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Glycomic profiling of developmental changes in bovine testis by lectin histochemistry and further analysis of the most prominent alteration on the level of the glycoproteome by lectin blotting and lectin affinity chromatography

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Summary. The emerging concept of the sugar code attributes functional significance to oligosaccharides of cellular glycoconjugates by protein (lectin)-carbohydrate interactions. Hence it follows that monitoring of glycan expression (glycomic profiling) is not only valuable to delineate characteristic (phenomenological) changes in the cell's glycosylation but will also come up with the localization of epitopes with potential in biorecognition. It is for this purpose that we have set up a panel of 16 markers (plant lectins and a carbohydrate-specific antibody). The selection met two criteria: a) to be able to detect the common constituents of natural glycans; and b) to place emphasis on detection of neutral carbohydrate units at the spatially accessible branch ends of glycan chains, which are known to be active as ligands for endogenous lectins *in situ*. Next, we incorporated recent insights into the importance of epitope clustering to turn less abundant oligosaccharides into potent ligands into our study design. To be able to focus on such high-affinity sites, we performed systematic titration studies aimed at defining the probe concentration at which carbohydrate-independent background staining is minimal while still yielding a clear signal. These requirements were met by marker concentrations of 1.25-2.5 µg/ml. Under these conditions, we defined cell-type- and differentiation-dependent changes in bovine testis. Sertoli cells lacked reactivity, whereas gonocytes were differentially reactive with the tested markers. The extent of staining intensity was subject to developmental changes, preferentially for Gal/GalNAc presentation and in this group most prominently with the galactoside-specific lectin from *Viscum album L.* (mistletoe). Of interest in this context, this lectin is known as a potent mitogen and signal inductor as well as haemagglutinin. The Gal/GalNAc-dependent signals decreased markedly in the course of development and staining was completely lost in the case of mistletoe lectin 12 weeks after gestation. Spermatids of adult testis presented respective glycan epitopes. In contrast to this developmental course of staining, endothelial cells either maintained a constant signal intensity or revealed a signal increase during development for Gal/GalNAc-specific lectins. Their binding of concanavalin A and the two phyto-haemagglutinins (PHA-E/L), which were not or only weakly reactive for gonocytes, served as inherent activity control. Based on lectin blot analysis with the mistletoe lectin as the marker which detected the most prominent change, the glycoprotein patterns from fetal and adult tissue specimens were qualitatively different, rendering changes in expression of the protein part of glycoproteins more likely than remodeling a glycoprotein's glycan chains. Methodologically, results of this procedure were compared to data obtained with lectin affinity chromatography and the combination of the two procedures. Differences in the profiles were discovered that can be assigned to the disparate ways to process the detergent extracts. When access to sample quantity is limited, as is possible in the case of fetal tissue, direct lectin blotting is recommended.

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Introduction

The emerging concept of the sugar code, attributing functionality in biological information storage and transfer to glycan epitopes of cellular glycoconjugates, rekindles interest in lectin histochemistry (Gabius, 2000; Solís et al., 2001; Gabius et al., 2002, 2004). In fact, oligosaccharides surpass any other type of biomolecule in the capacity for high-density coding, and the complex enzymatic machinery for glycan chain production enables us to swiftly reach an exceptional level of regulation and modulation of glycan signals without any change in gene expression (Brockhausen and Schachter, 1997; Laine, 1997; Sharon and Lis, 1997; Reuter and Gabius, 1999; Spiro, 2002). Truth be told, monitoring of the glycan profile by lectin histochemistry can thus no longer be viewed as phenomenological mapping of cell characteristics (Damjanov, 1987; Spicer and Schulte, 1992; Danguy et al., 1994). After all, the identification of regulated expression of defined epitopes carries the promise of tracking down functionally relevant determinants. In the first step of a respective study program, profiling with probes specific for common building blocks of glycan chains will be used to discover whether any relevant changes occur, for example in the course of development. Care is to be exercised to cover the array of naturally occurring oligosaccharides with their different anomeric positions and linkage points. In analogy to the terms *genome* and *proteome* this aim is referred to as mapping the cells' *glycome* (Gabius, 2000; Hirabayashi and Kasai, 2000; Rüdiger et al., 2000). Owing to fine-specificity differences among lectins with identical monosaccharide specificity, e.g. documented for N-acetyl-D-galactosamine (GalNAc)-specific lectins in biochemical binding assays and histochemical application (Sato and Muramatsu, 1985; Piller et al., 1990; Brinck et al., 1995, 1996; please see also Table 1 when comparing the entries of mono- and oligosaccharide specificity), it is advisable to include into the panel several markers that share monosaccharide specificity. Special attention should be given to probes detecting epitopes at spatially accessible positions in the glycan chains. These sites will not only be recognized by the histochemical tools but also probably by endogenous receptors, as shown for galectins (Gabius et al., 1991, 1993; Gabius and Gabius, 1992; Nagy et al., 2000; Wollina et al., 2000; Holiková et al., 2002; Plzák et al., 2002; Purkrábková et al., 2003; André et al., 2004a). Such mammalian carbohydrate-binding proteins, currently assigned to at least five families, are the missing link between the sugar signals and the cellular response (Ashwell and Morell, 1977; Gabius, 1987, 1997a,b, 2001a; Kaltner and Stierstorfer, 1998; Kilpatrick, 2002). Complexity at the level of lectins matches the already noted complexity at the level of code word generation by the array of isoenzymes for various glycosyltransferases, especially those which add ß-D-galactose (Gal) and α-D-GalNAc moieties to growing glycan chains. This accruing knowledge strongly implies functional significance for Gal/GalNAc-containing sequences. Consequently, probes with this specificity establish the core of our panel, presented in Table 1. This compilation also lists information on potent oligosaccharide ligands to document fine-specificity differences, as noted above.

Recent investigations have taught three important lessons, i.e. a) the potency of lectin-carbohydrate recognition to cause post-binding signaling; b) the crucial nature of multivalent and positively cooperative binding and threshold phenomena in lectin interactions; and c) the inherent selectivity of lectins to certain ligands in terms of sequence and conformation (Grant and Peters, 1984; Gabius, 1998; Mann and Waterman, 1998; Villalobo and Gabius, 1998; Yamazaki et al., 2000; Ahmad et al., 2002; Cairo et al., 2002; Dam and Brewer, 2002; Siebert et al., 2003a,b). Already ng-quantities/ml x10^6 cells can be sufficient to trigger lectin-dependent signaling with impact on mediator release and proliferation of immune/tumor cells (Gabius, 2001b; Gabius and Gabius, 2002). This low level of effector concentration should be taken into account in histochemistry. Traditionally, monitoring has been performed with a concentration of plant lectins between 10-40 µg/ml. This procedure will not distinguish between epitopes with varying degrees of abundance when plateau levels of staining are reached. To focus on localization of sites with high avidity, presumably relevant for biosignaling following the given reasoning on the importance of thresholds and regulation, we performed systematic titration of probe concentration and monitoring of signal-to-noise ratio in the first stage of our study. After this initial work, we determined the developmental course of the glycomic profile of bovine testis at the defined conditions.

It is tempting to analyze glycan expression in this organ system because of the intricate coordination of growth and differentiation of several cell types including the spermatogenic process. The extent to which lectin histochemistry has therefore already been examined in this respect reflects the principal interest to monitor glycan presence in this organ during development. In detail, testicular tissue has been subjected to lectin staining from rat (Söderström et al., 1984; Arya and Vanha-Perttula, 1984, 1986a; Malmi and Söderström, 1988; Malmi et al., 1990; Jones et al., 1992a,b, 1993; Martinez-Menarguez et al., 1993; Wine and Chapin, 1997), the musk shrew (Kurohmaru et al., 1995), mouse (Watanabe et al., 1981; De Felici, 1984; Lee and Damjanov, 1984; Arya and Vanha-Perttula, 1986b; Kanai et al., 1989; Sakumaki et al., 1989; Nagano et al., 1999), hamster and three other tetrapode vertebrates from birds, amphibians and reptiles (Ballesta et al., 1991), guinea pig, gerbil and nutria (Arya and Vanha-Perttula, 1986b), boar (Calvo et al., 2000; Pinart et al., 2001a,b), goat (Kurohmaru, 1991), bull (Arya and Vanha-Perttula, 1985), horse (Verini-Supplizi et al., 2000) and human specimens including tumors (Lee and Damjanov, 1985; Caselitz, 1987; Malmi and Söderström,

1987; Malmi et al., 1987; Wollina et al., 1989; Arenas et al., 1998; Xu et al., 2000; Gheri et al., 2003). The recent observation that a N-acetyl-D-glucosamine (GlcNAc)-terminated triantennary and fucosylated N-glycan of mouse spermatogenic cells, which is reactive with GSA II (please see Table 1 for further information on this lectin), plays a pivotal role in germ cell-Sertoli cell adhesion underscores the implied functional activities (Akama et al., 2002). This body of evidence and our previous work on lectins in bovine development (Kaltner et al., 1997, 2002) thus prompted us to perform the present glycomic profiling and ensuing biochemical analysis.

We aimed to pick up signals of presumedly functionally relevant epitopes of high avidity by using a deliberately non-saturating probe concentration instead of a visualization of all reactive epitopes at saturating marker concentration. Based on these histochemical measurements and the comparative assessment of staining patterns we detected a prominent developmental change for binding of a member of the group of galactoside-specific markers. Next, we proceeded to address the question as to whether the changes in intensity of staining in histochemistry in this case are accounted for by qualitative or quantitative alterations in glycoprotein modification/expression using biochemical methods, i.e. lectin blotting and lectin affinity chromatography. Processing by these two protocols also resolves the pertinent issue as to whether and to what extent the obtained results are comparable. The presented results of these experiments constitute an important methodological aspect of our work, especially when access to material (e.g. from early fetal stages) is limited. In this case, lectin blotting is advisable.

Materials and methods

Tissue material and reagents

Fresh specimens of adult bovine testes (three animals) and of testicular tissue from seven different stages of fetal development (total of 25 animals), grouped on the basis of crown-rump length into age categories according to Rüsse and Sinowatz (1991), namely 6.0-8.0 cm (equaling the 8th week of gestation, 3 fetuses), 9.0 cm (equaling the 9th week of gestation, 4 fetuses), 10.0-11.0 cm (10th week of gestation, 5 fetuses) 12.0-13.5 cm (11th week of gestation, 7 fetuses), 19.0 cm (12th week of gestation, 3 fetuses), 24.5-27.0 cm (13th week of gestation, 3 fetuses) and 29.5 cm (16th week of gestation, 3 fetuses) were obtained from a local slaughterhouse. For the glycomic profiling by histochemistry a panel of 14 biotinylated plant lectins covering a capacity to detect common building blocks of glycan chains of mammalian glycoconjugate specificities was purchased from Vector Labs. (distributed by Alexis Germany, Grünberg, Germany). The lectins and their individual mono- and oligosaccharide specificities are presented in Table 1. To localize Galα3Gal epitopes the α-galactoside-binding

Table 1. Lectins used in this study and their binding specificities to mono- and oligosaccharides.

LATIN NAME (COMMON NAME)	ACRONYM	MONOSACCHARIDE SPECIFICITY	POTENT OLIGOSACCHARIDE[a]
Arachis hypogaea (peanut)	PNA	Gal	Galß3GalNAc
Canavalia ensiformis (jack bean)	Con A	Man/Glc	GlcNAcß2Manα6(GlcNAcß2Manα3)Manß4GlcNAc
Dolichos biflorus (horse gram)	DBA	GalNAc	GalNAcα3GalNAcα3Galß4Galß4Glc
Erythrina cristagalli (coral tree)	ECA	Gal	Galß4GlcNAcß6(Galß4GlcNAcß2)Man
Glycine max (soybean)	SBA	GalNAc	GalNAcα3Galß6Glc
Griffonia simplicifolia I	GSA I	GalNAc	GalNAcα3Gal, GalNAcα3GalNAcß3Galα4Galß4Glc
Griffonia simplicifolia I-B_4	GSA I-B_4	Gal	Galα3Gal
Griffonia simplicifolia II	GSA II	GlcNAc	GlcNAcß4GlcNAc, glycans with terminal, non-reducing-end GlcNAc
Phaseolus vulgaris erythroagglutinin (kidney bean)	PHA-E	[b]	Bisected complex-type N-glycans: Galß4GlcNAcß2 Manα6(GlcNAcß2-Manα3)(GlcNAcß4)Manß4GlcNAc
Phaseolus vulgaris leukoagglutinin (kidney bean)	PHA-L	[b]	tetra- and triantennary N-glycans with ß6-branching
Ricinus communis (castor bean)	RCA I	Gal	Galß4GlcNAcß2Manα6 (Galß4GlcNAcß2Manα3)Manß4GlcNAc
Sophora japonica (pagoda tree)	SJA	GalNAc	GalNAcß6Gal
Ulex europaeus I (gorse seed)	UEA I	Fuc	Fucα2Galß4GlcNAcß6R
Vicia villosa (hairy vetch)	VVA	GalNAc	GalNAcα3(6)Gal, GalNAcß3Gal
Viscum album (mistletoe)	VAA	Gal	Galß2(3)Gal, Galα3(4)Gal, Galß3(4)GlcNAc, Fucα2Gal

[a]: based on previously compiled information (Rüdiger and Gabius, 2001), extended and modified; [b]: no monosaccharide known as ligand.

immunoglobulin G fraction from human serum (α-Gal IgG) was purified. This process required a series of chromatographic steps comprising passages over unmodified column resin (Sepharose 4B) to remove proteins with affinity to the matrix such as serum amyloid P component or agarose-binding antibodies, over melibiose-containing Sepharose 4B, synthesized after divinyl sulfone activation (Gabius, 1990), with affinity elution to separate proteins with affinity to α-galactosides and galactose from other serum components, over protein A-Sepharose 4B (Amersham Biosciences, Freiburg, Germany) with elution to remove proteins other than IgG and over lactose-presenting resin to separate the α-galactoside-specific subfraction in the flow-through fraction from bound IgG fractions with affinity to galactose without anomeric preference, as described in detail previously (Dong et al., 1995, 1997). Labeling of the purified material was performed under activity-preserving conditions with biotinamidocaproyl hydrazide, as described before (Dong et al., 1995, 1997), and the lack of detrimental influence of the chemical modification was ascertained by solid-phase assays using neoglycoproteins and thyroglobulin (Kirkeby et al., 2004). Second-step reagents for signal generation were purchased from different sources: goat anti-rabbit immunoglobulin G-peroxidase and streptavidin-peroxidase conjugates from Sigma (Munich, Germany); avidin/biotinylated horseradish peroxidase complex (ABC Kit) and biotin-blocking kits from Vector Labs. (distributed by Alexis Germany, Grünberg, Germany); and kit reagents of the enhanced chemiluminescence system (ECL™) from Amersham Biosciences (Freiburg, Germany).

Lectin purification, labeling and antibody production

The galactoside-specific lectin from mistletoe (*Viscum album* L. agglutinin, VAA) was purified from extracts of dried leaves by affinity chromatography on lactose-containing Sepharose 4B as crucial step, as described previously (Gabius, 1990). Purity was routinely checked by analytical gel filtration and one- and two-dimensional gel electrophoresis. Labeling was performed with the biotinyl-N-hydroxysuccinimide ester (Sigma, Munich, Germany) in the presence of 20 mM lactose to exclude the carbohydrate-binding site from biotin conjugation which reached a final yield of 12-15 biotin moieties per subunit of the AB-plant toxin, and the lack of a harmful influence of biotin attachment on lectin activity was determined by solid-phase and cell-binding assays (Gabius et al., 1992; André et al., 1999a, 2000, 2001, 2003). Preparation of antibodies could not be done without adequate precautions owing to the presence of glycosylation (Gabius et al., 1985). To preclude that the glycan part of the lectin could cause production of cross-reactive antibodies, periodate-induced oxidation (2 mM, treatment for 10 min at 4 °C) and reduction of resulting aldehydes by exposure to 20 mM sodium borohydride for 20 min at 4 °C was required. Owing to the potent toxicity of this mistletoe protein *in vivo,* a member of the family of ribosome-inactivating proteins (Rüdiger and Gabius, 2001) and further treatment with 2% formaldehyde for three days at room temperature preceded standard immunization of rabbits (details presented elsewhere) (Hajto et al., 1989). Analysis of the IgG fractions prior to and after injections by ELISA, immunospotting and blotting, as outlined previously (Gabius et al., 1983), ascertained the antibody specificity and also excluded any cross-reactivity to mammalian proteins in extracts.

Lectin (antibody) histochemistry

Tissue material was immediately treated with the fixative to limit post-mortem alterations. We used three different fixatives in the test series, i.e. methanol with 30% acetic acid, 4% buffered paraformaldehyde and Bouin's solution, which led to routine use of Bouin's solution. The paraffin-embedded sections (5 µm thick) were treated with methanolic hydrogen peroxide solution for 30 min to inhibit the activity of endogenous peroxidase, non-specific protein-binding sites were saturated by incubation with 10 mM Hepes buffer, pH 7.5, containing 1% (w/v) bovine serum albumin and any biotin-specific binding sites whose presence would result in false-positive (carbohydrate-independent) reactions with the biotinylated probes were blocked by application of the respective kit reagents, as described previously (Kaltner et al., 1997, 2002). The processed sections were then incubated overnight at 4 °C with a solution containing a biotinylated lectin (2.5 µg/ml for the commercial products, 1.25 µg/ml in the case of VAA) in 10 mM Hepes buffer, pH 7.5, containing 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$ and/or 0.1 mM $MnCl_2$ as additives, where necessary for lectin activity. After three washing steps to remove unbound marker a solution with ABC kit reagents was added, and sections were incubated for one hour at room temperature. In the last step of the procedure, the bound lectin was localized by incubation with 3,3'-diaminobenzidine tetrahydrochloride (0.05% (w/v) in phosphate buffer, pH 7.2) and hydrogen peroxide (0.01% (v/v)) as chromogenic substrates. Control reactions to ascertain carbohydrate-dependent binding of the markers were performed by competitive inhibition with the respective haptenic sugar and by omission of the incubation step with biotinylated lectin to exclude a contribution to staining by biotin-dependent lectin binding, carbohydrate-independent lectin binding via protein-protein interactions or by binding of kit reagents, i.e. the glycoproteins horseradish peroxidase or avidin, as described earlier (Sinowatz et al., 1989; Kuchler et al., 1990; Brinck et al., 1996).

Lectin blot analysis

Tissue samples which had been stored frozen at -80 °C were homogenized under conditions to minimize proteolysis and to facilitate proper solubilization of

membrane glycoproteins by the presence of a mixture of ionic and non-ionic detergents (0.1% sodium deoxycholate and 1% Triton X-100) and processed as described previously (Gabius et al., 1991). Extract (glyco)proteins were separated by discontinuous gel electrophoresis (4% stacking gel, 10% running gel) under denaturing and reducing conditions, and their electroblotting onto a nitrocellulose matrix was carried out as described previously (Kaltner et al., 1997). Following saturation of non-specific protein-binding sites on the nitrocellulose membrane by incubation in 50 mM Tris/HCl buffer, pH 7.5, containing 3% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20 overnight at 4 °C probing followed two protocols: a) successive incubation steps with reagents of the biotin-blocking kit to prevent binding of the streptavidin derivative, with labeled VAA for two hours at room temperature at a concentration of 0.25 µg/ml and with 0.5 µg/ml streptavidin-peroxidase conjugate for one hour at room temperature; and b) successive incubation steps with label-free VAA (0.25 µg/ml) for two hours at room temperature, with the VAA-specific rabbit polyclonal antibody fraction at a concentration of 0.4 µg/ml for one hour at room temperature and with goat anti-rabbit antibody-peroxidase conjugate (0.5 µg/ml) also for one hour at room temperature. Visualization of the bound lectin on the blots was accomplished with reagents of the enhanced chemiluminescence system according to the instructions of the producer. Control reactions were performed, as described in the section on lectin histochemistry, either by competitive inhibition with the haptenic sugar (100 mM lactose) or omission of the labeled lectin (protocol a) or omission of the lectin or lectin-specific antibody (protocol b). To further exclude probe-independent signal generation by binding of the high-mannose N-glycan of horseradish peroxidase and to ascertain the maintenance of the sugar-specific reaction of the lectin on the blot surface, a negative control was also performed with 100 mM D-mannose.

Lectin affinity chromatography

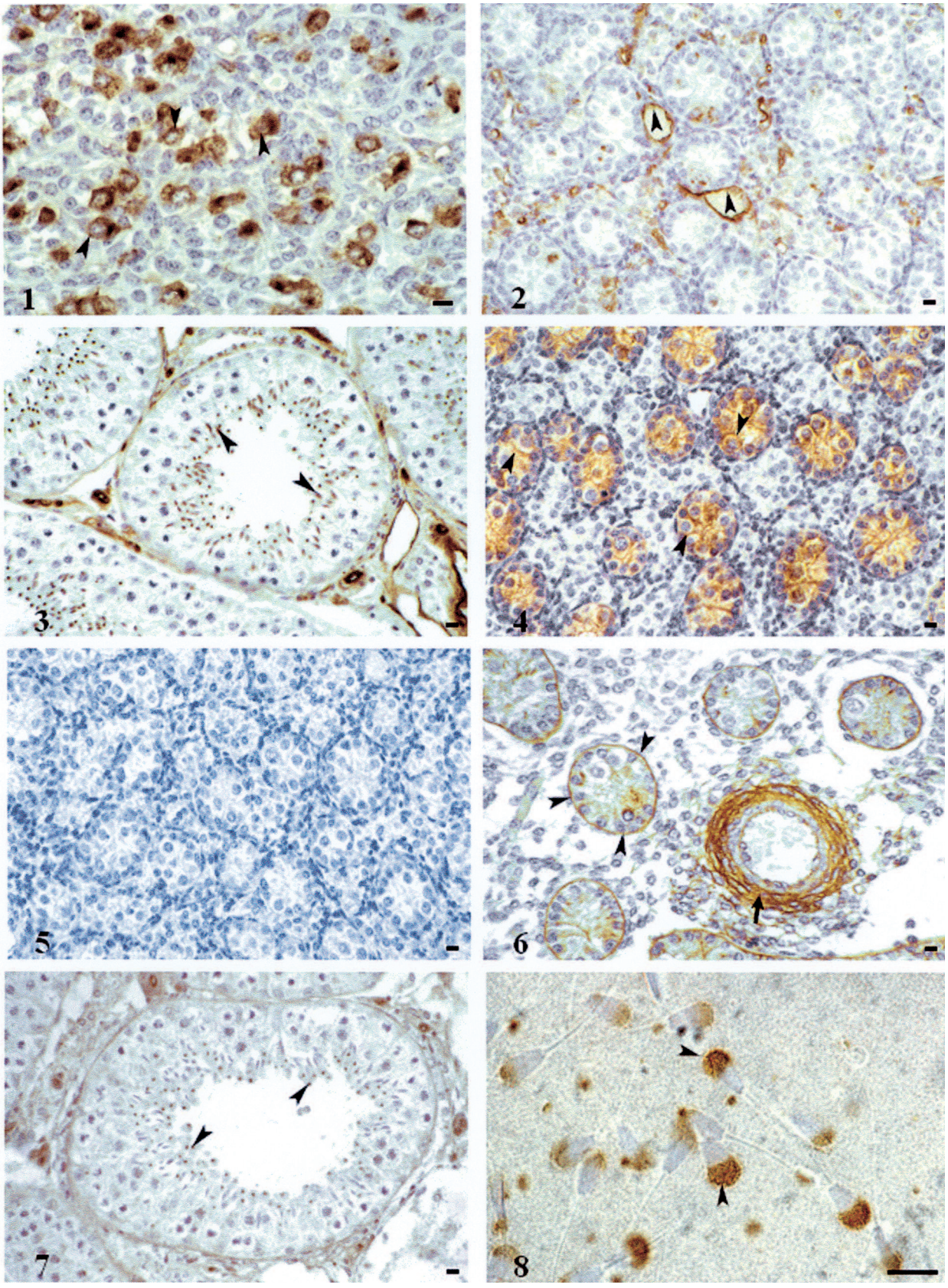
VAA was covalently coupled to divinyl-sulfone-activated Sepharose 4B at a density of 1 mg/ml resin with more than 95% yield and checked for binding activity with asialofetuin as described previously (Gabius et al., 1991). Extract samples were prepared as described for the samples used in lectin blot analysis except for the absence of 2 mM ß-mercaptoethanol in the buffer, and were incubated with the lectin-containing resin overnight at 4 °C. Then the column was washed free of non-binding material and elution was performed with 0.3 M lactose as affinity eluant, as described in detail previously (Gabius et al., 1991). The purified fractions treated with chloroform to remove the non-ionic detergent whose presence would interfere with further processing were analyzed by standard gel electrophoretic analysis and silver staining or by lectin blotting analysis as described in the preceding section.

Results

Our study is divided into a histochemical and a biochemical part. First, we defined the glycomic profile of gonocytes and endothelial cells in fetal testis at different stages of development as well as in seminiferous tubules and interstitium in adult testis by lectin histochemistry. The pronounced developmental changes in reactivity with Gal/GalNAc-specific markers, especially VAA, prompted biochemical analysis. We performed lectin blotting and lectin affinity chromatography including a combination of both techniques to monitor the glycoproteome, comparatively focusing on VAA-reactive epitopes.

Lectin histochemistry (glycomic profiling)

To ensure that all neutral building blocks of glycan chains of cellular glycoconjugates can be detected, the panel of plant lectins must cover this range including GlcNAc, Man, Fuc, Gal and GalNAc. In addition, common variations of anomeric positions for Gal/GalNAc command attention. These requirements are met by the lectins summarized in Table 1. Furthermore, an antibody with specificity to α-galactosides was added. With this series of probes we monitored the presence of glycans with the aim to histochemically elucidate the way the glycomic profile is altered during development in tissue sections. To exclude a major impact of the type of fixative used on epitope localization, e.g. by fixation-dependent artifacts which cause ligand redistribution or loss due to extraction by the solvent, as noted for gangliosides (Schwarz and Futerman, 1997), we first systematically studied three different fixation protocols. Based on the criteria of preservation of morphological features and mode of reaction in the rigorous specificity controls (please see below) Bouin's fixative yielded material of comparatively the best quality. As a result, we decided to routinely work with this solution in tissue fixation. In the next step, we explored the relation between marker concentration and ratio of intensity of specific staining vs. the background in order to visualize sites with preferential reactivity at subsaturating concentrations. A common measure at 2.5 µg/ml (1.25 µg/ml in the case of VAA) was found to be optimal. Figures 1-16 illustrate the localization of lectin (antibody)-reactive glycans in the sections including specificity controls on which special emphasis was placed. To make sure that signal generation exclusively depended on the lectin-carbohydrate interaction, we routinely blocked non-specific protein-binding and biotin-binding sites in the sections. We also systematically performed competitive inhibition by the haptenic sugar, a means by which to delineate protein-protein recognition (except for potential binding to glycomimetic peptides with the -Y-X-Y- sequence; please see Arnusch et al., 2004) as a source for erroneous data interpretation if not pinpointed. Furthermore, we carried out the staining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protocol without the incubation step with the labeled marker, a means by which to detect any lectin-independent signal production, e. g. by binding of kit reagents such as the glycoproteins of the ABC kit to receptor sites in the section, a pitfall noted in sections from cerebellum (Kuchler et al., 1990). An example of what a routine control section obtained after lectin histochemical processing with competitive inhibition looks like is given in Fig. 5.

Having defined the experimental conditions and documented the quality standard of the lectin histochemical analysis, we analyzed tissue samples at seven different fetal stages as well as those from adult testes (at least three independent specimens per group). Dominating morphological structures in fetal testis were the solid testis (seminiferous) cords, which developed into the seminiferous tubules after birth. The gonocytes, which will give rise to spermatogenic cells, were surrounded by immature Sertoli cells. As thorough microscopical assessment of the processed sections revealed, this cell type was not reactive with any lectin at the given concentration, whereas the gonocytes were stained by probes specific for D-galactose, N-acetyl-D-galactosamine and N-acetyl-D-glucosamine (Table 2, Figs. 1, 4 and 9-12). Looking at the sections, staining of endothelial cells of blood vessels was also prominent with several of the lectins in the panel (Table 3, for example Figs. 2, 6, 14, 15). As summarized in Table 2, the extent of the staining reaction was subject to developmental control in the gonocytes. In general, there appeared to be a tendency for a decrease in the extent of reactivity with increasing numbers of weeks of gestation from eight to 16; PNA, DBA, GSA II and PHA-L binding deviating from this course. It was most pronounced for the intensity of staining by the mistletoe lectin, illustrated in Figs. 9-14. As internal control for the validity of this result it is noteworthy that application of the structurally related agglutinin from *Ricinus communis* (RCA) resulted in a rather similar developmental pattern (Table 2). Probes with specificities to N-acetyllactosamine (ECA) or the Thomsen-Friedenreich disaccharide (PNA) let a dissimilar signal profile or intensity, respectively, develop. This was an indication that these two types of ß-galactosides were not preferentially represented in the group of VAA-reactive epitopes (Table 2). In the case of VAA, with its binding to α- and ß-galactosides in solid-phase and haemagglutination assays, it is expedient to flank its application in histochemistry by probes with narrow selectivity. Hereby, the level of insight on the actual reactive determinants from the large group of galactosides is improved. The importance of α3(4)-linked galactose was supported by the results with α-galactoside-binding GSA I-B_4 and also the α-Gal antibody (please see Table 1, Table 2). Staining of

Table 2. Presence of lectin (antibody)-reactive glycans in gonocytes.

LECTIN/	WEEKS OF GESTATION						
ANTIBODY	8	9	10	11	12	13	16
Gal							
VAA	+++	+++	++	+	-	-	-
RCA	++	++	++	(+)	(+)	(+)	(+)
GSA I-B_4	+++	++	(+)	-	-	-	-
PNA	(+)	(+)	(+)	+	+	+	+
α-Gal IgG	+	(+)	(+)	-	-	-	-
ECA	+	-	-	-	-	-	-
GalNAc							
DBA	+	++	+	+	+	+	+
SBA	++	+	+	+	+	-	-
GSA I	+++	+	+	-	-	-	-
VVA	+	+	+	+	(+)	(+)	(+)
SJA	+	-	-	-	-	-	-
GlcNAc							
GSA II	-	-	(+)	(+)	(+)	(+)	(+)
Man/Glc							
Con A	-	-	-	-	-	-	-
L-Fuc							
UEA I	-	-	-	-	-	-	-
complex-type N-glycans							
PHA-L	(+)	(+)	(+)	(+)	(+)	(+)	(+)
PHA-E	-	-	-	-	-	-	-

The intensity of staining is grouped into the following categories: - no staining, (+) very weak but specific and significant, + weak, ++ medium, +++ strong, independently assessed by two observers; the lectins are listed in groups of identical monosaccharide specificity and then based on their staining intensity.

Figs. 1-8. Localization of lectin(antibody)-reactive glycan epitopes in sections of fetal and adult bovine testis. **Fig. 1.** Seminiferous cords of a fetus (8th week of gestation). Intense staining of gonocytes in the center of the solid cords (arrowheads) using biotinylated GSA I-B_4. Supporting cells (pre-Sertoli cells) and interstitial tissue are not reactive. Bar: 10 µm. **Fig. 2.** Localization of GSA I-B_4-binding sites in endothelial cells of interstitial blood vessels of a fetal testis in the 13th week of gestation (arrowheads). Bar: 10 µm. **Fig. 3.** Seminiferous tubules of adult bovine testis. Reactivity to GSA I-B_4 is restricted to spermatids (arrowheads) in the seminiferous epithelium and to endothelial cells of blood vessels in the interstitium. Bar: 10 µm, **Fig. 4.** Membranes of gonocytes in seminiferous cords of fetal testis (13th week of gestation) are reactive with labeled PNA (arrowheads), whereas the developing interstitial cells are completely negative. Bar: 10 µm. **Fig. 5.** Binding of PNA was completely blocked by competitive inhibition with 100 mM D-galactose (control to Fig. 4). This photomicrograph also illustrates lack of staining by probe-independent binding of kit reagents. Bar: 10 µm. **Fig. 6.** Detection of PNA-reactive glycans in fetal testis (16th week of gestation). The *lamina propria* of the seminiferous cords (arrowheads) surrounding the tubules is clearly accentuated by staining, and the *tunica adventitia* of blood vessels (arrow) shows an intense reaction. Bar: 10 µm. **Fig. 7.** Seminiferous tubules from adult bovine testis are reactive with the α-galactoside-specific immunoglobulin G subfraction (αGal IgG) from human serum. Presence of respective α-galactosides in glycans is restricted to late spermatids (arrowheads) and to endothelial cells of blood vessels in the interstitium (please see Fig. 3 for comparison to the staining pattern with GSA I-B_4). Bar: 10 µm. **Fig. 8.** Acrosomal caps of bull sperm are strongly reactive with PNA. Bar: 10 µm.

gonocytes at the same fetal stage with labeled GSA I-B_4 and VAA is presented in Figs. 1, 9 and 10. To address the question as to whether endothelial cells were also subject to developmental control of glycosylation, staining intensity arising from carbohydrate-dependent binding by the probes was likewise semiquantitatively graded for this cell type.

The gonocyte-characteristic course of development of VAA (RCA)-dependent staining was not observed (Table 3). The intensity of staining was at a medium level in material at the eighth week of gestation and - in contrast to a decrease reaching zero level in gonocytes - even eventually increased to the category "strong" (Table 3, Figs. 9-14). An increase was also noted for PNA, PHA-L and Con A. These reactions of Con A and PHA-L excluded a false-negative interpretation of their lack of reactivity or weak binding to gonocytes at this marker concentration. It is noteworthy that four out of five lectins sharing monosaccharide specificity to GalNAc were negative. Their reactivity with gonocytes underscored the differential changes of glycan epitopes during development in these two cell types (Table 2, Table 3). Having monitored changes in fetal stages, we next processed specimens from adult testes in order to comparatively define their glycophenotype.

Table 3. Presence of lectin (antibody)-reactive glycans in endothelial cells of blood vessels in fetal testis.

LECTIN/	WEEKS OF GESTATION						
ANTIBODY	8	9	10	11	12	13	16
Gal							
VAA	++	++	++	++	++	+++	+++
GSA I-B_4	++	++	++	++	++	+++	+++
RCA	++	++	++	++	++	++	++
PNA	-	-	-	-	+	++	++
α-Gal IgG	-	-	(+)	+	+	+	++
ECA	-	-	-	-	-	-	-
GalNAc							
GSA I	+	+	+	+	++	+	(+)
SJA	-	-	-	-	-	-	-
DBA	-	-	-	-	-	-	-
SBA	-	-	-	-	-	-	-
VVA	-	-	-	-	-	-	-
Man/Glc							
Con A	(+)	(+)	(+)	+	+	+	+
GlcNAc							
GSA II	-	-	-	-	-	-	-
L-Fuc							
UEA I	-	-	-	-	-	-	-
complex-type N-glycans							
PHA-E	++	++	++	++	++	++	++
PHA-L	(+)	+	+	+	+	+	++

The intensity of staining is grouped in the following categories: - no staining, (+) very weak but specific and significant, + weak, ++ medium, +++ strong, independently assessed by two observers; the lectins are listed in groups of identical monosaccharide specificity and then based on their staining intensity.

Spermatids, spermatozoa and endothelial cells were the most prominently stained cell types (Table 4, Figs. 3, 7, 8, 15, 16). Looking at galactosides, the emerging presence of Galß4GlcNAc-containing epitopes reactive with ECA (please see Table 1) indicated a shift in the relative proportion between probe-accessible α- and ß-galactosides. Of interest regarding cell-type-dependent changes, ECA binding was seen in spermatids but not endothelial cells (Table 4). The non-uniform detection of GalNAc-containing determinants revealed another case of particular monosaccharide-defined sugar epitopes with distinctive representation in cells, while UEA I and a bean isolectin (PHA-L) were not reactive under these experimental conditions.

To summarize this glycomic profiling at a non-saturating concentration, marked cell-type-characteristic alterations were observed between gonocytes and endothelial cells. Developmental changes were primarily determined in gonocytes. The most conspicuous change from strong staining to signal negativity in gonocytes and then to medium reactivity in spermatids/spermatozoa was attributable to VAA. The medium to strong signal intensity in fetal stages from the ninth to tenth weeks of gestation and in adult tissue prompted us to try to answer the question regarding the nature of glycoproteins binding this plant lectin. To resolve the issue as to whether and to what extent the patterns of VAA-binding glycoproteins from fetal and adult tissue material are different we performed lectin blot analysis.

Lectin blot analysis

Detergent extracts were prepared under conditions to minimize proteolysis, and the use of a mixture of non-ionic/ionic detergents aimed to reach adequate solubility of membrane glycoproteins. Such a mixture had the added benefit of doing very limited harm to the lectin's

Figs. 9-16. Localization of VAA-reactive glycan epitopes in sections of fetal and adult bovine testis. **Figs. 9, 10.** Solid seminiferous cords at the 8th week of gestation. Intense staining of gonocytes (arrowheads) presented at two levels of magnification. Bars: 10 µm. **Figs. 11-14.** Gradual decrease of reactivity of gonocytes in the seminiferous cords of fetal bovine testis to VAA at different developmental stages (arrowheads). Moderate staining intensity is characteristic for the specimen from the 10th week of gestation **(Fig. 11)**. At the 11th week of gestation staining intensity is reduced **(Fig. 12)**, and material from the 13th week of gestation reveals a complete loss of gonocyte reactivity. Clusters of interstitial cells including endothelial cells, however, maintain moderate to strong staining intensity **(Fig. 13)**. Bars: 10 µm (please see also Tables 2, 3). **Fig. 14.** VAA-binding glycans in fetal testis at the 16th week of gestation are nearly exclusively present in endothelial cells of blood vessels in the interstitium (arrowheads), whereas gonocytes and pre-Sertoli cells are completely negative. Bar: 10 µm. **Figs. 15, 16.** Spermatids of two different stages in the seminiferous cycle (arrowheads) express VAA-reactive glycans in sections from adult testis. Bars: 10 µm.

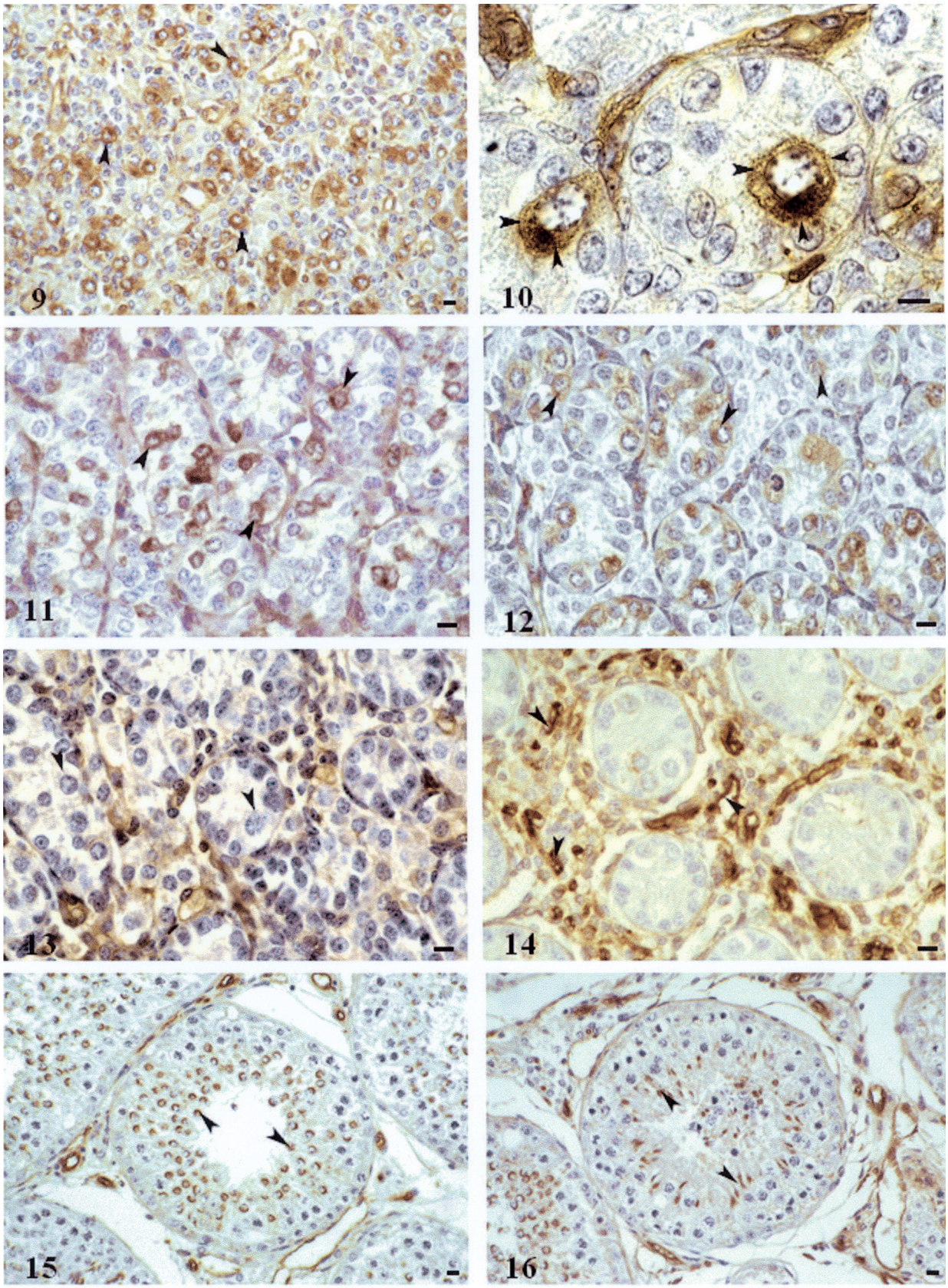
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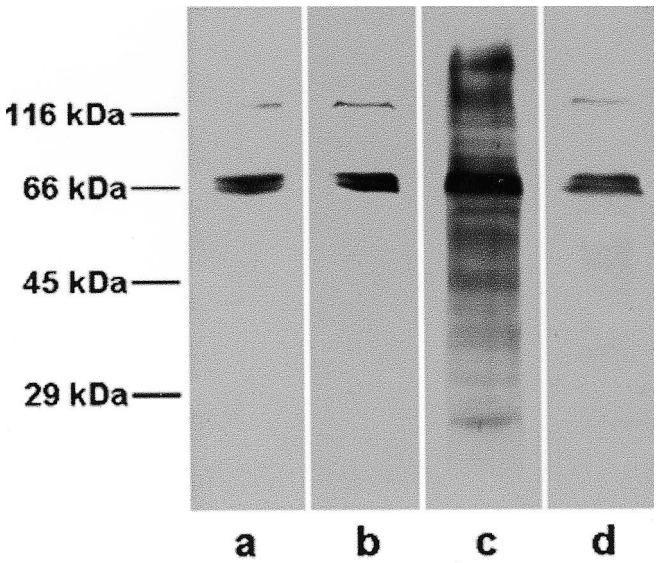
Table 4. Localization of lectin (antibody)-reactive glycans in cells of the seminiferous tubules and the interstitium in adult testis.

LECTIN/ANTIBODY	SEMINIFEROUS TUBULES			INTERSTITIUM	
	Spermatids		Sertoli cells	Leydig cells	Endothelial cells
	cap phase	acrosome phase			
Gal					
GSA I-B_4	+++	++	-	-	+++
ECA	++	+++	-	-	-
PNA	++	+++	-	-	-
α-Gal IgG	++	++	-	-	++
VAA	++	++	-	-	++
RCA	++	++	-	-	+
GalNAc					
SJA	++	++	-	-	-
SBA	++	++	-	-	+
GSA I	+	++	-	-	++
VVA	+	++	-	-	-
DBA	-	-	-	-	-
GlcNAc					
GSA II	+	++	-	-	-
Man/Glc					
Con A	-	-	-	-	+
L-Fuc					
UEA I	-	-	-	-	-
complex-type N-glycans					
PHA-E	-	-	-	-	+
PHA-L	-	-	-	-	-

The intensity of staining is grouped into the following categories: - no staining, (+) very weak but specific and significant, + weak, ++ medium, +++ strong, independently assessed by two observers; the lectins are listed in groups of identical monosaccharide specificity and then based on their staining intensity.



activity in affinity chromatography under identical conditions (Lotan and Nicolson, 1979). When probing the nitrocellulose membrane after electrophoretic transfer of the extract (glyco)proteins (100 µg) with biotinylated lectin, two strong bands and an additional weak signal already developed after 15 seconds of exposure of the X-ray film to the blot on which the chemiluminescence reaction had been initiated (Fig. 17a). The previous standard use of the biotin-blocking kit reagents intimated a lectin-dependent reaction. However, the staining intensity of these bands was not diminished when the incubation step with the biotinylated lectin was omitted from the blot processing (Fig. 17b). Running a 200 µg extract (glyco)protein in the lectin blot analysis revealed further bands (film exposure for 10 seconds), and the control reaction with competitive inhibition by 100 mM lactose showed that

Fig. 17. Lectin blot analysis of VAA-binding glycoproteins from detergent extracts of adult bovine testis using biotinylated lectin as probe. Aliquots of extract protein (**a, b:** 100 µg; **c, d:** 200 µg) are subjected to the processing as described in Methods. The effects of omission of the incubation step with the labeled lectin from the processing protocol **(b)** and of the presence of 100 mM lactose, the competitive inhibitor in carbohydrate-dependent binding of VAA, in the incubation step with the lectin **(d)** are shown as controls to lanes **a** and **c** in order to distinguish carbohydrate-dependent binding of VAA from other mechanisms to generate staining. Blots are routinely calibrated with the following molecular weight markers: ß-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa) and carbonic anhydrase (29 kDa).

only these emerging signals were sensitive to interfering with the lectin binding, thereby pointing to underlying lectin-carbohydrate interactions in these cases (Fig. 17c, d). Evidently, the dense presentation of extract (glyco)proteins on the nitrocellulose membrane after their concentration in bands by gel electrophoresis led to a situation which was different from histochemical staining. In that case, control reactions had excluded any carbohydrate-independent generation of staining. Although we were able to detect lectin-reactive bands under these conditions, the main signal without connection to our question marred the illustration. We thus turned to an alternative two-step procedure using label-free VAA and VAA-specific antibody for marking positions of VAA-reactive glycoproteins in the blots. This procedure fully eliminated the problem of lectin-independent staining. There was a satisfactory concentration-dependence excluding differential loss of certain glycoproteins from the membrane and a complete carbohydrate-dependence of the reaction (Fig. 18a-c). Staining was completely sensitive to the presence of lactose (Fig. 18c), but not mannose as an inhibitor, precisely following the lectin's specificity (not shown). Lanes equally negative to that of Fig. 18c were obtained when omitting the incubation step with either the lectin or the lectin-specific antibody or when running samples from fetal tissue using competitive inhibition of lectin binding (not shown). Under these conditions adult and fetal glycoprotein profiles could be reliably compared. They were found to differ significantly in quantitative and qualitative terms (Fig. 18a, b, d). The overall staining intensity with a 100 µg sample was stronger for fetal than adult samples, and the molecular weight distributions revealed clear differences as well. Changes in glycoprotein expression and not remodeling on the level of a glycoprotein's glycan chains thus apparently account for the quantitatively similar staining intensity in lectin histochemistry in fetal specimens of the 9^{th}-10^{th} week of gestation and in adult samples. Although lectin blot analysis is a common, widely applied technique, we proceeded to compare this result with data when performing lectin affinity chromatography and probing the purified fractions with gel electrophoresis/silver staining or lectin blotting. A drawback of this method is the requirement for larger tissue quantities than for lectin blotting due to e. g. loss of material by adsorption from very dilute solutions. The next paragraph will answer the question regarding comparability of the two methods with respect to the lectin-reactive glycoprotein profile.

Lectin affinity chromatography

We performed the isolation of glycoproteins with affinity for VAA on a resin with immobilized lectin (about 1 mg/ml) under conditions not harmful to the lectin's activity. Silver staining of gels with the glycoprotein fractions from adult testicular tissue obtained after affinity elution gave a complex pattern of

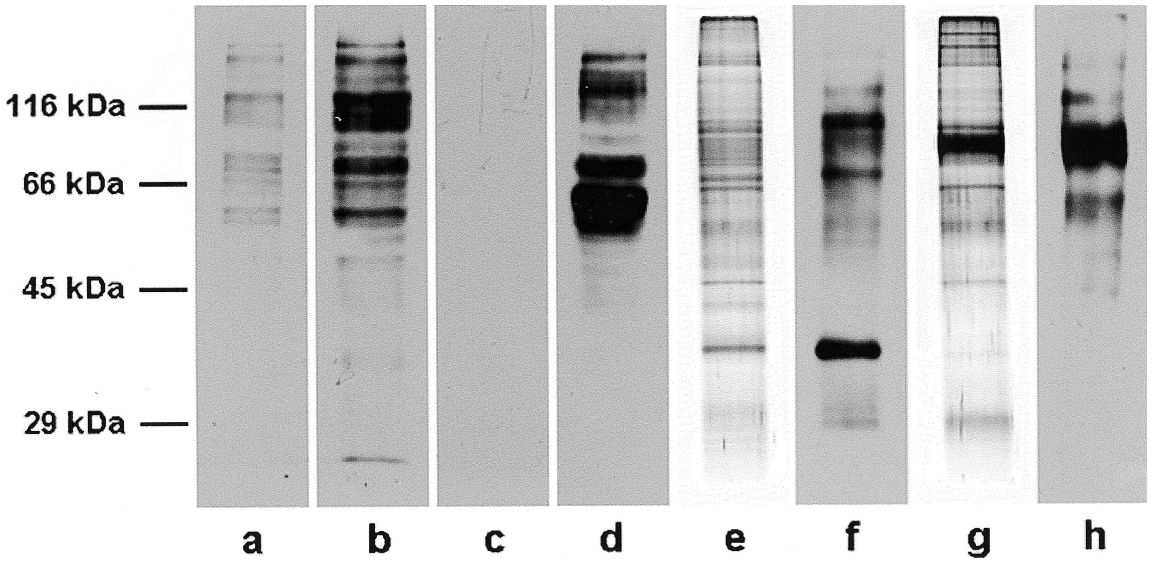


Fig. 18. Lectin blot analysis of VAA-binding glycoproteins from detergent extracts of fetal and adult bovine testis using lectin/anti-lectin antibody as probes and a comparison to the pattern of glycoproteins purified by lectin affinity chromatography. Aliquots of extract protein (**a, d:** 100 µg; **b, c:** 300 µg) are subjected the processing as described in Methods. The effects of the amount of extract protein per lane (**a:** 100 µg; **b:** 300 µg), of the presence of the haptenic sugar (**c:** 100 mM lactose) and of the developmental stage of the sample (**d:** fetal tissue from the 9^{th} week of gestation) are shown. The profiles of glycoproteins purified by lectin affinity chromatography from detergent extracts of adult testis, as obtained by standard gel electrophoretic analysis including silver staining (4 µg; **e**) or by lectin blot analysis (7.5 µg; **f**), enable a comparison between the two methods to define lectin-reactive glycoproteins. The control result of an analysis on detergent extracts from a different organ (i.e. adult heart) is shown in lane **g** (gel electrophoresis and silver staining of glycoproteins; 4 µg) and lane **h** (lectin blot of glycoproteins purified by lectin affinity chromatography; 1 µg). Blots and gels are routinely calibrated with the set of molecular weight markers listed at the end of the legend to Fig. 17.

bands stained with weak-medium intensity (Fig. 18e). It is likely that the presence of bulky glycans interferes with development of silver staining, and therefore we proceeded to analyze these samples in parallel by lectin blot analysis. Here, three major bands were developed (Fig. 18f). A control preparation with adult heart tissue proved tissue-type-specific differences (Fig. 18g, h). The comparison of lanes b, f in Fig. 18 illustrates the impact of the method of preparation on the glycoprotein profile. A direct comparison, when access to material especially for fetal tissue is limited, is valid only with processing under identical conditions (Fig. 18a, d). The comparison of results from different protocols should be interpreted cautiously (Fig. 18b, e, f). It is also noteworthy that lectin blot analysis of extract (glyco)proteins will not necessarily cover the entire panel of lectin-reactive glycoproteins which bind to the lectin in affinity chromatography and are thus significantly enriched (Fig. 18b, f). Keeping these warnings in mind, adequately controlled lectin blotting remains a suitable choice for gaining information on the profile of strongly reactive glycoproteins following electrophoretic separation and presentation on a matrix.

Discussion

Our glycomic profiling by lectin histochemistry routinely worked with Bouin-fixed sections. This decision was based on the experience in test series with three different fixatives and is in accordance with previous reports (Söderström et al., 1984; Wollina et al., 1989; Danguy et al., 1991; Kaltner et al., 2002). Due to the noted fine-specificity differences among lectins using the same monosaccharide structure as primary target we tested more than one marker for those sugar units (letters in the sugar code) that are often positioned at spatially accessible terminal positions in glycan branches. As noted in the introduction, an elaborate enzymatic system has evolved which facilitates signal diversity, especially at these sites (Brockhausen and Schachter, 1997; Reuter and Gabius, 1999). The results obtained confirm the validity of our assumption that the differences in oligosaccharide ligand selection given in Table 1 indeed translate into distinct binding patterns in this organ. The differences presented provide an instructive example of how a display of distinct lectin-reactive ligands is non-randomly regulated. They are keys to proceed from mapping of glycan complexity to functional considerations within the framework of the concept of the sugar code. Admittedly, our analysis, which was rigorously controlled for specificity, was confined to those marker-accessible epitopes which would not be subject to a loss by solvent extraction during processing.

In contrast to previous lectin histochemical studies in this context we focused localization on lectin-reactive sites with comparatively high avidity by using a relatively low marker concentration. Reasons which motivated us to do this included a) the intention to avoid a saturating concentration in order to be able to separate such sites from more common and functionally probably less relevant low-density presentation, and b) the notion that endogenous lectins will likewise, very selectively, be reactive at a low dose with particular ligands whose presentation is spatially restricted. A graphic example for this assumed selectivity is set by galectins (for an introduction to lectin nomenclature in animals, please see Gabius, 1997a). These tissue lectins target only a few distinct glycans on cell surfaces at physiologically relevant concentrations; for example, the pentasaccharide of ganglioside GM_1 or of integrins in the case of galectin-1, despite the abundance of other ß-galactosides (Kopitz et al., 1998, 2001, 2003; André et al., 1999b; Siebert et al., 2003a). Following systematic titration assays the marker concentration of 2.5 µg/ml was found to be suitable for profiling, with the exception of VAA where a concentration of 1.25 µg/ml was already sufficient. Under these conditions we observed clear evidence for cell-type-selective and developmentally regulated expression of certain classes of epitopes, especially those harboring Gal/GalNAc moieties. These results directed our selection of a marker for ensuing biochemical analyses of lectin-reactive glycoproteins to VAA.

Interestingly, fucose-containing glycans were below the detection limit at this concentration. In mice, the targeted deletion of α2-fucosyltransferases FUT1/FUT2 did not appear to affect sperm maturation or fertility, intimating that this type of fucosylation is not essential in this species. However, a documented role of fucosylated oligosaccharides in adhesion *in vitro* between rat spermatogenic cells and Sertoli cells precludes the general reference to this glycan part as not eminently active (Raychoudhury and Millette, 1997). Regarding regulation of presentation of Gal/GalNAc residues it is noteworthy that evidence is available for the presence of potential receptors *in situ*. They are galectin-1, which has been mentioned in the previous paragraph due to its selectivity to home in on cell-surface epitopes, in human and rat testis and also human testicular tumors, as well as a C-type lectin related to the hepatic asialoglycoprotein receptor in human and rat testis and also GalNAc-binding sites in human testicular tumors (Gabius et al., 1987; Abdullah and Kierszenbaum, 1989; Goluboff et al., 1995; Wollina et al., 1999; Xu et al., 2000; Dettin et al., 2003; Kayser et al., 2003; Martinez et al., 2004). The latter set of results was obtained with neoglycoproteins, a convenient way to visualize glycan-binding epitopes in tissue sections (Gabius et al., 1993, 1998; Gabius, 2001a; Hittelet et al., 2003). Because carrier-immobilized carbohydrates had thus already been instrumental for detection of sites complementary to certain carbohydrate determinants in testicular tissue, they established an option for the clarification of the suggested presence of a Sertoli cell lectin for the special functionally crucial N-glycan, termed 310.11 on mouse germ cells, as referred to in the introduction (Akama et al., 2002).

A pronounced developmental regulation with complete loss of the strong reactivity in gonocytes in early stages, seen from the 12th week of gestation onwards, and the reappearance of intense staining in seminiferous tubules in adult testis was observed for VAA. This lectin is a potent glycan cross-linker and mitogen for immune and, notably, tumor cells and an elicitor of mediator release as well as an inducer of cell-cell adhesion (Hajto et al., 1990; Gabius et al., 1992, 2001; Gupta et al., 1996; Timoshenko et al., 1999, 2000, 2001; Dettmann et al., 2000; Kunze et al., 2000). Thus, a correlation between the measured decrease in lectin staining and the migration of the primordial germ cells is suggestive. Its binding to galactosides shows no marked selectivity for the anomeric linkage, because anomeric isomers Galß2(3)Gal and Galα3(4)Gal are efficient ligands (Lee et al., 1992, 1994; Galanina et al., 1997; Bharadwaj et al., 1999; Alonso-Plaza et al., 2001). The differential course of developmental changes of ECA/PNA-reactive sites in combination with qualitative similarity to the profile of GSA I-B_4 reactivity suggests the importance of α-galactoside-containing epitopes. Conceptually, it is clearly advantageous to run assays with several lectins sharing specificity for the same monosaccharide to sort out likely ligand structures. Regarding Galα3Gal structures a report on mice failed to detect mRNA for the α3-galactosyltransferase in spermatids (Johnston et al., 1995). As already noted above, species differences in this aspect have not yet been fully resolved. With regard to the definition of the lectin-reactive epitope(s) by lectin histochemistry a new aspect besides the sequence of the sugar ligand is emerging.

This aspect concerns ligand density. The mode of spatial presentation causes marked modulation of ligand properties. Epitope density by glycan branching and clustering is increasingly being recognized as a crucial factor for regulating lectin avidity. A focus of current research is given to membrane lectins governing serum glycoprotein turnover and soluble endogenous or dietary lectins binding to surface glycans (Gabius, 1991, 2004; Wu et al., 2001, 2002, 2003, 2004; Weigel and Yik, 2002). To continue this line of research, i. e. the detailed analysis of the influence of spatial parameters on the extent of lectin reactivity with complex natural ligands, is sure to advance our knowledge on operative *in situ* binding partners. In this context we are beginning to learn how the introduction of substitutions into glycan chains such as core fucosylation or bisecting GlcNAc, implants regulatory switches. They have been delineated to have a bearing on the glycan's conformation and, notably, on lectin (galectin-1 and VAA) affinity, another level of regulation of ligand properties (André et al., 1997, 2004b; Unverzagt et al., 2002). Consequently, the histochemical results of glycomic profiling, especially at low marker concentrations, will benefit from thorough specificity analysis of the markers, taking clustering, branching and presence of substitutions into account. In our study, we have further analyzed the profile of glycoproteins reactive with VAA to answer the question as to whether and to what extent it is subject to qualitative changes.

Lectin blot analysis clearly revealed marked alterations in this aspect between fetal and adult tissue samples. In contrast to the cases of heart, kidney and liver samples (Kaltner et al., 1997), this biochemical monitoring of VAA-binding epitopes defined more than quantitative changes in the profile of protein bands. The methodological comparison between results obtained by lectin blot, lectin affinity chromatography and combined lectin affinity chromatography/blot analysis delineated differences when working with either method. When faced with limited availability of material, our results tend to recommend lectin blot analysis. In aggregate, the glycomic profiling by lectin histochemistry at low marker concentration characterized cell-type-selective and developmental regulation in bovine testis. Fittingly, a lectin known for its potent activity to trigger biosignaling and adhesion/agglutination detected pronounced alterations in the course of development and in relation to adult tissue. Biochemical analysis attributed qualitative changes in the glycoprotein profile to the histochemically observed alterations. As noted above, when access to tissue is a problem lectin blot analysis appears to be preferable to lectin affinity chromatography. Nonetheless, the occurrence of differences in results between the two methods, which we have described in detail based on our gel electrophoretic analyses, should be kept in mind.

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