Soybean Agglutinin Binding in Germ Cells during Spermatogenesis of the Cricket, Gryllus bimaculatus

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Soybean Agglutinin Binding in Germ Cells during Spermatogenesis of the Cricket, *Gryllus bimaculatus*

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ABSTRACT—The sugar chain expression in germ cells of a cricket, *Gryllus bimaculatus*, during spermatogenesis was cytochemically followed using soybean agglutinin (SBA) as a probe. Affinities to SBA were tested on paraffin sections of the testes fixed in Bouin's solution with biotinylated SBA and streptavidin-peroxidase. SBA showed spermatogenesis stage-specific binding. The most intense binding activity appeared at the early meiotic prophase of the spermatocytes, with the activity decreasing at later stages of spermatocytes. Through electron microscopic analysis, it was elucidated that SBA bound to the cell surface and some clusters of dense bodies with a multilaminar structure. At the stage of round spermatids, SBA bound to Golgi apparatus. Treatment of the section with α-*N*-acetylgalactosaminidase before cytochemistry resulted in the disappearance of most of the binding activity. These results suggest that glycoconjugates with terminal α-*N*-acetylgalactosamine take part in cricket spermatogenesis during meiotic prophase and acrosome formation.

INTRODUCTION

Cells have a number of glycoconjugates such as glycoproteins, glycolipids and proteoglycans, some of which are known to play important roles in cellular events [10]. The same statement has been verified in several processes of male germ cells. For example, the binding of spermatozoa to an egg is likely mediated by carbohydrate moieties of the egg surface [22]. Mammalian sperm surfaces are variously modified during maturation processes [11], something that has been thought to play a role in the capacitation of fertility. It has also been thought that glycoproteins of developing germ cells have a function in the interaction between germ cells and Sertoli cells [1, 18].

Although investigations into insect spermatogenesis have been accumulated, our knowledge about the glycoconjugates of male germ cells of insects is quite limited. Investigation on *Drosophila melanogaster* has shown the presence of concanavalin A (Con A)-binding activity on the surface of spermatozoa [17]. Esponda and Guerra [6] showed the distribution of labelling with ConA and wheat germ agglutinin (WGA) on the surface of Orthopteran spermatids. These previous studies were devoted mainly to the glycoproteins in the acrosome or those on mature spermatozoon, which might be concerned in fertilization. We are interested in how sugar chains are expressed in the entire process of the spermatogenesis.

In the present study, we investigated cricket spermatogenesis from spermatogonia to early spermatids using a histochemical approach with a lectin, soybean agglutinin (SBA). We make description of the stainability during spermatogenesis and of the cellular structures on which SBA-binding activities were located. These observations and results of enzyme-treatment experiments suggested that some glycoconjugates with terminal α-*N*-acetylgalactosamine participate in the spermatogenesis of crickets.

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MATERIALS AND METHODS

Tissue processing

Crickets, *Gryllus bimaculatus*, were reared on an artificial mouse diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan). Testes were dissected from animals, washed in PBS and processed as follows: For light microscopy, they were fixed in Bouin's solution for 2–3 hr, dehydrated in ethanol, embedded in paraffin, and 5-μm sections were obtained. They were then dewaxed and treated for lectin cytochemistry. For lectin-gold electron microscopy, testes were fixed in picric acid-paraformaldehyde solution [19] overnight at 4°C and washed with 50 mM NH_4Cl in PBS for 30 min. The specimens were dehydrated in ethanol and embedded in Lowicryl K4M (Chemische Werke Lowi, Waldkraiburg, Germany) at 4°C. Ultrathin sections were mounted on nickel grids.

Lectin cytochemistry

Light microscopy: The dewaxed sections were soaked in Tris-HCl buffered saline (TBS) pH 7.4 and the non-specific sites were blocked with TBS containing 1% BSA (BSA-TBS) for 30 min. After the pretreatment, the sections were incubated for 1 hr with biotinylated SBA (Vector Laboratories, Inc., Burlingame, CA, USA) at 20 μg/ml diluted in BSA-TBS. After washing with TBS, they were then incubated with peroxidase-labeled streptavidin (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) at 1:200 dilution for 30 min. Development was carried out after final washing with TBS using 0.02% 3-amino-9-ethyl-carbasol in 50 mM acetate buffer pH 5.0 containing 0.03% H_2O_2. All incubations were carried out at room temperature. Sections were counterstained with Mayer's hematoxylin, if necessary.

Electron microscopy: Ultrathin sections on nickel grids were incubated for 15 min on a drop of BSA-TBS and then with biotiny-

lated SBA for 1 hr. After washing with TBS, they were incubated on anti-biotin antibody conjugated with 15 nm-gold particle (BioCell Research Laboratories, Cardiff, UK) diluted in TBS (1:20) for 30 min. After gold-labelling, they were washed in TBS and distilled water. Thin sections were counterstained with uranyl acetate (5 min) and Millonig's lead acetate [16] (1 min), and observed in a JEOL 100 S electron microscope at 80 kV.

Pretreatments of the paraffin sections

Enzyme treatment: Paraffin sections were incubated with various enzymes before lectin cytochemistry. The enzymes used in the experiments were as follows: 50 mU/ml of Peptide N-glycosidase F from *Flavobacterium meningosepticum* (Oxford GlycoSystems Ltd, Oxon, UK) in 20 mM phosphate buffer, pH 7.5, containing 50 mM EDTA (24 hr, 37°C); 20 mU/ml of glycopeptidase A from almond (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) in citric acid-phosphate buffer, pH 5.0 (24 hr, 37°C); 1.0 U/ml of α-*N*-acetylgalactosaminidase from squid liver (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) in 50 mM sodium citrate buffer, pH 3.5; 2.5 U/ml of β-*N*-acetylhexosaminidase from jack beans (Sigma Chemical Co., St. Louis, MO, USA) in 50 mM sodium citrate buffer, pH 3.5 (24 hr, 37°C); 0.5% hyaluronidase from bovine testes (Sigma) in 0.1 M phosphate buffer, pH 6.0 (18 hr, 37°C). Control sections were incubated with enzyme-free buffers.

Alkaline treatment: The β-elimination reaction for the removal of *O*-glycosidically linked oligosaccharide side chains [4] was done on the paraffin section. Sections were incubated in 0.1 N NaOH (48 hr at 37°C or 15 hr at 45°C). After extensive rinses with TBS the sections were treated for lectin cytochemistry.

Chloroform/methanol treatment: In order to extract the glycolipids from the sections, they were treated with chloroform/methanol (2:1, by vol) for 10 min [21]. After serial rinses with methanol and water they were treated for lectin cytochemistry.

RESULTS

SBA binding to paraffin sections of germ cells

Gryllus spermatogenic cells develop synchronously in each discrete spermatocyst. Figure 1 shows the paraffin sections of the 6th- (Figs. 1a and 1b), 7th- (Fig. 1c) and the last (8th-) instar (Fig. 1d) testis stained by SBA-cytochemistry. Spermatogenesis stage-specific binding of SBA was apparent. Spermatocysts including cells at earlier stage were located in the outer zone of testes, and stained intensely by SBA. The lectin also bound to the tissues of the

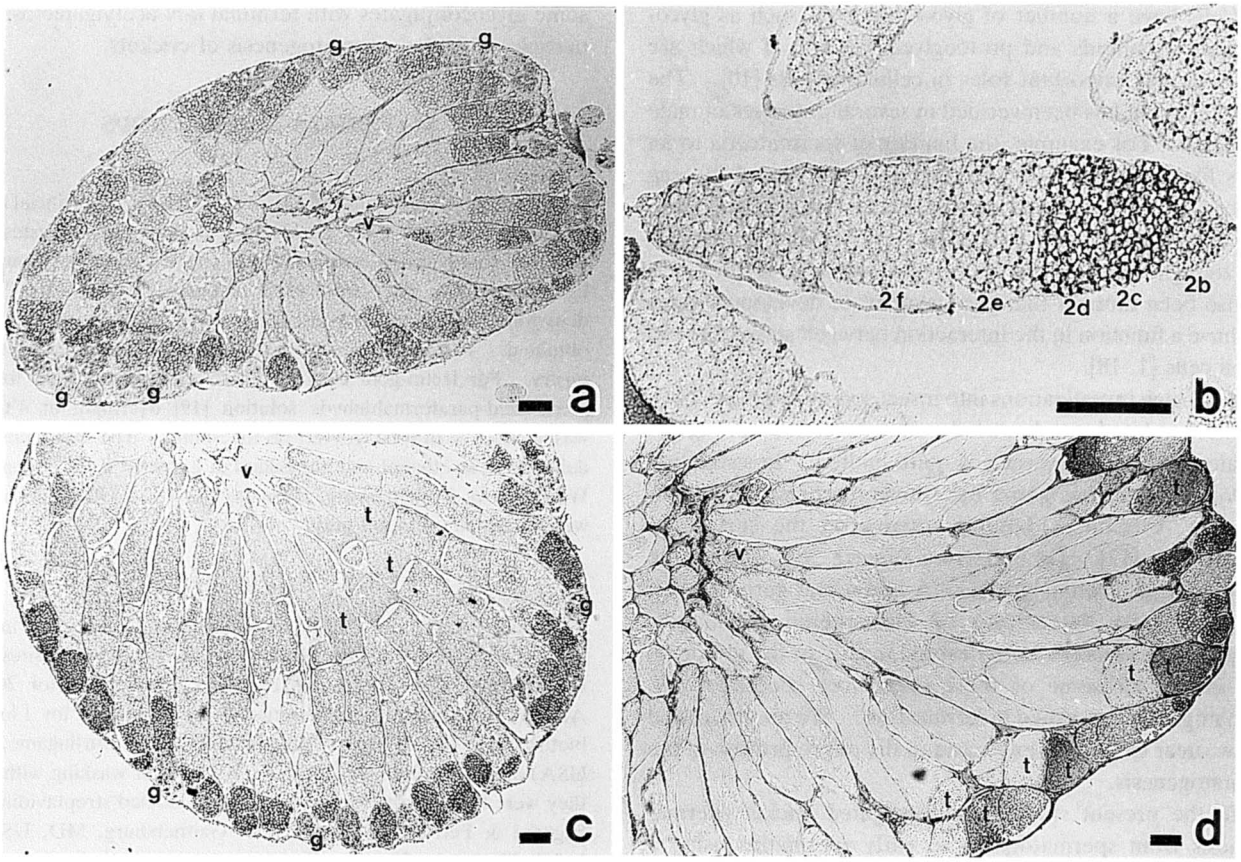

FIG. 1. *Gryllus* testes of 6th- (a and b), 7th- (c) and 8th- (last) instar (d), stained by biotinylated SBA and streptavidin-peroxidase without counterstaining. A large number of testis follicles are bundled in a testis sheath. Each follicle contains some spermatocysts. Spermatocysts of spermatogonia (*g*) are located in the outermost zone and vas efferens (*v*) are arrayed in the internal region. In the 6th-instar testis, most spermatocysts contain primary spermatocytes at prophase. Inner cysts are more developed and stained less than earlier (outer) ones. Spermatids (*t*) appear in the 7th- and 8th-instar testis. The most part of 8th-instar testis are occupied by spermatozoa with long tails which are not stained. Fig. 1b shows a series of cysts in a follicle of a 6th-instar testis. The cysts designated as 2b-2f correspond to those of adjacent section shown in Figs 2b-2f. Scale bars, 100 μm.

testis sheath, testis follicle, cyst walls and tracheae. The long tails of the spermatozoon were not stained (Fig. 1d). Control sections that omitted the incubation with lectin were not stained. Preincubation of biotinylated SBA with *N*-acetylgalactosamine inhibited the binding (Figs. 5a and 5b). The followings are the details about the SBA-binding to male germ cells at each spermatogenic stage.

Spermatogonia: Spermatogonia had a polymorphic nuclei and cytoplasmic polarity. We could often observe in an early cyst that several cells arrayed like a rosette, and that the cytoplasm facing to the center were stained (Fig. 2a). Some stained granules were also found in the cytoplasm at metaphase. The cell surface of spermatogonia was not stained.

Spermatocytes: There are a large number of spermato-

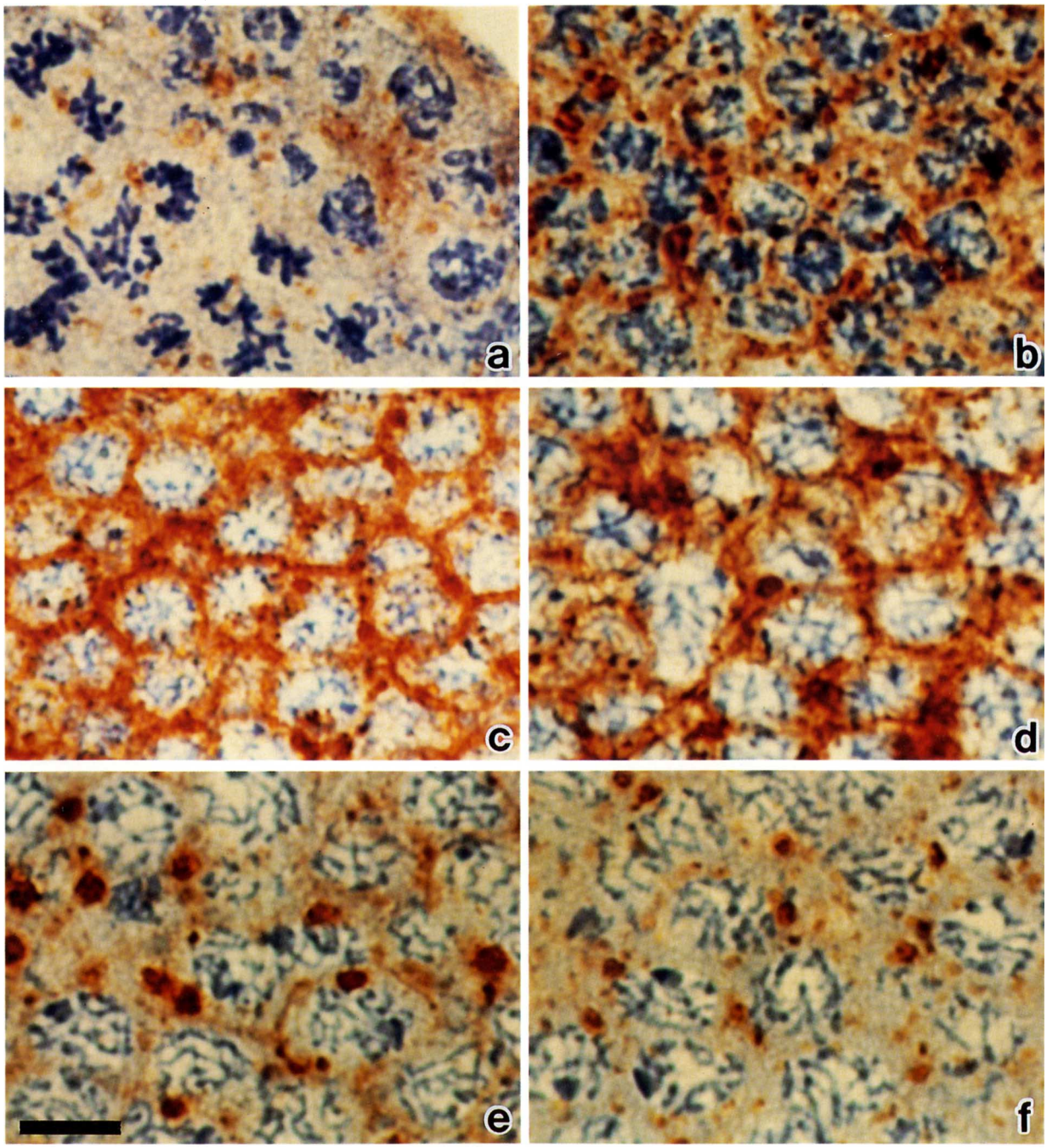

FIG. 2. Spermatogonia (a) and primary spermatocytes (b-f) stained by biotinylated SBA/streptavidin-peroxidase and hematoxylin. a Several cysts of spermatogonia from a 7th-instar testis. Central region of a rosette of spermatogonia is stained (upper right). Spermatogonia at mitotic metaphase are seen on the left side. b-f A series of five cysts in a follicle from a 6th-instar testis (cf. Fig. 1b). The nuclei of preleptotene spermatocyte (b) are similar in shape as spermatogonia. The most intense bindings of SBA are seen in Fig. 2c. After this stage, the intensity of binding tended to reduce and apparently restricted to granular structures (Figs. 2d-f). Scale bar, 10 μm.

cytes in a cricket spermatocyst. When we tried to examine their number in serial sections of two cysts at late prophase, we could count 856 and 984 cells, respectively. Thus more than nine times of sequential mitoses of a spermatogonium lineage in each cyst likely occurred to prepare the spermatocytes. Figures 2b, c, d, e and f show a series of five cysts in a testis follicle. Preleptotene spermatocyte was about 9–10 μm in the major axis and had a polymorphic nucleus as that of a spermatogonium. We could observe many granules intensely stained around the nucleus at this stage (Fig. 2b). The peak of binding intensity of SBA was observed in spermatocytes at an early meiotic prophase stage (Fig. 2c). In these cells, many granules around the nucleus were stained more strongly than in earlier stages. The major axes of

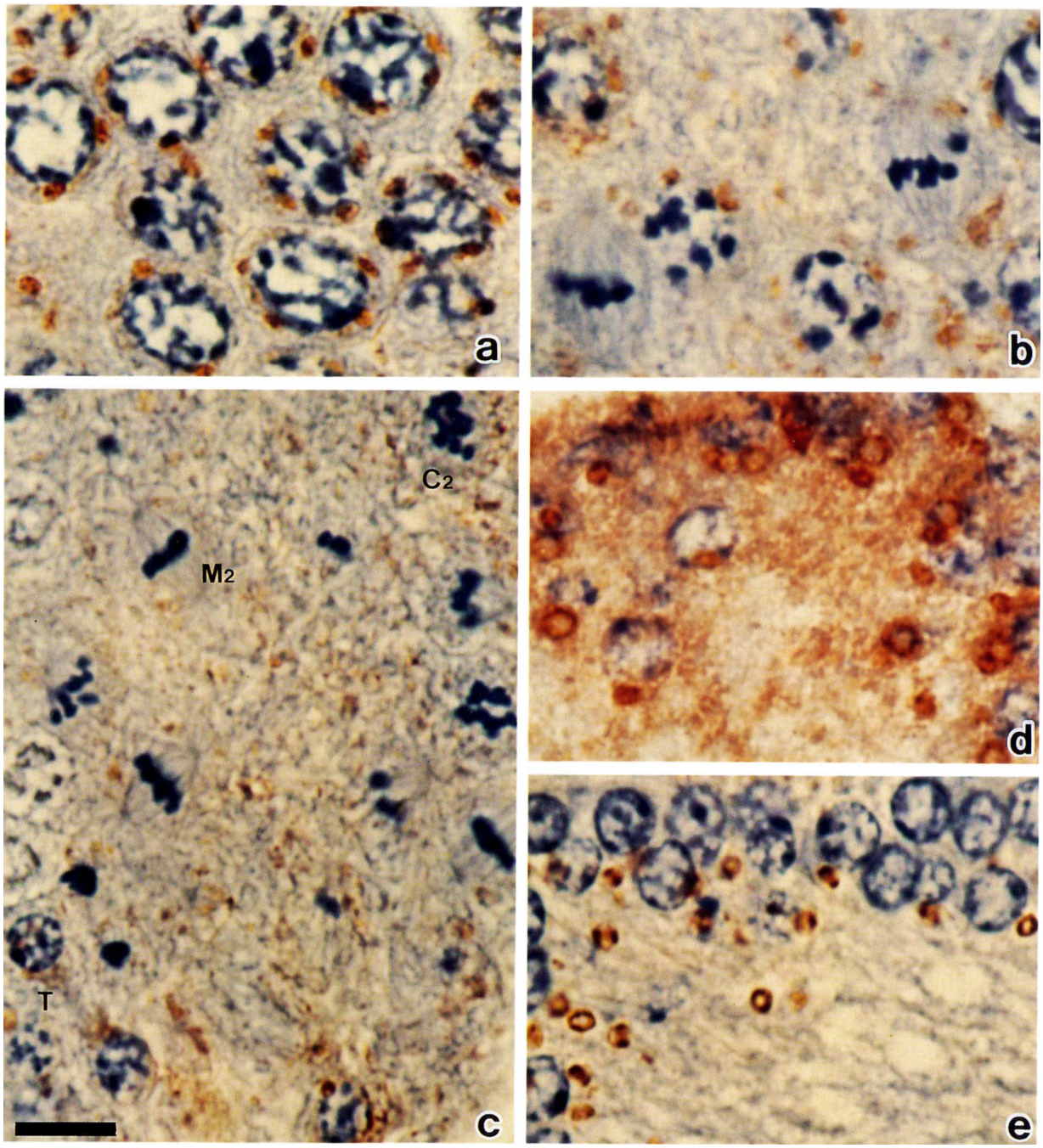

Fig. 3. Spermatogenic cells from late meiotic prophase to early spermatids stained by biotinylated SBA/streptoavidin-peroxidase and hematoxylin. a, b, c and e are from an 8th-instar; d from a 7th-instar testis. a Spermatocytes at later stage than those in Fig. 1f. b Spermatocytes at diakinesis and metaphase of first meiotic division. c Secondary spermatocytes (*C2*), metaphase of secondary devision (*M2*) and spermatids just after meiosis(*T*) exist in a cyst. d Spermatids begining tail elongation. Besides the intense staining of round bodies, the cytoplasm or cell surface is diffusely stained. e Spermatids progressed tail elongation. Scale bar, 10 μm.

these cells and the slightly ellipsoidal nuclei were about 11 μm and 8 μm, respectively. The staining intensity was reduced at later stages (Figs. 2d-f). In cells at the late meiotic prophase, however, the staining of several large granules was remarkable (Figs. 2e and 2f). At these stages, spermatocytes and their nuclei became very large to reach about 18 μm and 13 μm, respectively.

From spermatocytes to early spermatids: SBA-binding in the primary meiotic division was mostly restricted to large granules or vacuolar bodies around the nucleus (Figs. 3a and b), and much the same in the secondary division and in the spermatid which had just completed meiosis (Fig. 3c). In spermatids beginning tail elongation a large vesicular body (ca. 3.5-4 μm in diameter) and another smaller body (ca. 2.5 μm in diameter) near the nucleus were strongly stained. The whole cytoplasm was also stained diffusely (Fig. 3d).

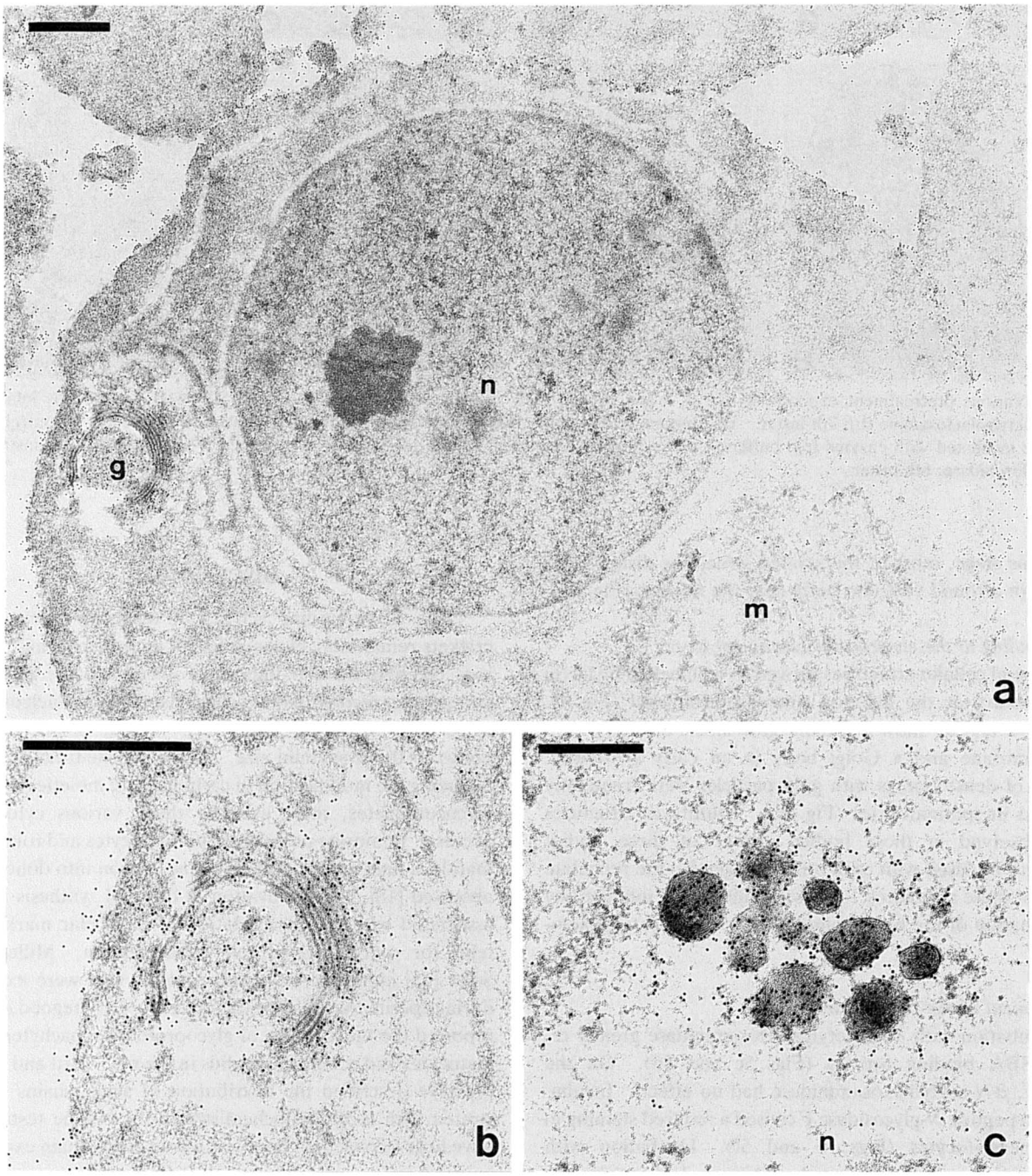

FIG. 4. K4M sections labelled with biotinylated SBA and anti-biotin antibody conjugated with 15 nm-gold particle. a Early spermatid. Golgi body (*g*) and cell surface are intensely labelled. *m*, mitochondrion. *n*, nucleus. Scale bar, 1.0 μm. b Higher magnification of *g* in a. Scale bar, 1.0 μm. c Preleptotene spermatocyte. A cluster of dense bodies near nucleus intensely labelled with gold particles. Scale bar, 0.5 μm.

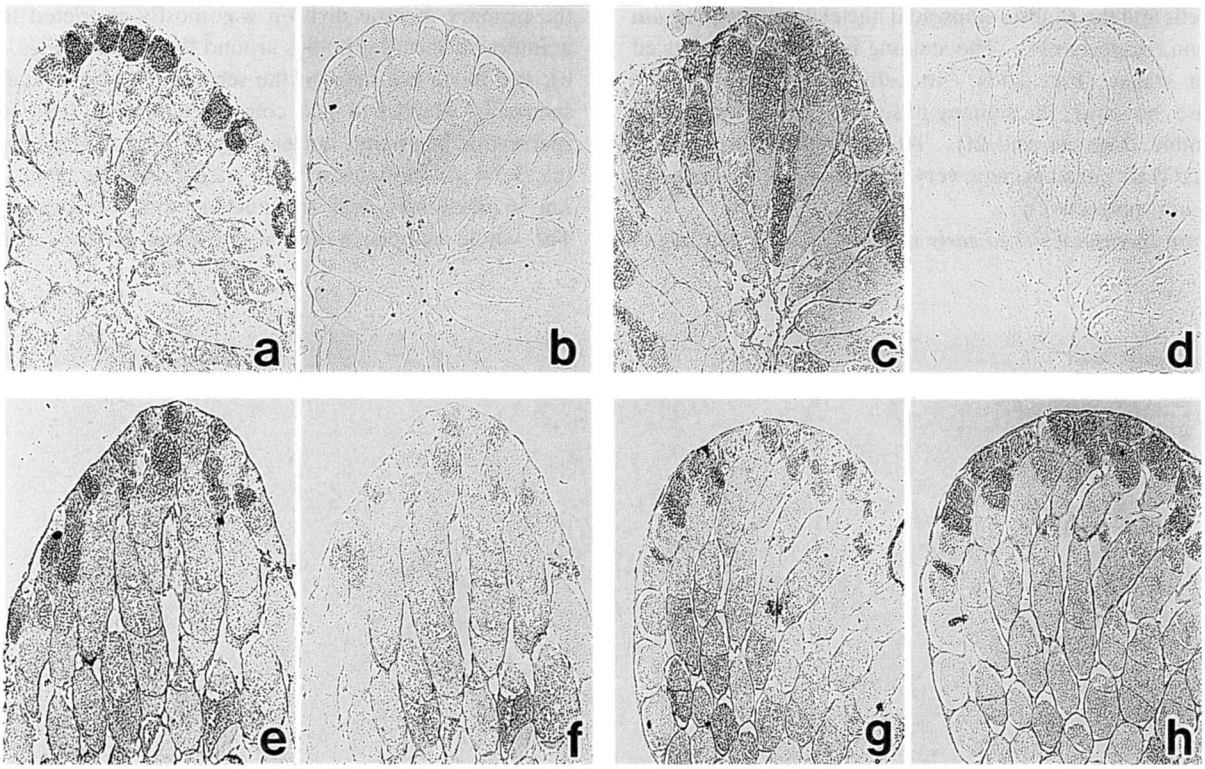

FIG. 5. Various pretreatment experiments (b, d, f and h) and their controls (a, c, e and g). a, b SBA staining (a) was inhibited by *N*-acetylgalactosamine (b); 7th-instar. c-h Enzyme treatments on the paraffin section before the incubation with lectin. Control sections were incubated with enzyme-free buffers. c, d α-*N*-acetylgalactosaminidase; 6th-instar. e, f Peptide *N*-glycosidase F; 7th-instar. g, h Hyaluronidase; 6th-instar.

After this stage, most of the stained structures disappeared except for a round vesicular body near the nucleus (Fig. 3e).

SBA-binding at the electron microscopical level

Several cellular structures proved to be labelled by lectin cytochemistry at the electron microscopical level (Fig. 4). Figures 4a and 4b show abundant gold labels distributed on cell membrane and a Golgi body in an early spermatid. Groups of dense bodies with gold particles were frequently observed in spermatocytes (Fig. 4c). Multilayer structures were observed in these bodies. Some of these bodies seemed to be fused with each other, suggesting the reticulate nature of these structures. These binding were inhibited by preincubation of the biotinylated SBA with *N*-acetylgalactosamine.

Pretreatment of the paraffin sections

Incubation with α-*N*-acetylgalactosaminidase greatly reduced SBA binding activity (Figs. 5c and 5d). On the contrary, β-*N*-acetylhexosaminidase had no effect. Incubation with peptide *N*-glycosidase F caused a reduced staining in early spermatocytes (Figs. 5e and 5f). Incubation with hyaluronidase caused a reduced staining in later spermatocytes (Figs. 5g and 5h). Glycopeptidase A, alkaline treatment, and chloroform/methanol treatment had no effect on the staining intensity under the conditions in this experiment.

DISCUSSION

The SBA binding activity of testicular germ cells of crickets remarkably changed in the meiotic prophase. Various complicated and important phenomena, e.g. synapsis and genetic recombination, take place in the nucleus at this stage. At the same time, the volume of spermatocyte grows to the maximum size. Many biochemical processes, including the modification of carbohydrate moieties of certain glycoconjugates, must underlie these various cytoplasmic events. In murine pachytene spermatocytes and round spermatids, a high rate of acetate incorporation into dolichol was observed [18]. This elevation of dolichol synthesis is likely associated with the glycosylation of particular marker proteins for spermatogenic cell differentiation. Millette and Scott [15] identified such glycoproteins that were expressed during spermatogenesis in the mouse. Grootegoed *et al.* [9] reported the fucosylation of glycoproteins in pachytene spermatocytes and round spermatids in the rat. Ertl and Wrobel [5] have described the distribution of sugar chains demonstrated with lectin histochemistry in the bovine testis. We have little knowledge, however, about sugar chain expression in insect testes. Our observations in the present experiment suggest that the carbohydrate moieties of certain glycoconjugates change their structure during spermatogenesis also in insect.

The glycoconjugates recognized by SBA in the cricket testes are considered to have terminal α-*N*-acetylgalactosamine residue because the lectin binding activity could be removed with the treatment of sections with α-*N*-acetylgalactosaminidase. Terminal α-*N*-acetylgalactosamine residue is known to exist in the ceramide pentasaccharide from the green-bottle fly, *Lucilia caesar* [20], and that from blowfly, *Calliphora vicina* [3]. Although no description on the sugar chain from Orthopteran insects is available now, and non-glycolipid nature was suggested on our SBA binding sites, the data on the glycolipids from other insects might be useful to structural analysis of sugar chains from cricket testes.

Electron microscopical analysis clearly showed that clusters of dense bodies with a multilaminal structure exhibited SBA-binding activity. It is possible that they are similar structures as those termed "yolk-granules" or "Y-granules" [7, 8], "rubrophile granula" [12], "rubrophile bodies" [13], and "neutral red bodies" [23]. Several authors have previously described these structures at the electron microscopical level [2, 14]. Although Gatenby [7] regarded them at first as male yolk-granules because of their histochemical nature, their function and origin are still unknown. We hope that the present finding of clusters of dense bodies accumulating some glycoconjugates gives a clue to their function. Whether the structure plays a role in the degradation or storage of glycoconjugates is the subject of future studies.

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