogenesis (Labate and Desantis, 1995) is likely the only one that mentioned certain changes in the intensity of lectin binding

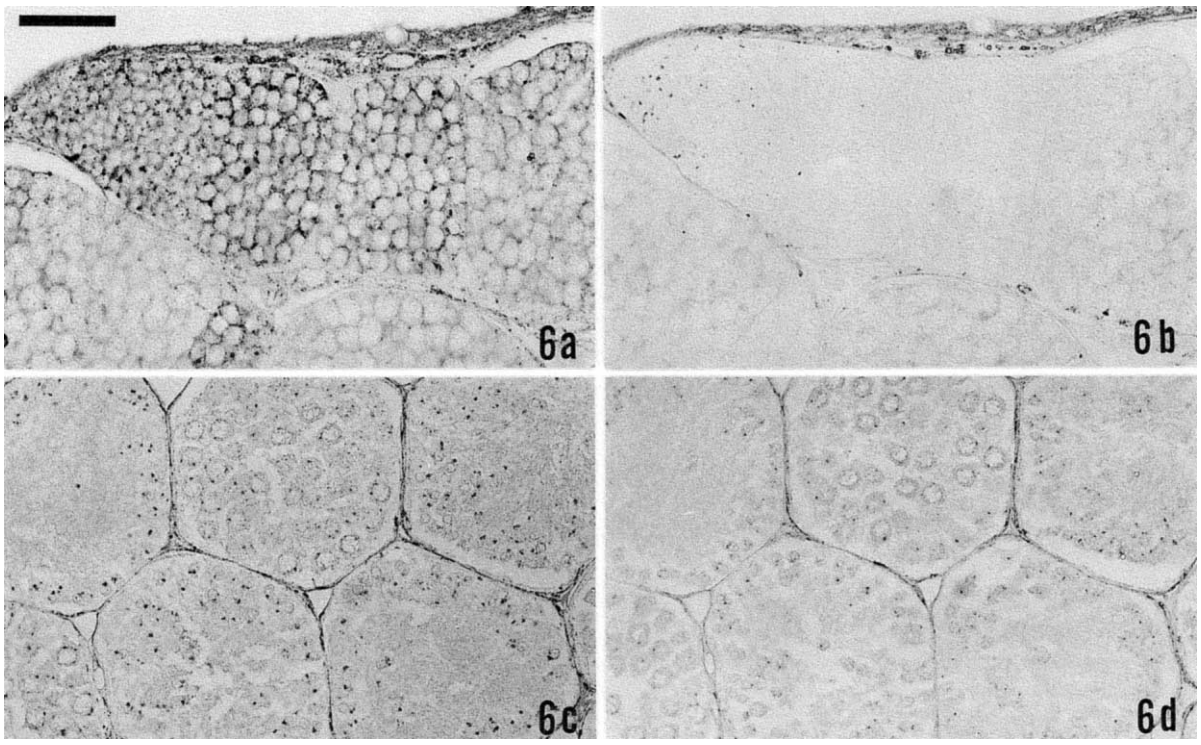

Fig. 6. Enzyme treatment and RCA-I staining. Endo F/PNGase F (**b**) and the control (**a**), hyaluronidase (**d**) and the control (**c**). Scale bar, 50 μm.

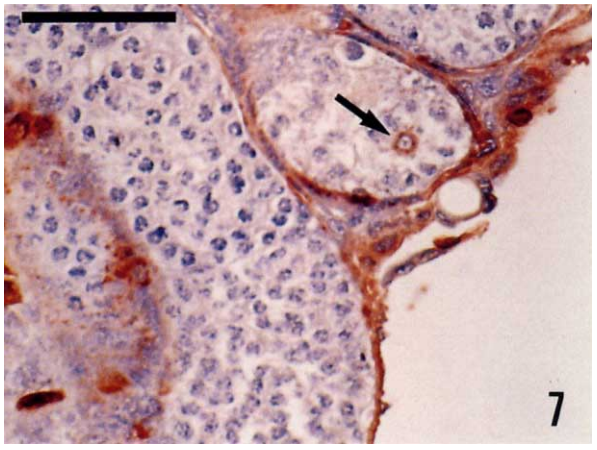

Fig. 7. At the tip of a follicle, a cell (arrow) showed positive response with PNA. Germ cells and cyst cells showed no positive response with this lectin. Counterstained with hematoxylin. Scale bar, 50 μm.

during spermatocytes. Thus the present study focused on the early stages of spermatogenesis, from spermatogonium to early spermatid. We employed two kinds of enzymes, PNGase F and hyaluronidase, and a panel of lectins. Pretreatment with these enzymes provided useful informations about the type of the carbohydrate chain. Consequently, the present observation offers the following new perspectives for spermatogenesis research.

Early-spermatocytes are conspicuous by their N-linked carbohydrates with terminal αGalNAc

Several *N*-acetylgalactosamine-binding lectins such as DBA, SBA, HPA and LBA showed early-spermatocyte-specific binding patterns. The most typical pattern was observed with DBA. These stage-specific staining patterns were sensitive to PNGase F. This endoglycosidase shows broad substrate specificity and hydrolyzes major oligosaccharide classes of *N*-linked glycans (Tarentino and Plummer, 1994). It was noted in early-spermatocytes that the binding activity to mannose-binding lectins, Con A, LCA and PSA, was also sensitive to this enzyme treatment, and the negative pattern was similar to showing a negative film of the DBA-positive pattern (Figs. 3a, 9b). These facts suggest that the glycoconjugates in early-spermatocytes showing this staining pattern with DBA, SBA, HPA and LBA have either complex- or hybrid-type *N*-linked oligosaccharide containing terminal α-*N*-acetylgalactosamine residue. The similarity of the staining pattern with RCA-I in the spermatocytes suggests that several glycoconjugates that showed the early-spermatocyte-specific pattern have a terminal βGal.

Although DBA showed no positive response in late-spermatocytes, other GalNAc-responsive lectins, such as SBA and HPA, bound to these cells. Such differences in the GalNAc-responsibe lectins may be explained by the expression of different carbohydrates in cricket testes. DBA binds strongly to Forssman and blood group A antigens, but it has little affinity with terminal GalNAcα1-4Galβ1-, βGalNAc- and βGal-, which are recognized by SBA and HPA (Piller *et*

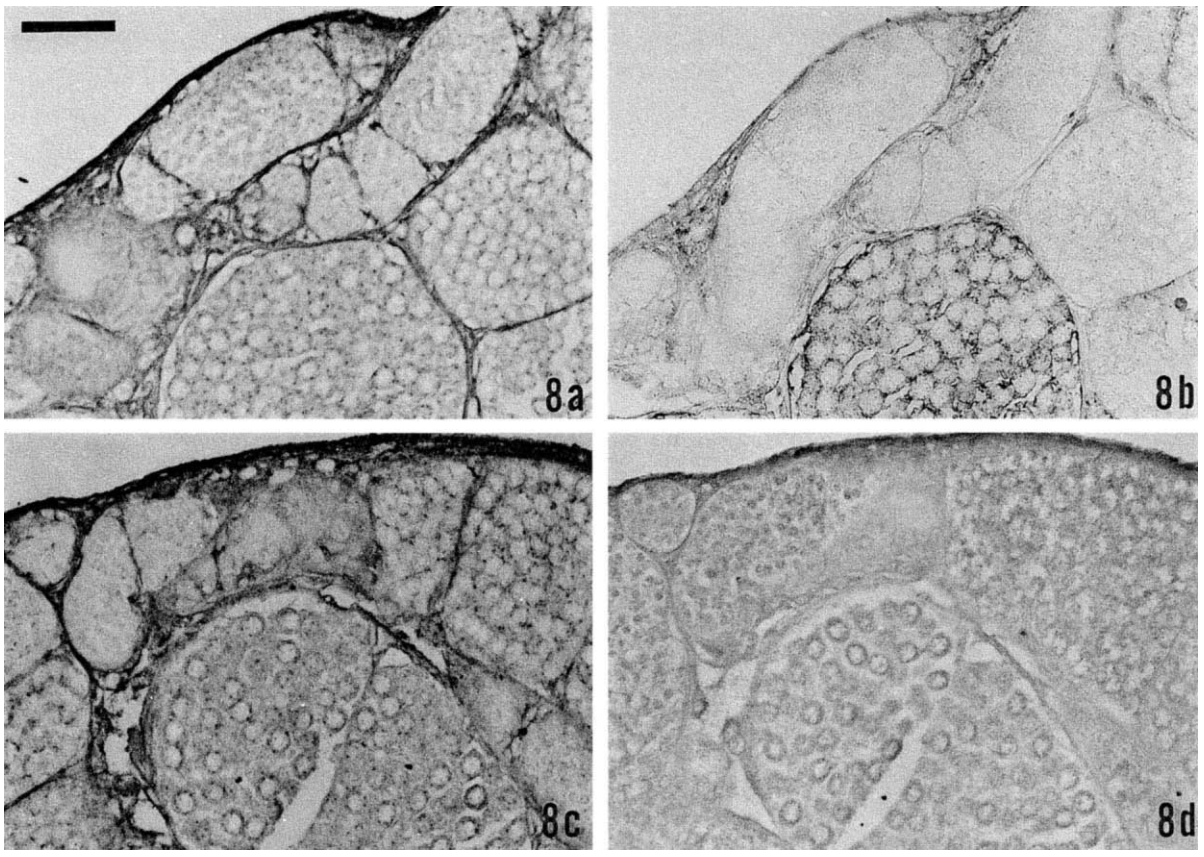

Fig. 8. Enzyme treatment and Con A staining. Endo F/PNGase F (**b**) and the control (**a**), hyaluronidase (**d**) and the control (**c**). Scale bar, 50 μm.

al., 1990).

Mid- and late-spermatocytes express transitory PNGase F-resistant glycoconjugates

The staining patterns in mid- and late-spermatocytes with lectins as LCA, PSA, Con A, AAA and UEA-I after PNGase F or Endo F/PNGase F treatment, gave unexpectedly remarkable results. LCA and PSA are well known to be specific to complex-type *N*-linked oligosaccharides (Debray *et al.*, 1981; Kobata and Yamashita, 1993), which are mostly susceptible to PNGase F (Tarentino and Plummer, 1994). Consequently, the LCA/PSA-positive glycoconjugates which are expressed after the mid-spermatocyte stage may have an uncommon type of sugar chain. The similarity of the LCA/PSA-pattern and AAA/UEA-I-pattern suggests the presence of fucosyl residue in these glycoconjugates. The α1-3-linked fucose that substituted on the asparagine-proximal GalNAc is the only known structural feature of an oligosaccharide moiety that confers resistance to PNGase F (Tretter *et al.*, 1991; Tarentino and Plummer, 1994). This structure, which is uncommon in mammals, may be widely distributed in insects (Kubelka *et al.*, 1995). Therefore, the α1-3-linked fucose in the core region is one of the possiblities for explaining the staining pattern. We must not forget, however, an alternate possibility that the degree of hydrolysis to release the oligosaccharides from glycoproteins may depend on the size and conformation of the peptide portions.

Hyaluronidase-sensitive substance in the nucleus

The weak staining of nuclei with LBA is also of interest in respect to its susceptibility to hyaluronidase. The present result clearly shows that certain hyaluronidase-sensitive substances exist in germ cell nuclei. Though the staining intensity with LBA in spermatogonial nuclei seemed to be stronger than those of spermatocytes at later stages, it may be due to the high degree of chromatin condensation. PNA, Con A and LCA, which had not shown any binding to nuclei, showed faint binding to nuclei of spermatogonia and early-spermatocytes after hyaluronidase treatment. These phenomena also suggest the existence of hyaluronidase-sensitive substances that cover these cryptic binding sites.

As reviewed by Hart *et al.* (1989), the presence of nuclear and cytoplasmic glycosaminoglycans has been claimed by several authors. Busch *et al.* (1992) proposed a model for the nuclear glycosaminoglycan modulation of transcription factor activity. Recently a novel hyaluronan-binding protein revealed its homology to Cdc37, a cell cycle control factor from yeast and *Drosophila* (Grammatikakis *et al.*, 1995). Thus the distribution of hyaluronidase-sensitive substances in the nuclei may suggest that certain glycosaminoglycans participate in

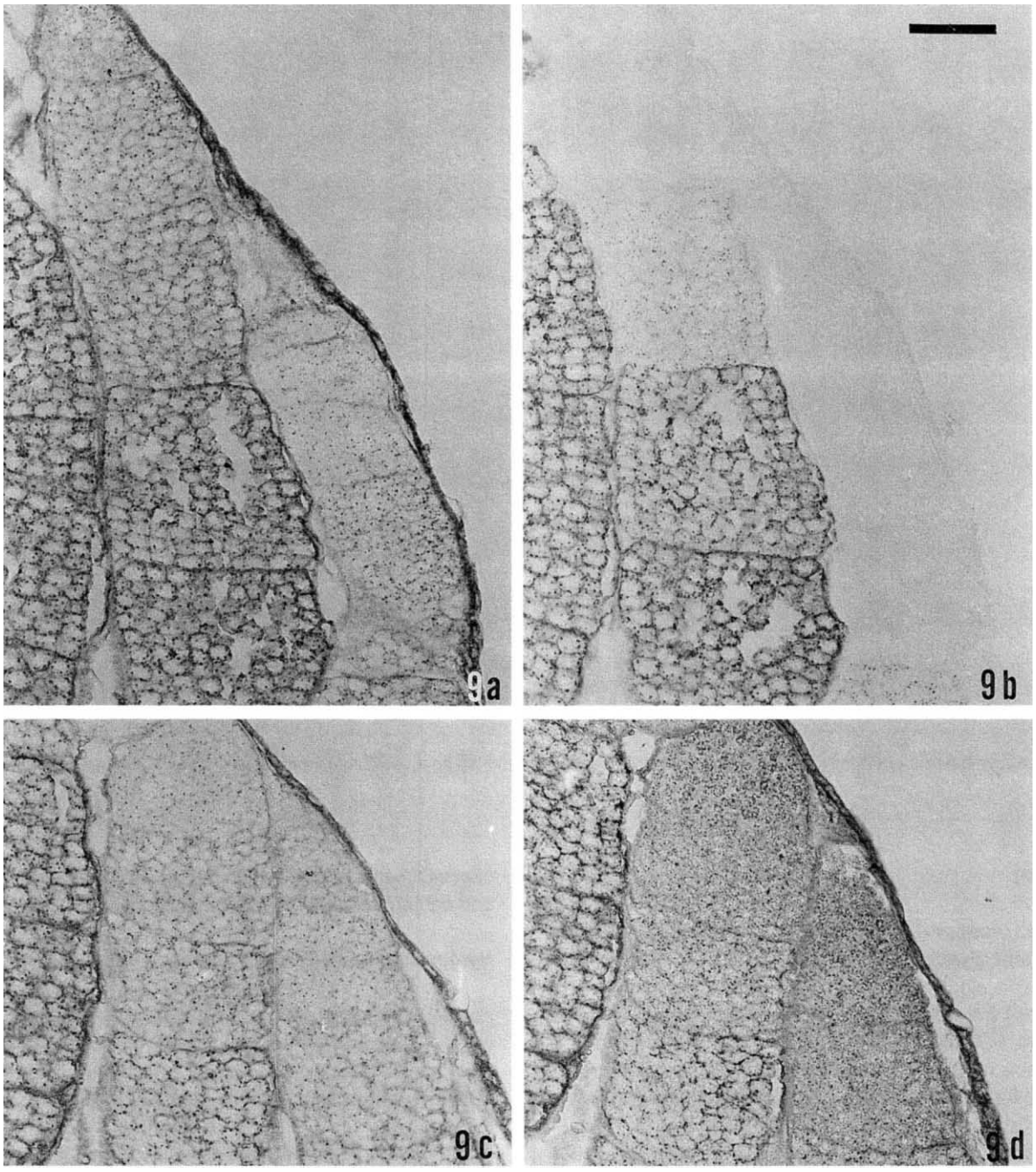

Fig. 9. Enzyme treatment and LCA staining. PNGase F (**b**) and the control (**a**), hyaluronidase (**d**) and the control (**c**). Scale bar, 50 μm.

spermatogonial mitosis and meiosis. It is not yet clear, however, which moiety of the substance was recognized by LBA, because it has been known that LBA has adenine binding sites and some hydrophobic binding properties (Roberts and Goldstein, 1983) as well as specific binding to blood group A oligosaccharide chain (Sikder *et al.*, 1986).

Considerations on negative responses

In contrast to the stainability with other GalNAc/Gal-binding lectins, GS-I-B_4 and SJA showed no binding to any part of cricket testis. As GS-I-B_4 has a strong affinity with terminal Galα1-3Galβ1-4GlcNAcβ1- sequence (Wood *et al.*, 1979; Kobata and Yamashita, 1993), cricket testis may have no glycoconjugates with this terminal sequence. PNA never showed affinity with testicular germ cells. This lectin, however, showed very specific binding patterns on somatic cells except cyst cells. This specific stainability may afford a unique marker to study the interaction between somatic cells and testicular germ cells. Although PNA has relatively strict specificity with terminal Galβ1-3GalNAc sequence (Hennigar *et al.*, 1987),

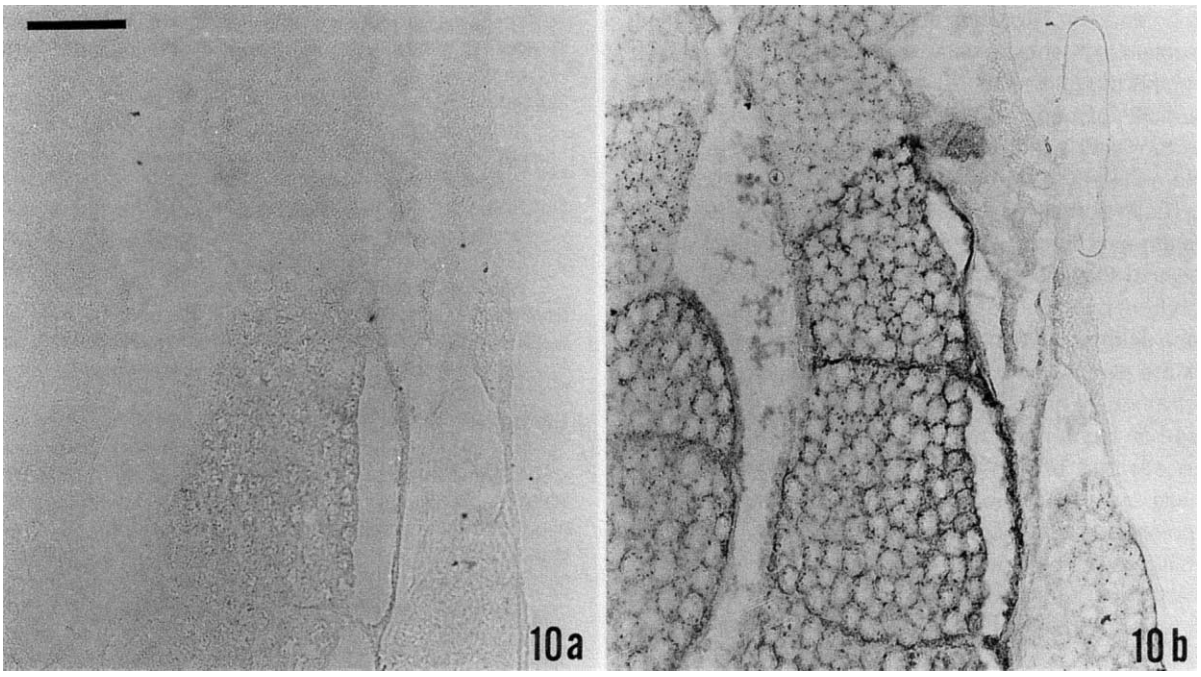

Fig. 10. AAA staining after treatment without (**a**) and with (**b**) Endo F/PNGase F. Scale bar, 50 μm.

Table 3. Binding of fucose-specific lectin to mid- and late-spermatocytes

Enzyme treatment[a]		Inhibitor[b]	Reactivity to lectin	
1st	2nd		AAA	UEA-I
1) –	–	–	+	–
–	–	Fuc	–	–
2) Fucosidase	–	–	+	–
None	–	–	+	–
3) EndoF/PNGaseF	–	–	++	+
EndoF/PNGaseF	–	Fuc	–	–
None	–	–	+	–
4) EndoF/PNGaseF	None	–	++	+
EndoF/PNGaseF	Fucosidase	–	+	–

[a] Sections were treated with fucosidase or EndoF/PNGase F before lectin labelling. None, control incubation with corresponding enzyme-free buffer; –, no treatment.
[b] Lectins (10 μg/ml) were preincubated without (–) or with 0.1 M L-fucose (Fuc) for 30 min before use.

the negative results with SJA, which also has affinity with a similar sequence, suggest a presence of another kind of oligosaccharide chain in somatic cells.

We observed no positive response with LFA in the germ cells. Although weak binding was shown in testis sheath cells, we did not investigate the LFA-binding further in this experiment because the staining was not inhibited by 0.1 M *N*-acetylneuraminic acid. Sialic acids are the major component of vertebrate glycoproteins, but its occurrence in insects had long been doubtful until recent demonstrations in *Drosophila* embryo (Roth *et al.*, 1992) and an immune protein from *Manduca sexta* (Kyriakides *et al.*, 1995). There are some reports about the absence of sialic acid in the glycoprotein expressed in insect cell lines, followed by a recent work (Davis and Wood, 1995) that reports sialic acid in certain cell lines from Lepidoptera origin. The last work, however, only observed the binding of sialic acid-binding lectin to the proteins blotted on membrane without any control experiments. We should give careful consideration to the result of our lectin staining, especially that of sialic acid-binding lectin. As far as the *Gryllus* testes fixed with Bouin's solution are concerned, there is little possibility of existence of sialic acids in their germ cells.

Glycoconjugates expressed in early stages of spermatogenesis

Although the cell localization of lectin-binding substances suggests their important role in cricket spermatogenesis as described above, the molecular nature of such glycoconjugates

is yet to be studied. There are only a few reports concerning glycoproteins that show similar stage-specific behavior as shown in this study. Koshimizu *et al.* (1993) characterized a stage-specific glycoprotein by monoclonal antibodies. The glycoprotein was expressed on mouse spermatocytes at zygotene and early pachytene, but was not detected at later stages. Their results show a similar transition process to the DBA-positive pattern in the cricket testis. O'Brien (1987) reported that three proteins were synthesized during the transition from leptotene to zygotene and five proteins during pachytene spermatocytes, while it is unknown whether these proteins are glycosylated. C-*kit* protein, a receptor tyrosine kinase having *N*-linked carbohydrates, is known to be expressed on the surface of type A to type B spermatogonia and the earliest preleptotene spermatocytes of mice (Yoshinaga *et al.*, 1991). Some nuclear proto-oncogenes were also expressed in type B spermatogonia and may play a role in switching the cell cycle into meiotic prophase (Wolfes *et al.*, 1989). After entry into meiosis, a male germ cell-specific calcium-binding protein is expressed in the cytoplasm from pachytene spermatocyte to elongated spermatid stages (Watanabe *et al.*, 1994). Although these molecules cited above are all from mammalian testicular germ cells, it is possible that cricket glycoconjugates expressed in early spermatogenic stages can be their insect homologues. We are further exploring the molecular nature of glycoconjugates. Their precise localization at the electron microscopic level is also being investigated.

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