Different Glycoconjugate Content in Mucus Secreting Cells of the Rat Fundic Gastric Glands

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ABSTRACT

The fundic glands of the stomach contain two types of mucous cells: Surface mucous cells (SMCs) located at the surface of the stomach and the pits, and mucous neck cells (MNCs) situated in the neck of the glands. They produce mucins, highly glycosylated proteins. Very little is known about the glycan composition of these mucins and of gastric secretion in general. We used several lectins combined with deglycosylation pretreatments to analyze the glycan composition of SMCs and MNCs. The results showed the presence of terminal sialic acid and subterminal Gal and GalNAc, which is consistent with previous knowledge about glycosylation in mucins. Our results also support previous reports that showed a different expression of mucins in the SMCs, depending on their superficial or deep location in the pit. Some lectins labeled only the perinuclear region of the SMCs, but not the apical region, where the secretory granules are stored. This suggests that the lectins are labeling sugar residues that are accessible

to lectins during the first steps of glycan synthesis, which occurs in the endoplasmic reticulum and Golgi apparatus. Our results indicate that SMCs and MNCs produce a mucus secretion with a different glycoconjugate composition. The secretion is more varied in SMCs. As our results coincide with what we know about glycosylation of mucins, we can conclude that most of the glycans detected belong to mucins, and the differences in glycosylation observed in each cell type may be due, mainly, to the different secreted mucins. Anat Rec, 301:2128–2144, 2018. © 2018 Wiley Periodicals, Inc.

ABBREVIATIONS: AAL = Aleuria aurantia lectin; ABC = avidin-biotin-peroxidase complex; BSA = bovine serum albumin; BSI-B4 = Bandeiraea simplicifolia-I-B4 lectin; Con A = concanavalin A; DAB = diaminobencidine; DBA = Dolichus biflorus agglutinin; DIG = digoxigenine; DSA = Datura stramonium agglutinin; Fuc = Fucose; Gal = Galactose; GalNAc = N-Acetylgalactosamine; GlcNAc = N-Acetylglucosamine; GNA = Galanthus nivalis agglutinin; HPA = Helix pomatia agglutinin; HRP = horseradish peroxidase; Le = Lewis antigen; LFA = Limax flavus agglutinin; LTA = Lotus tetragonolobus agglutinin; MAH = Maackia amurensis hemagglutinin; Man = Mannose; MNC = mucous neck cell; MPA/MPL = Maclura pomifera lectin; MUC = mucine; NeuAc =sialid acid; PBS = phosphate buffered saline; PNA = peanut agglutining; PNGase F = peptide-N-glycosidase F; RCA-I = Ricinus communis agglutinin-I; SBA = soybean agglutinin; SMC = surface mucous cell; SNA = Sambucus nigra agglutinin; TBS = Tris buffered saline; UEA-I = Ulex europaeus agglutinin-I; WGA = wheat germ agglutinin. Grant sponsor: Fundación Séneca, Comunidad Autónoma de la Región de Murcia; Grant number: 04542/GERM/06; Grant sponsor: The University of the Basque Country UPV/EHU; Grant numbers: UFI 11/44, EHUA15/26, EHUA13/15.
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INTRODUCTION

The rat stomach can be divided into three different regions: the proximal region is covered by a squamous stratified epithelium without glands, the second región forms the corpus, which contains fundic or gastric glands, and the third region is the pyloric antrum, which has antral or pyloric glands. The corpus and the antrum form the glandular stomach (Lee et al., 1982). The gastric glands open at the bottom of the pits, which are invaginations of the surface of the glandular stomach. The pit and the glands that open at the bottom form a gastric unit. In the corpus, there are two types of mucus-producing glandular cells: surface mucous cells (SMCs), which cover the surface of the stomach and the pits, and mucous neck cells (MNCs), which are localized in the upper part of the corpus glands (Mills and Shivdasani, 2011; Hoffmann, 2013). SMCs are cylindrical and bulge into the lumen of the stomach. The nucleus is basal with a lateral or supranuclear Golgi apparatus. The apical secretory granules are larger in the cells of the upper part of the pit than in the cells of the bottom. MNCs are thin and cylindrical cells whose basal pole, which contains the nucleus, is compressed by neighboring parietal cells, and whose apical cytoplasm is wide. Secretory granules occupy a larger part of the cell than in the SMCs (Wattel et al., 1977). Both cell types produce a secretion rich in highly glycosylated mucins, which forms the mucous gel that covers the gastric surface to protect it from the acidic pH of gastric juice and other noxious agents (Laine et al., 2008; Corfield et al., 2001; Khoder et al., 2016).

The mucin (MUC) protein family includes 22 different proteins that can be either transmembrane (membranebound) or secreted (gel-forming) (Hollingsworth and Swanson, 2004; Kaur et al., 2013). Gastric glandular cells produce both transmembrane and secreted mucins, and it has been shown that SMCs and MNCs secrete different mucins (Reis et al., 2000; Duarte et al., 2016), thus we should expect a different glycan composition in both cell

types. The properties of mucins directly correlate with the protective properties of the mucus secretion (Khoder et al., 2016). In addition, pathological disorders alter the production of mucins by gastric cells (Corfield et al., 2001; Duarte et al., 2016).

The composition of the gastric mucous secretion has been studied by several approaches, both in physiologic and pathological conditions (Dekker et al., 1989; Klomp et al., 1994; Corfield et al., 2001; Holmén Larsson et al., 2013; Duarte et al., 2016), including the analysis of glycoconjugates by means of lectin histochemistry (Madrid et al., 1990, 1998a). The use of lectin histochemical procedures combined with deglycosylation pretreatments allows in situ analysis of the glycan composition of glycoconjugates (Spicer and Schulte, 1992; Brooks and Hall, 2012; Valbuena et al., 2010, 2011a,b, 2012, 2016). We investigated the glycan composition of gastric parietal cells by lectin histochemistry combined with deglycosylation pretreatments (Gómez-Santos et al., 2017a); but reports about the glycan composition of mucous secreting cells of the stomach using deglycosylation pretreatments are lacking. The aim of this work was to analyze the glycan composition of gastric mucus-secreting cells, SMCs and MNCs, in the rat fundic glands, by means of lectin histochemistry combined with deglycosylation pretreatments. We used for the first time a battery of lectins, which recognize some of the most common carbohydrate moieties: Fucose (Fuc), N-acetygalactosamine (GalNAc), galactose (Gal), sialic acid (NeuAc), N-acetylglucosamine (GlcNAc) and mannose (Man), in combination with deglycosylation pretreatments. The data obtained allow us to increase our knowledge about gastric secretion and glycan production of SMCs and MNCs, which could add to our understanding of the protective role of this secretion in physiological and pathological situations.

MATERIAL AND METHODS

Reagents

We used the following reagents from EY (San Mateo, CA): peroxidase-labeled agglutinin from Limax flavus (LFA), biotinylated Aleuria aurantia lectin (AAL), Galanthus nivalis agglutinin (GNA), Glycine max agglutinin (SBA), Ulex europaeus agglutinin-I (UEA-I) and Canavalia ensiformis agglutinin (Concanavalin A, Con A).

Reagents supplied from Sigma Aldrich (Spain) were: type III glucose oxidase from Aspergillus niger, Bovine serum albumin (BSA), 3,30-diaminobenzidine (DAB), peroxidase-labeled agglutinins from Helix pomatia (HPA) and Maclura pomifera (MPA/MPL), and biotinylated Arachis hypogaea (PNA) and Lotus tetragonolobus (LTA) agglutinins.

Other reagents were obtained from Roche (Spain): the enzyme Peptide-N-glycosidase F (PNGase F) from Flavobacterium meningosepticum and expressed in Escherichia coli, peroxidase-labeled anti-digoxigenin antibody (HRPanti- DIG), digoxigenine-labeled Datura stramonium (DSA) and Sambucus nigra (SNA) agglutinins.

Finally, some reagents were purchased from Atom (Barcelona, Spain): avidin-biotin-peroxidase complex (Vectastain ABC kit peroxidase standard), Avidin-biotin blocking kit, biotinylated Maackia amurensis haemagglutinin (MAH), Bandeiraea simplicifolia lectin I-B4 (BSIB4), Ricinus communis agglutinin-I (RCA-I), Dolichos biflorus (DBA) and Triticum vulgaris (WGA) agglutinins.

Samples

We used paraffin-embedded samples of rat stomach from our archives, which came from a previous study (Gómez-Santos et al., 2007) and were obtained during the years 2002 and 2003 from six adult male Sprague–Dawley rats weighing between 250 and 300 g. For the controls of deglycosylation techniques, we also used samples of gall bladder, testis and intestine from our archives (Madrid et al., 1994). At the time the samples were obtained, we followed all the applicable international, national and institutional guidelines for the care and use of animals. Samples were stored until use.

Histochemical Techniques

Table 1 shows the 17 lectins used, their origins, abbreviations, and specificities. We obtained 4-μm-thick sections from the paraffin embedded samples, removed paraffin, hydrated the sections, and immersed them in 1% (v/v) H2O2 in Tris buffered saline (TBS) to block the endogenous peroxidase. Then, for peroxidase-labeled lectins, we washed the sections with TBS and incubated with the lectin diluted in TBS at room temperature in a moist chamber for 1 h 30 min. We diluted each lectin as indicated: 6 μg mL−1 HPA, 20 μg mL−1 MPA/MPL and 25 μg mL−1 LFA. Then, we washed the sections with TBS and developed the peroxidase with 0.1% (v/v) H2O2 and 0.25 mg mL−1 DAB in TBS for 7 min. Finally, we counterstained the sections with hematoxylin.

For digoxigenin-labeled lectins, to block unspecific binding of the antibody we incubated the sections in a moist chamber at room temperature for 10 min with 1% (w/v) BSA in TBS. Then, we incubated the sections with the lectin diluted in 1% (w/v) BSA in TBS for 1 hr 30 min. The dilution of each lectin was as follows: 10 μg mL−1 DSA and 30 μg mL−1 SNA. After washing

with TBS, we incubated the sections with 0.6 U mL−1 HRP-anti-DIG in 1% BSA in TBS. Finally, we developed the peroxidase and counterstained the sections as indicated above.

The procedure for biotinylated lectins differed because we replaced TBS by phosphate buffered saline (PBS), but the blocking of the endogenous peroxidase and unspecific binding sites for antibodies, and the incubation with the lectins were similar. The dilutions were 5 μg mL−1 RCA-I, WGA and MAH, 3 μg mL−1 BSI-B4, 50 μg mL−1 DBA, PNA, LTA and SBA, 10 μg mL−1 AAL, UEA-I and Con A, and 60 μg mL−1 GNA. After incubation with the lectin, we washed the sections with PBS and incubated them with ABC kit for 1 hr. Finally, we developed the peroxidase and counterstained the sections as previously indicated.

Deglycosylation Pretreatments

We carried out the histochemical procedure with every lectin in three ways, each in a different section: (1) without previous treatment, (2) after previous chemical deglycosylation (β-elimination) procedure, and (3) after previous enzymatic deglycosylation with PNGase F. For some lectins, we also carried out the histochemical procedure after previous treatment of β-elimination and PNGase F in the same section.

The chemical deglycosylation by β-elimination removes O-linked oligosaccharides (Ono et al., 1983). We immersed the hydrated sections in 0.5 N NaOH in 70% ethanol at 4 _C. We performed this technique for every lectin in different sections for 1 and 5 days, to discriminate both labile and resistant O-glycans (Gómez-Santos et al., 2007).

We removed N-linked oligosaccharides from tissues by incubation of sections with 40 U mL−1 PNGase F in a moist chamber at 37 _C (Martínez-Menárguez et al., 1993).

When deglycosylation pretreatments were performed, we blocked endogenous biotin by Avidin-biotin blocking kit, because previous unpublished observations have shown that both β-elimination and incubation with PNGase F enhance endogenous biotin labeling by ABC kit in gastric gland cells.

To determine the subterminal sugars to terminal sialic acid (NeuAc), we also tested some lectins (Con A, GNA, AAL, LTA, PNA, RCA-I, BSI-B4, MPA/MPL, HPA, DBA, SBA, and DSA) after removal of terminal NeuAc. To this end, we immersed the sections in 0.1 N HCl at 82–84 _C for 3 hr before the lectin histochemical procedure (Madrid et al., 1994). We also employed this technique with WGA, a lectin that recognizes both N-acetylglucosamine (GlcNAc) and NeuAc, to determine which carbohydrate was labeled by this lectin.

We also used the procedure of methylationsaponification with some lectins (Con A, GNA, AAL, LTA, RCA-I, BSI-B4, MPA/MPL, HPA, DBA, and DSA) to show the presence of sulfated terminal sugars. For this, we immersed the deparaffined sections in pure ethanol for 4 min and then in 1% (v/v) HCl at 60_C in methanol for 5 hr. After this, we hydrated the sections and immersed them in 1.8% (w/v) barium hydroxide at 4 _C for 1 hr (Martínez-Menárguez et al., 1992).

To verify if Con A is labeling Glucose (Glc) or Mannose (Man), we carried out a preincubation with 50 U mL−1 type III Glucose oxidase at 37 _C overnight, a method that converts Glc into gluconic acid, which is not recognized by Con A (Alonso et al., 2006).

Controls

We used the following controls for the lectin histochemical procedure: (1) substitution of the lectins or the enzyme by buffer alone, (2) preincubation of the lectins with the corresponding hapten sugar inhibitor –fucose (Fuc) for AAL, LTA and UEA-I; N-acetylgalactosamine (GalNAc) for HPA, DBA, SBA and MPA/MPL; galactose (Gal) for PNA, BSI-B4 and RCA-I; lactose for MAH and SNA; NeuAc for LFA, chitotriose for WGA and DSA (Cummings and Etzler, 2009); and Man for GNA and Con A– at a concentration of 0.2 M, and (3) staining of sections of other tissues of known altered binding pattern for each of the chemical and enzymatic pretreatments.

We carried out β-elimination combined with HPA staining of rat testis sections as specific control of the β-elimination procedure. This tissue loses the HPAlabeling when β-elimination works correctly (Martínez-Menárguez et al., 1993). The same tissue loses the AALlabeling after incubation with PNGase F (Martínez- Menárguez et al., 1993), thus we used this as a specific control of deglycosylation with this enzyme. To verify if the acid hydrolysis procedure worked properly, PNA histochemistry was performed in human gall bladder sections, whose principal cells show labeling in their secretory granules after the removal of terminal NeuAc (Madrid et al., 1994). As control of the methylationsaponification procedure, we carried out PNA histochemistry after this desulfation technique in rat intestine to verify the labeling of microvilli and goblet cells (Martínez-Menárguez et al., 1992).

Semi-Quantitative Evaluation of the Staining

Three independent observers evaluated the staining intensity in the mucous gastric cells of three histological sections from each sample and histochemical procedure with each lectin and with each deglycosylation procedure. We classified staining intensity into six categories: no

labeling (0), very weak (1), weak (2), moderate (3), strong (4), and very strong (5). When there was a different staining intensity of the cells in different sections, or in different fields of the same section, it was classified within a range indicated with the minimum and máximum values.

RESULTS

Table 2 summarizes the results. Both SMCs and MNCs are typical mucous glandular cells with a basal region, which contains the nucleus and perinuclear cytoplasm with most of the organelles, and a large apical región with plenty of mucous secretion granules. Usually, the lectins labeled all the cytoplasm of the cells, but with some lectins, the apical granular region was negative or weakly stained and the perinuclear (basal) region was positive (indicated with an asterisk in Table 2). Sometimes, the apical surface of the SMCs was stained more intensely (indicated with a hash in Table 2).

The three specific lectins labeling Fuc stained the SMCs with different intensities, AAL and LTA very weakly or weakly and UEA-I moderately (Fig. 1). After most of the pretreatments, the labeling patterns obtained with AAL were very similar to those obtained with LTA. However, after desulfation by methylation-saponification, LTA only labeled the apical Surface of the cells. Staining with both AAL and LTA increased after β-elimination or after acid hydrolysis, but with AAL the SMCs of the high and top region of the pit were more strongly labeled tan SMCs of the mid and low region (Fig. 1a–j). Labeling with UEA-I was similar after all the treatments (Fig. 1k–n).

AAL and LTA never labeled MNCs (Fig. 2a–c), whereas UEA-I moderately stained them after the histochemical procedure alone or after deglycosylation by incubation with PNGase F or after the β-elimination method (Fig. 2d–f).

Three of the GalNAc binding lectins, HPA, DBA and MPA/MPL, labeled the perinuclear region of the SMCs. In addition, HPA and MPA/MPL also stained the apical surface. However, SBA, the remaining GalNAc binding lectin labeled the entire cytoplasm of the SMCs, with more intensity in the cells of the high and top regions of the pit (Fig. 3a,f,j,m). After incubation

with PNGase F, the staining remained unaltered for HPA, DBA and MPA/MPL, but was slightly increased for SBA (Fig. 3b,g, k). The β-elimination procedure produced a decrease in HPA- and DBA-staining, but an increase in labeling with SBA—especially in the cells of the high and top regions of the pit—and in the staining of the apical granular región of the cells with MPA/MPL (Fig. 3c,h,l,n). Acid hydrolysis and methylation-saponification methods did not modify the labeling patterns, except for MPA/MPL, which also stained the secretory region of the cells (Fig. 3d,e,i,o).

The MNCs showed a different reactivity to GalNAcbinding lectins (Fig. 4). DBA never stained the MNCs (Fig. 4d–f). The MNCs were always positive for HPA and MPA/MPL alone or after any of the deglycosylation pretreatments, although some of these could increase or decrease the intensity of the staining (Fig. 4a–c,j–l). However, SBA labeled the MNCs, except after β-elimination (Fig. 4g–i).

Gal binding lectins showed different staining patterns of the SMCs (Fig. 5). PNA very weakly labeled only the perinuclear region, BSI-B4 did not label the cells, and RCA-I weakly labeled them (Fig. 5a,f,k). Staining with PNA was not modified by incubation with PNGase F, whereas after β-elimination for 1 day (but not after β-elimination for 5 days), acid hydrolysis and, to a minor degree, after methylation-saponification, the secretory granules became positive. The cells of the high and top regions of the pit were more strongly stained after β-elimination for 1 day and after acid hydrolysis (Fig. 5b– e). Although SMCs were negative for BSI-B4 or when combined with PNGase F or β-elimination, this lectin weakly stained the perinuclear region after acid hydrolysis and the secretory granules after methylationsaponification (Fig. 5g–j). Staining with RCA-I was stronger in cells of the high and top regions of the pit and increased

with different intensity after most of the deglycosylation procedures used (Fig. 5k–n), but was unaltered after methylation-saponification (Fig. 5o).

PNA and RCA-I alone, but not BSI-B4, stained the MNCs with different intensity (Fig. 6a,d,g). Labeling with PNA and RCA-I remained unaltered after deglycosylation pretreatments, except after β-elimination for 5 days, when the labeling almost disappeared (Fig. 6b,c,h–k). MNCs were negative for BSI-B4, but the methylationsaponification method turned the MNCs weakly positive to this lectin (Fig. 6d–f).

Two NeuAc binding lectins, MAH and SNA, did not label the SMCs, but LFA did (Fig. 7). Deglycosylation pretreatments did not modify the labeling pattern with MAH or SNA (Fig. 7a–f). Staining of the SMCs with LFA was stronger in the cells of the high and top regions of the pit and remained or even increased slightly after pretreatments with PNGase F or β-elimination (Fig. 7g–i).

MAH and SNA did not label the MNCs, whereas LFA was positive (Fig. 8a,d,g). None of the deglycosylation pretreatments modified the labeling pattern of each of the lectins, except for β-elimination, which resulted in a decrease in labeling with LFA (Fig. 8b,c,e,f,h,i).

Both WGA and DSA labeled the apical surface of the SMCs (Fig. 9). After all deglycosylation pretreatments, the apical granular cytoplasm was also labeled by WGA with different intensities: moderate after PNGase F, strong after β-elimination, and very strong after acid hydrolysis (Fig. 9b–d). In a similar way, the staining of the apical granular cytoplasm of the SMCs increased with DSA after β-elimination for 1 day, acid hydrolysis and methylation-saponification (Fig. 9f–i). With both lectins, the cells of the high and top regions of the gland were more strongly stained than cells of the mid and low regions (Fig. 9c,g).

WGA very weakly stained the MNCs, but the staining remained unaltered or slightly increased after every deglycosylation pretreatment (Fig. 10a–c). In a similar way, MNCs were negative for DSA but they turned moderately positive after any deglycosylation procedure (Fig. 10d–g).

The granular cytoplasm of the SMCs was negative after histochemistry with any of the Man binding lectins, GNA and Con A, but the perinuclear region was strongly labeled (Fig. 11). The labeling pattern with GNA remained almost unaltered after any of the deglycosylation pretreatments, although labeling intensity decreased after PNGase F (Fig. 11b–e). The apical cytoplasm of the SMCs was slightly stained by Con A after β-elimination or acid hydrolysis (Fig. 11g,h). Con A and GNA showed staining at the apical surface after methylationsaponification (Fig. 11e,i). Preincubation with glucose oxidase decreased the intensity of perinuclear Con A-staining (Fig. 11j).

MNCs were always negative for GNA (Fig. 12a–d). Con A labeled the cells, most of the pre-treatments slightly increased the staining, but this decreased after incubation with glucose oxidase (Fig. 12e–i).

DISCUSSION

The use of lectin histochemistry combined with deglycosylation pretreatments allows us to obtain more information on the composition of the glycoconjugates of a tissue. However, these results are sometimes difficult to interpret.

If labeling with a lectin disappears after incubation with PNGase F, we can conclude that the carbohydrate identified by the lectin is an N-linked oligosaccharide. If the labeling disappears after 1 day of β-elimination, then the sugar is in labile O-linked oligosaccharides, and if a 5 day treatment is required, it is in resistant O-linked oligosaccharides (Gómez-Santos et al., 2007). Sometimes the results are confusing, since the labeling may remain after both pretreatments (PNGase F and β-elimination) and it can be interpreted that sugar is in both types of glycan, while at other times a new staining can appear and it may be thought that some hidden carbohydrates are unmasked by the deglycosylation pretreatment (Valbuena et al., 2012; Gómez-Santos et al., 2007, 2017a).

Acid hydrolysis eliminates the terminal residues of NeuAc (Madrid et al., 1994) and the methylationsaponification method removes the sulfate groups from the sugars (Martínez-Menárguez et al., 1992), so if after any of these pretreatments the labeling increases (or new labeling appears), it can be deduced, respectively, that we identify carbohydrates subterminal to NeuAc or sulfated carbohydrates.

Previous studies have shown that the lectin labeling in the rat stomach identifies mainly the glycans of the mucins (Kodaira et al., 2000). Mucins are glycosylated proteins that are highly expressed in mucous glandular cells (Brockhausen et al., 2009). However, lectins can also

recognize other glycoproteins, glycosphingolipids and, in a few cases, sulfated glycosaminoglycans, then making it difficult to draw definitive conclusions (Varki et al., 2009).

The gastric mucosa expresses MUC1, a transmembrane mucin that can also be released from the cell surface, and two secreted mucins, MUC5AC and MUC6. SMCs express MUC5AC and MUC1, whereas MNCs express MUC6 and MUC1 (Carlstedt et al., 1995; De Bolós et al., 1995; Ho et al., 1995; López-Ferrer et al., 2000; Duarte et al., 2016). Most of the oligosaccharides in mucins are attached by O-links to the protein core (Brockhausen, 1999). MUC5AC O-glycans frequently carry terminal structures classified as type 1 Lewis antigens, including Lea –Galβ(1,3)[Fucα(1,4)]GlcNAcβ(1-)–, Leb Fucα(1,2)Galβ(1,3)[Fucα(1,4)]GlcNAcβ(1-)–, and sialyl-Lea –NeuAcα(2,3)Lea–. On the other hand, the O-linked oligosaccharides of the MUC6 mucin carry terminal structures classified as type 2 Lewis antigens, for example Lex –Galβ(1,4)[Fucα(1,3)]GlcNAcβ(1-)–, Ley – Fucα(1,2)Galβ(1,4)[Fucα(1,3)]GlcNAcβ(1-)–, and sialyl-Lex –NeuAcα(2,3)Lex– (Duarte et al., 2016). Mucins also include a small number of oligosaccharides N-linked to the protein core (Corfield et al., 2001), including high-mannose, complex and hybrid-type N-linked oligosaccharides (Ho et al., 2003; Parry et al., 2006).

Some of the results of lectin histochemistry shown here are consistent with previous knowledge about glycosylation in mucins. Some results agree with the expected terminal structures of mucin glycans. For example, the staining with PNA and RCA-I, which can label sequences of carbohydrates present in the Lea and Lex antigens, suggests the presence of these sequences in masked Oglycans in the SMCs and in resistant O-linked oligosaccharides in MNCs. Also, we would expect to localize Fuc in both SMCs and MNCs. This was the case for SMCs, but MNCs were only labeled with UEA-I, which recognizes Fuc bound by α(1,2) linkage, found in Leb and Ley antigens. However, the results obtained with Fuc-binding lectins were consistent with

previous results described in rat and human stomach (Madrid et al., 1998b; Kodaira et al., 2000). Mucins also have sialic acid as peripheral residues in α(1,3), α(1,6) and α(1,8) linkage (Corfield et al., 2001), and our results showed that SMCs contain Fuc, Gal and GalNAc subterminal to NeuAc, as shown by AAL, LTA, MPA/MPL, PNA, and RCA-I after acid hydrolysis. However, only LFA, a lectin that labels NeuAc bound by any linkage, labeled SMCs, while MAH, which labels α(2,3) linked NeuAC, and SNA, which recognizes α(2,6), did not. It is difficult to explain why MAH and SNA were negative, but we can hypothesize that the mucins are oligomeric proteins and this oligomerization could produce a high packing that leaves the sugars inaccesible to some lectins. In fact, our results have shown that the labeling with some lectins increased after some deglycosylation treatments, suggesting that these treatments unmask glycans and make them accessible to lectins. However, MUC5AC in mouse stomach shows a low level of sialylation (Holmén Larsson et al., 2013), and this could happen in rat stomach as well.

Our results are also in agreement with the differential expression of mucins by gastric cells, because we have seen a different pattern of glycosylation in SMCs and MNCs. For example, HPA, MPA/MPL, PNA, and Con A, labeled the secretion of MNCs, but only labeled the secretion of SMCs after some of the deglycosylation pretreatments. Previously, Suganuma et al. (1985) considered HPA as a specific marker of MNCs in the rat fundic mucosa. On the other hand, the lectins AAL, LTA and BSI-B4 labeled the SMCs, but not the MNCs. Another lectin, DSA, which labeled the SMCs, only labeled the MNCs after pretreatments. These differences in labeling pattern between SMCs and MNCs indicate that both cells produce a mucus secretion with a different glycoconjugate composition, which can reflect the different mucin expression stated before. Previous studies have shown that SMCs and MNCs of the mouse stomach are

labeled with different lectins, however these lectins are different to those used in the present work, and both species have also different glycosylation patterns (Falk et al., 1994).

Previous reports have subdivided the rat fundic pit regions of the glands into four segments (low, mid, high and top), and the characteristics of the pit cells vary according to their situation (Yang et al., 1996; Sawaguchi et al., 2002). Our results have shown that the SMCs at the high and top regions of the pit are more strongly labeled with some lectins. These results support the hypothesis that the secretion of the SMCs varies as these cells are migrating from the low to the top regions of the pit. The SMCs at the high and top regions of the pits express Gal or GalNAc moieties (Sawaguchi et al., 2002), which coincides with the SBA, PNA and RCA-I labeling shown in the present work. However, our results did not show a higher expression of sulfated glycans by SMCs at the low region of the pit as previously suggested (Yang et al., 1996; Sawaguchi et al., 2002).

Some of our results suggest the existence of N-linked glycans, which is the last conclusion consistent with previous knowledge about glycosylation in mucins. For example, an increase in AAL and LTA labeling after β-elimination suggests that there are Fuc moieties in Nlinked oligosaccharides masked by O-linked glycans. This idea is supported by the fact that AAL preferentially labels Fuc in N-linked oligosaccharides (Osawa and Tsuji, 1987).

Furthermore, some lectins, such as HPA, DBA, MPA/MPL, GNA and Con A, labeled the perinuclear region, but not the apical secretory region of the SMCs. The results with HPA, DBA and MPA/MPL suggest that GalNAc residues are accessible to lectins during the first steps of glycan synthesis, which occurs in the endoplasmic reticulum and Golgi apparatus in the perinuclear region, but are then masked as the synthesis continues and glycans pack inside

the secretory granules. This was described earlier in some previous work with HPA in human SMCs (Madrid et al., 1990). Some of these lectins, such as MPA/MPL, labeled the apical granular region of the SMCs after β-elimination, which suggests that some GalNAc moieties in the secretory granules are unmasked after β-elimination. Others, such as HPA, stained the apical surface of the SMCs, and this could be due to the decompression/unpacking of the mucins during exocytosis of the secretory granules, which could make some sugars accessible to the lectins. Regarding the Man binding lectins (GNA and Con A), the observation that they labeled only the perinuclear region can be explained by the fact that many glycans have Man terminal moieties during their synthesis in the endoplasmic reticulum and the Golgi apparatus.

In conclusion, both SMCs and MNCs, the two mucussecreting cells, showed a different pattern of labeling with some lectins. In short, AAL, LTA, and BSI-B4 marked the SMCs but not the MNCs; HPA, MPA/MPL, PNA, and Con A, which labeled the MNCs, only labeled the entire cytoplasm of the SMCs after some pretreatments; and DSA, which weakly labeled the SMCs, only labeled the MNCs after pretreatments. Taken together, the results indicate that both cell types produce a mucus secretion with a different glycoconjugate composition, a secretion that seems to be richer and more varied in the SMCs. Moreover, in some cases, the glycosylation of SMCs is dependent on the cell location in the pit, which could suggest a relationship with cell maturation.

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AUTHOR CONTRIBUTIONS

LG-S and EA contributed to design, acquisition of data, data analysis and interpretation, and critical revision of the manuscript. JFM contributed to concept and design, data interpretation, critical revision of the manuscript and approval of the article. LD-F contributed to data analysis, critical revision of the manuscript and approval of the article. FJS contributed to design, data analysis and interpretation, drafting and revision of the manuscript, and approval of the article.

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Table Legends

TABLE 1. Binding specificity of the lectins used

Lectin	Abbreviation	Oligosaccharide affinity[a]
Orange peel (*Aleuria aurantia*) lectin	AAL[b]	Fucα(1,6) > Fucα(1,2) > Fucα(1,3)
Asparagus pea (*Lotus tetragonolobus*) agglutinin	LTA	Fucα(1,3) > Fucα(1,2)
Gorse seed (*Ulex europaeus*) agglutinin-I	UEA-I	Fucα(1,2)
Snail (*Helix pomatia*) agglutinin	HPA	GalNAcα(1,3) > > Galβ(1,3)
Horse gram (*Dolichos biflorus*) agglutinin	DBA	GalNAcα(1,3)
Soybean (*Glycine max*) agglutinin	SBA	αGalNAc > αGal > βGalNAc
Osage orange tree (*Maclura pomifera*) lectin	MPA/MPL	GalNAc, Gal
Peanut (*Arachis hypogaea*) agglutinin	PNA	Galβ(1,3) > Galβ(1,4) > Gal
Griffonia (*Bandeiraea simplicifolia*)I-B_4 lectin	BSI-B_4	Galα(1,3) > Galα(1,2)
Castor bean (*Ricinus communis*) agglutinin I	RCA-I	N-acetyllactosamine > neo-N-acetyllactosamine > αGal
Amur maackia (*Maackia amurensis*) hemagglutinin	MAH[c]	NeuAcα(2,3)
Elderberry bark (*Sambucus nigra*) agglutinin	SNA	NeuAcα(2,6)
Yellow slug (*Limax flavus*) agglutinin	LFA	NeuAc
Wheat germ (*Triticum vulgaris*) agglutinin	WGA	NeuAc > $(GalNAc)_{2-3}$ >> > GlcNAc
Thorn apple (*Datura stramonium*) agglutinin	DSA	$(GlcNAc)_{2-3}$ > GlcNAc > Galβ(1,4)
Snowdrop (*Galanthus nivalis*) agglutinin	GNA	Manα(1,3)Man > Man
Concanavalin A or Jack bean (*Canavalia ensiformis*) agglutinin	Con A	Man, Glc

Table 1: “Greater than” symbols indicate decreased affinity. aReferences in Valbuena et al. (2010, 2011a,b, 2012, 2016). bAAL is sometimes abbreviated in other works as AAA, but then could be confused with the lectin from fresh water eel (Anguilla anguilla), which is usually abbreviated as AAA. cMAH is also abbreviated in other works as MAA-II, MAA-2 and MAL-II. MAH should not be confused with MAL (Maackia amurensis leukoagglutinin, also abbreviated as MAM, MAL-I and MAA-I) or MAA (an undefined mixture of MAL and MAH).

TABLE 2. Semiquantitative evaluation of lectin-binding lectin labeling of the surface mucous cells (SMCs) and mucous neck cells (MNCs) in the rat fundic gastric glands

Lectin		Alone	PNGase F	β-elimination (1d)	β-elimination (5d)	Acid hydrolysis	Methylation-saponification	Glucose oxidase
AAL	SMCs	1–2	2	4	4	3	2	
	MNCs	0	0	0	0	0	0	
LTA	SMCs	1–2	2	4	4	2	0[a]	
	MNCs	0	0	0	0	0	0	
UEA-I	SMCs	2–3	2–3	3	3			
	MNCs	3	3	3	3			
HPA	SMCs	0[a,b]	0[a,b]	2	0	0[a,b]	0[a,b]	
	MNCs	5	5	5	3	4	4	
DBA	SMCs	0[b]	0[b]	0	0	0[b]	0[b]	
	MNCs	0	0	0	0	0	0	
SBA	SMCs	3	3–4	5	4	2–3		
	MNCs	2	2	0	0	1–2		
MPA/MPL	SMCs	0[a,b]	0[a,b]	3	3	2–3[b]	1–2[a,b]	
	MNCs	3	3	3–4	3	2–3	3	
PNA	SMCs	0[b]	0[b]	5	0–1	4	2[a,b]	
	MNCs	5	4–5	5	0	4–5	5	
BSI-B_4	SMCs	0	0	0	0	0[b]	3–4	
	MNCs	0	0	0	0	0	1	
RCA-I	SMCs	2[a]	2–3[a]	5	3	3	2[a]	
	MNCs	3	3	3	0	3	3	
MAH	SMCs	0	0	0	0			
	MNCs	0	0	0	0			
SNA	SMCs	0	0	0	0			
	MNCs	0	0	0	0			
LFA	SMCs	3	3–4	3	3			
	MNCs	3–4	4	1–2	1–2			
WGA	SMCs	1[a]	2–3	4	3–4	5		
	MNCs	1	2	2	3	3		
DSA	SMCs	0–1[a]	0–1[a]	4	0–1	3–4	2–3	
	MNCs	0	3	3	2	1–2	3	
GNA[c]	SMCs	0[b]	0[b]	0[b]	0[b]	0[b]	0[a,b]	
	MNCs	0	0	0	0	0	0	
Con A	SMCs	0[b]	0[b]	2[b]	0–1[b]	2[b]	0[a,b]	0[b]
	MNCs	1–2	2–3	3	2–3	2–3	2	0–1

Table 2. The table shows the staining intensity of the apical secretory cytoplasm of the cells evaluated and classified into six categories: no labeling (0), very weak (1), weak (2), moderate (3), strong (4), and very strong (5). When there was a variation in staining of the same cell type, the range of staining is indicated with the minimum and maximum values separated by a dash. When there is no value, it means that the procedure was not done. aThe apical surface of the cells was labeled. bThe perinuclear region was labeled. cSome data of GNA histochemistry of MNCs has been previously published (Gómez-Santos et al., 2017b).

Figure Legends

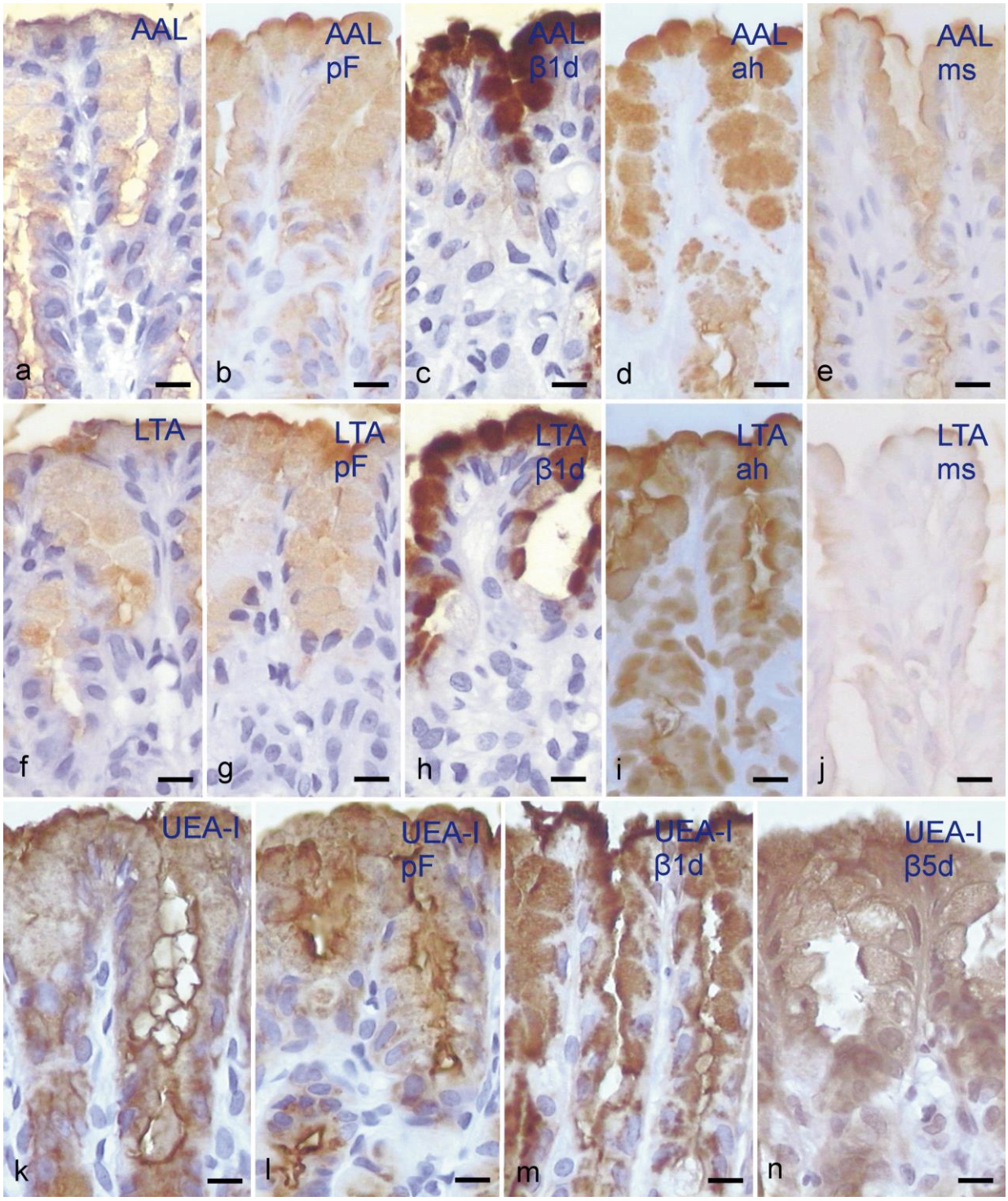

Figure. 1. Histochemical staining of SMCs with Fuc-binding lectins. The lectins are abbreviated as indicated in Table 1. The SMCs were weakly labeled by AAL, except when β-elimination was previously done (a–e), which showed that the cells of the high and top regions of the pit were strongly labeled (c). Similar results were observed with LTA (f–j). UEA-I moderately labeled the cells in all procedures used (k–n). Abbreviations: ah: acid hydrolysis;

ms: methylation-saponification; pF,incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

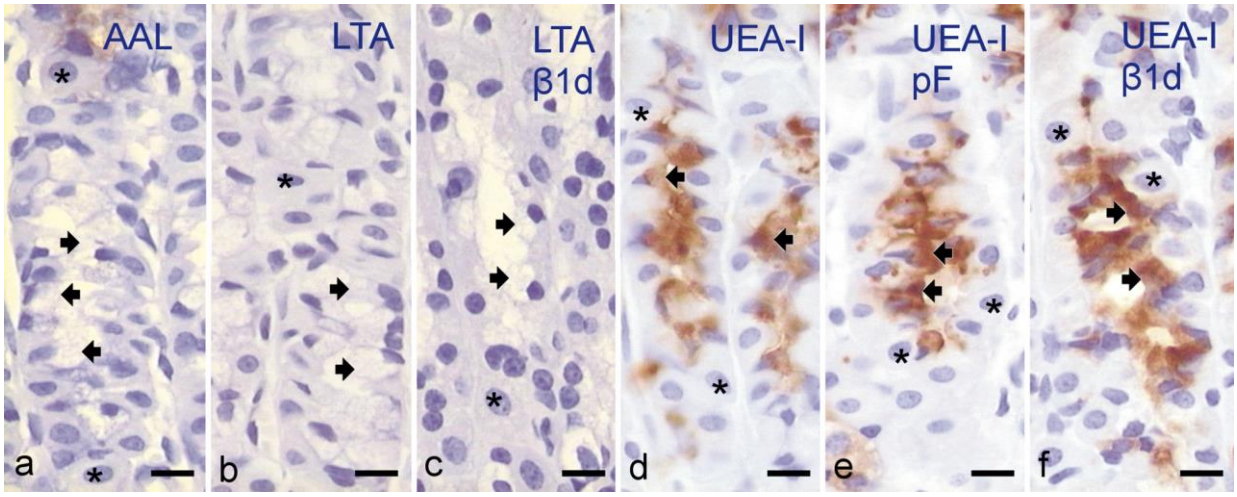

Figure. 2. Histochemical staining of MNCs with Fuc-binding lectins. The lectins are abbreviated as indicated in Table 1. The MNCs were always negative for AAL and LTA (a–c), but moderately labeled by UEA-I after all procedures employed (d–f). Some MNCs are marked by arrows, and some of the interspersed parietal cells are tagged with asterisks. Abbreviations: pF, incubation with PNGase F; β1d, β elimination for 1 day. Scale bars, 20 μm.

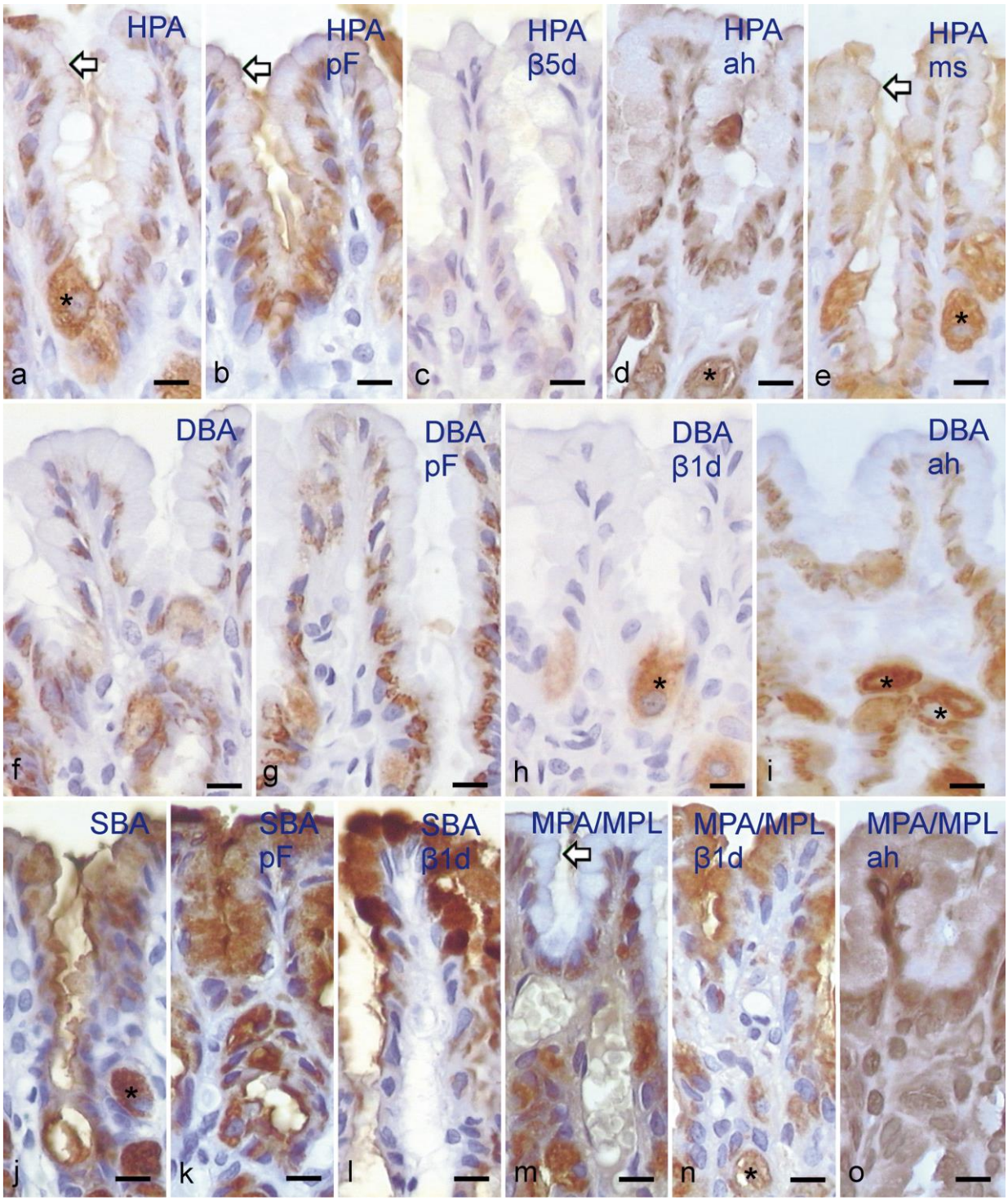

Figure. 3. Histochemical staining of SMCs with GalNAc-binding lectins. The lectins are abbreviated as indicated in Table 1. Most labeling of the SMCs with HPA was in the perinuclear region, but no labeling was observed after β-elimination for 5 days (a–e). Labeling with DBA showed a similar pattern, but no labeling was seen after β-elimination for 1 day in the SMCs (f–i). The moderate labeling with SBA increased after β-elimination pretreatments,

and was stronger in the cells of the high and top regions of the pit (j–l). MPA/MPL labeled the perinuclear region of the SMCs in a similar way to HPA and DBA, but, in contrast to them, the apical secretory region of the cells was stained after some pretreatments, such as β-elimination or acid hydrolysis (m–o). Sometimes, the apical surface of the SMCs was stained with different intensity (white arrows). In some cases, the interspersed parietal cells were labeled by the lectins (asterisks). Abbreviations: ah: acid hydrolysis; ms: methylationsaponification; pF, incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

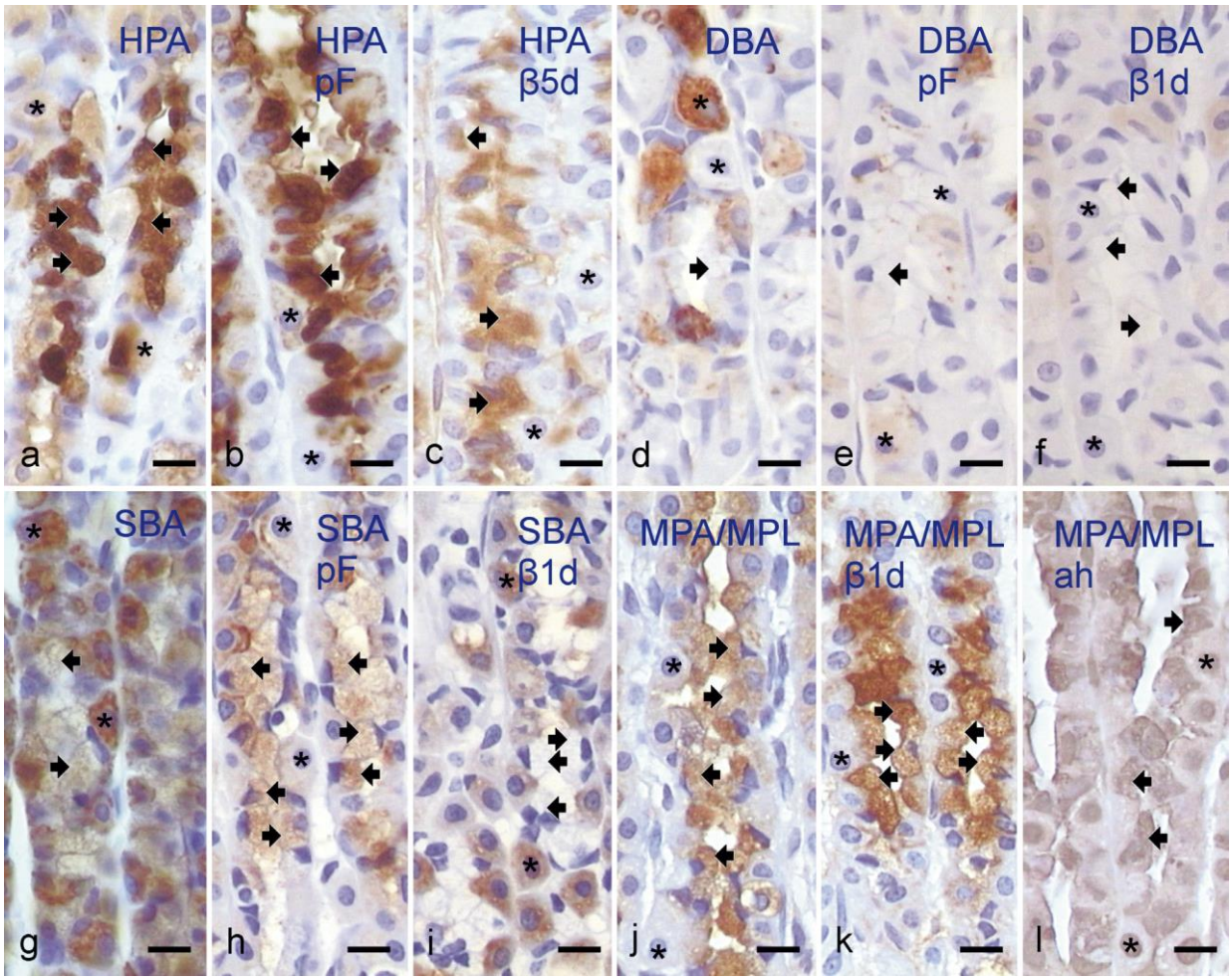

Figure. 4. Histochemical staining of MNCs with GalNAc-binding lectins. The lectins are abbreviated as indicated in Table 1. HPA labeled the MNCs in all the cases (a–c). By contrast, these cells were always negative for DBA (d–f). Labeling with SBA disappeared only after β-elimination (g–i). Finally, MPA/MPL always labeled the MNCs (j–l). Some MNCs are marked by arrows, and some of the interspersed parietal cells are tagged with asterisks. Abbreviations: ah: acid hydrolysis; pF, incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

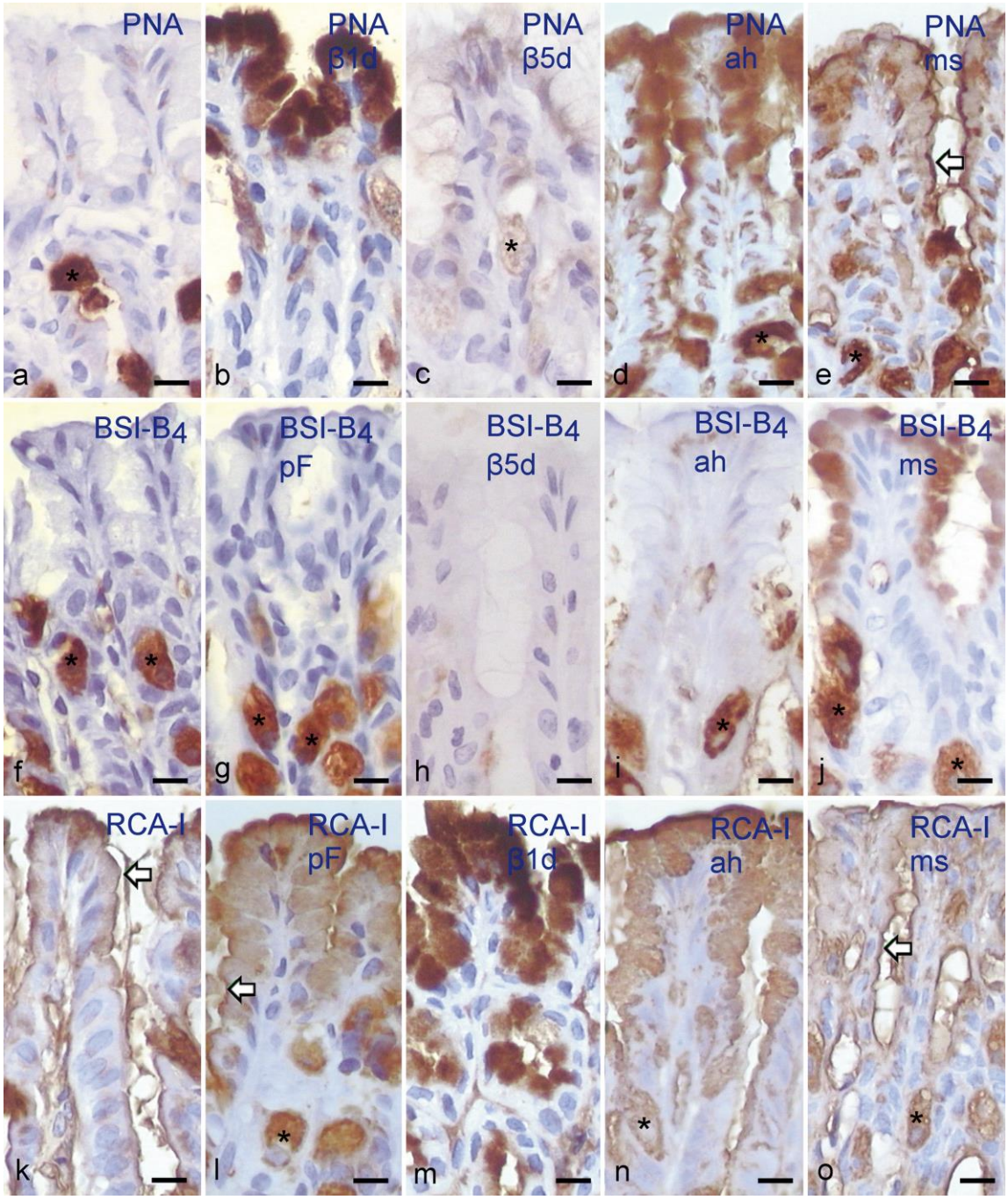

Figure. 5. Some more important results obtained after the histochemical staining of SMCs with Gal-binding lectins. The lectins are abbreviated as indicated in Table 1. PNA without pretreatments only very weakly labeled the perinuclear region of some SMCs, but strongly labeled the cells after some pretreatments (a–e). The cells of the high and top regions of the pit showed stronger staining than the cells of the low and mild regions, both after β-elimination for 1 day and acid hydrolysis (b,d). The SMCs were negative for BSI-B4, except at the perinuclear region after acid hydrolysis, and the apical secretory cytoplasm after methylation-

saponification. With RCA-I lectin, the apical surface and, at the high and top regions of the pit, the apical secretory cytoplasm of the SMCs were positive (k). Labeling of the cytoplasm increased after the deglycosylation pretreatments, except for methylation-saponification (l–o). Sometimes, the apical surface of the SMCs was stained with different intensity (White arrows). In some cases, the interspersed parietal cells were labeled by the lectins (asterisks). Abbreviations: ah: acid hydrolysis; ms: methylation-saponification; pF, incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

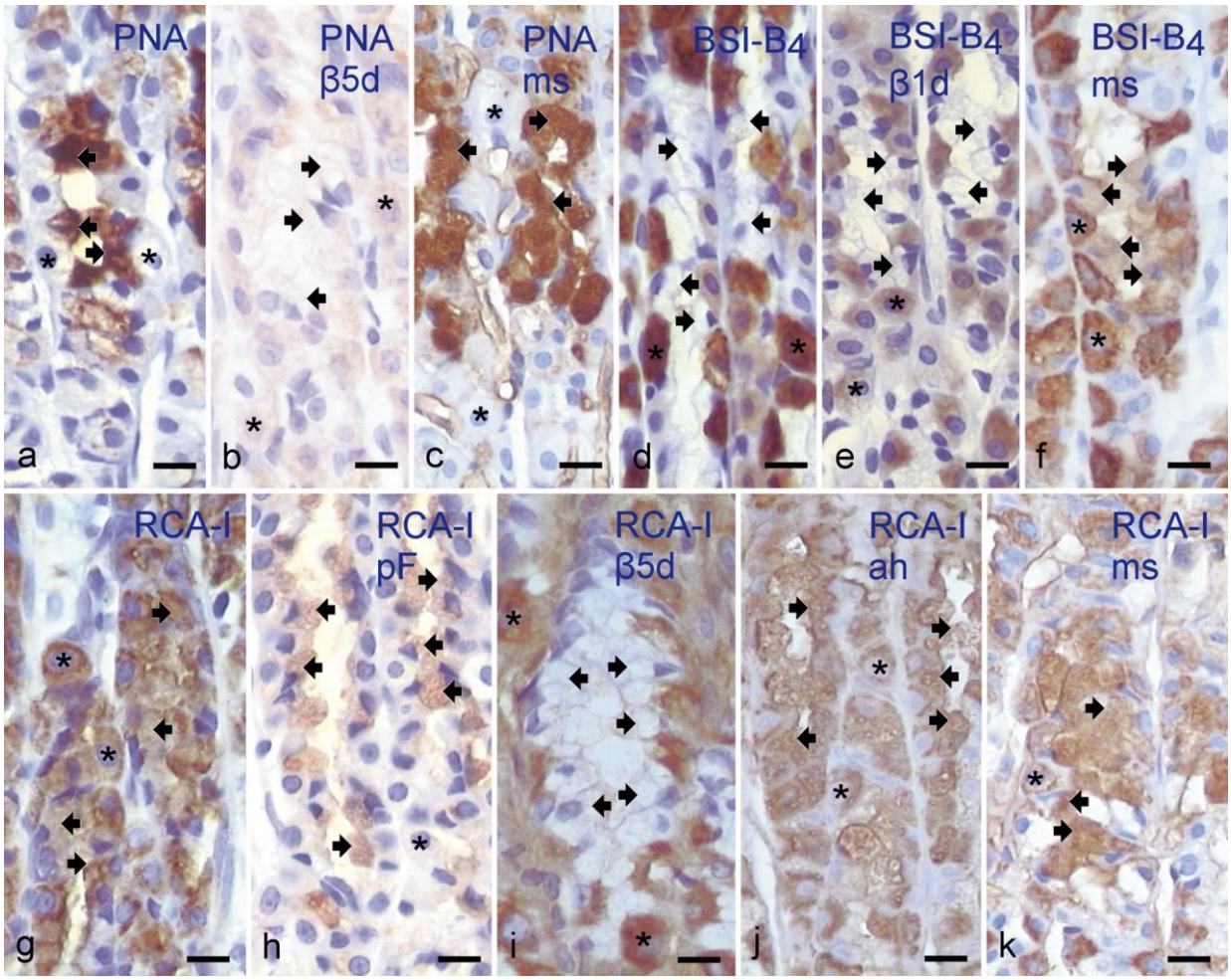

Figure. 6. Some more important results obtained after histochemical staining of MNCs with Gal-binding lectins. The lectins are abbreviated as indicated in Table 1. MNCs were positive for PNA, but there was no labeling after β-elimination for 5 days (a–c). These cells were positive for BSIB4 only after methylation-saponification pre-treatment (d–f). Like PNA, labeling with RCA-I only was impaired after β-elimination for 5 days (g–k). Some MNCs are marked by arrows, and some of the interspersed parietal cells are tagged with asterisks. Abbreviations: ah: acid hydrolysis; ms: methylation-saponification; pF, incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

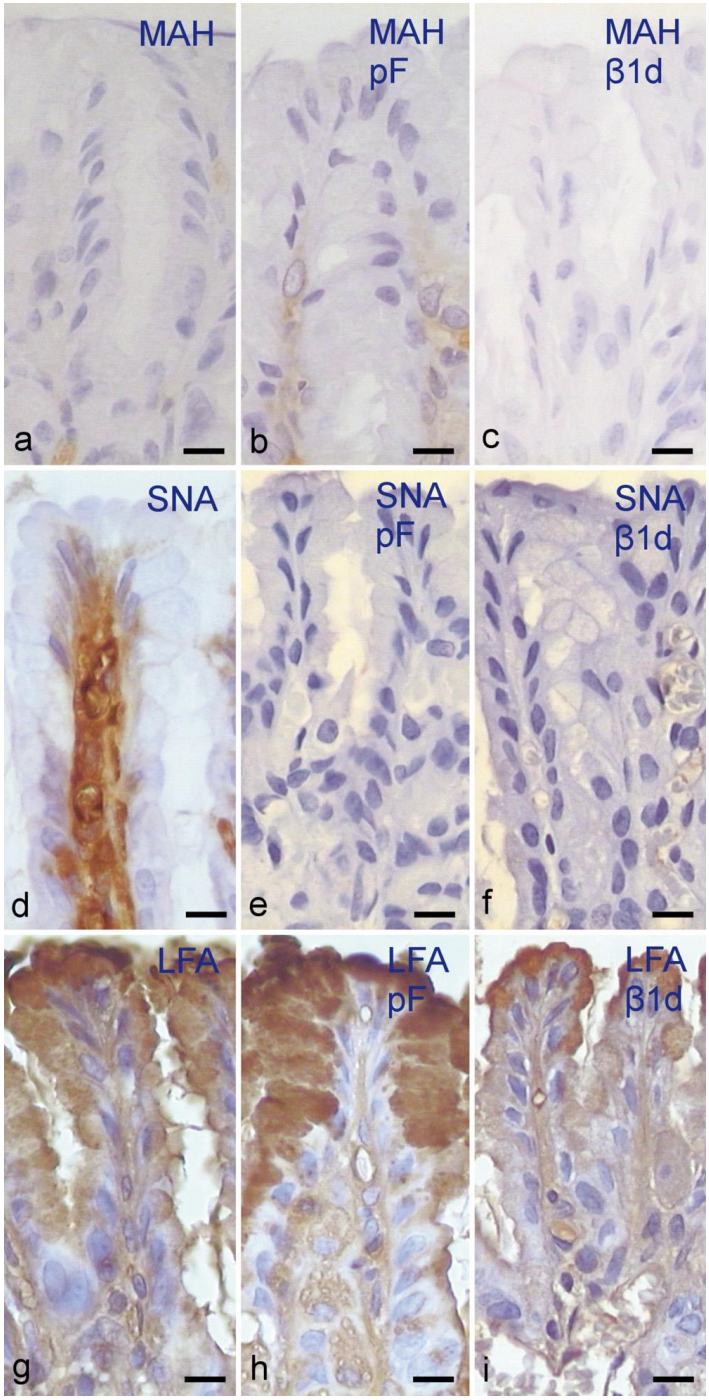

Figure. 7. Representative micrographs of some of the results obtained after the histochemical staining of SMCs with NeuAc-binding lectins. The lectins are abbreviated as indicated in Table 1. SMCs were always negative for both MAH and SNA (a–f). By contrast, the cells were always positive for LFA, and staining was stronger in the cells of the high and top regions of the pits (g–i). Abbreviations: pF, incubation with PNGase F; β1d, β-elimination for 1 day. Scale bars, 20 μm.

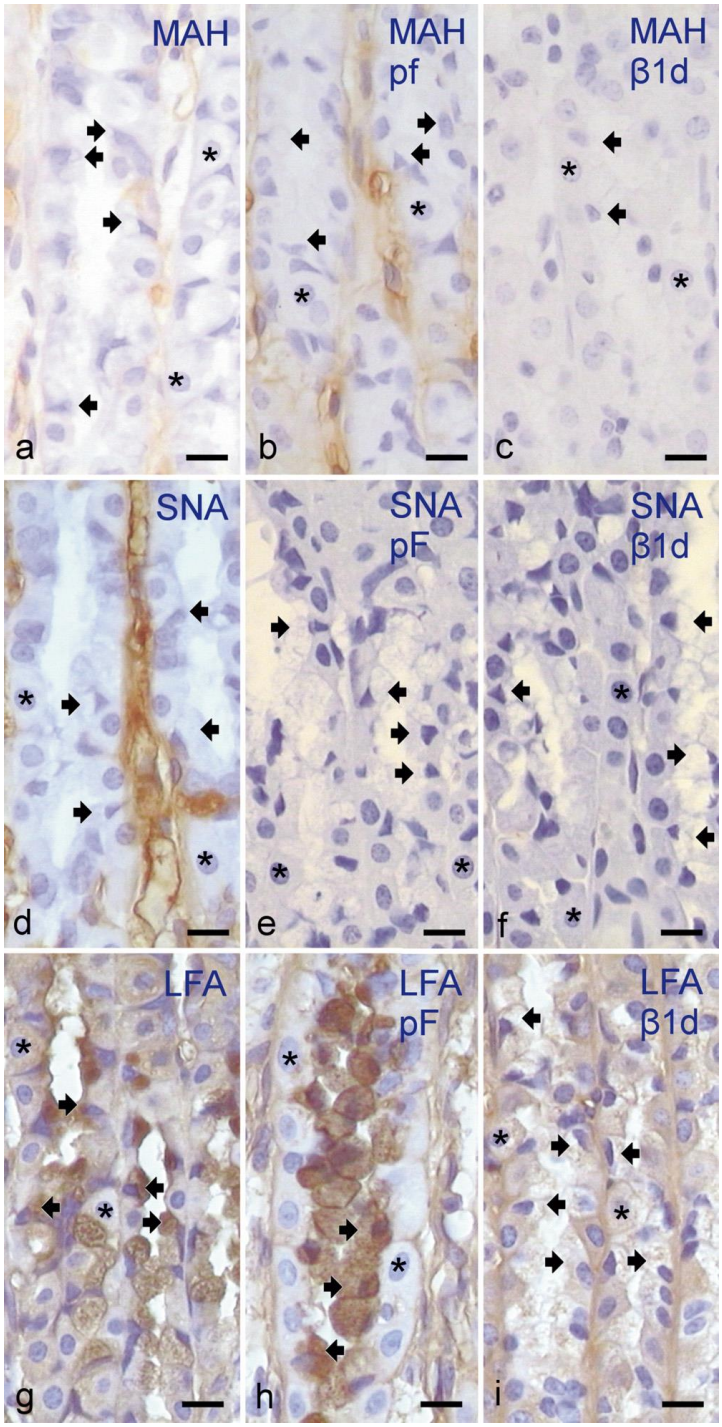

Figure. 8. Representative micrographs of some of the results obtained after the histochemical staining of MNCs with NeuAc-binding lectins. The lectins are abbreviated as indicated in Table 1. Both MAH and SNA did not label the MNCs, whatever the pretreatment used (a–f). Labeling with LFA was also observed after incubation with PNGase F, but after β-elimination, labeling of the cytoplasm showed a reticular pattern (g–i). Some MNCs are marked by arrows, and

some of the interspersed parietal cells are tagged with asterisks. Abbreviations: pF, incubation with PNGase F; β1d, β-elimination for 1 day. Scale bars, 20 μm.

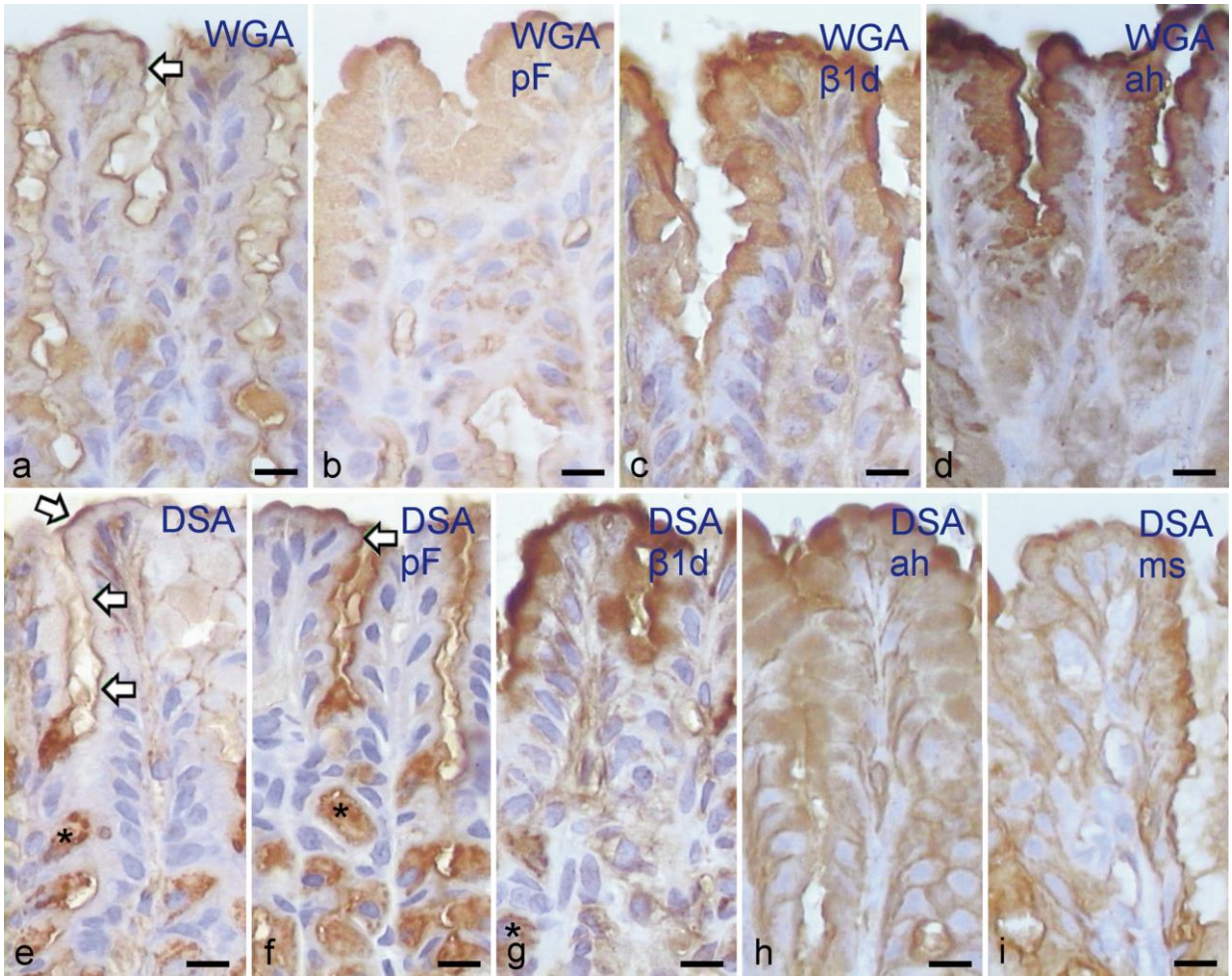

Figure. 9. Histochemical staining of SMCs with GlcNAc-binding lectins showing the more representative results. The lectins are abbreviated as indicated in Table 1. WGA alone mainly labeled the apical surface of the SMCs (a). The labeling in the cytoplasm increased after the deglycosylation pretreatments, and, in some of them, was stronger in the cells of the high and top region of the pit (b–d). The SMCs were very weakly labeled by the DSA lectin alone, except for the apical surface, but the labeling increased after some of the pretreatments. Occasionally, the cells of the high and top region of the pit showed stronger labeling (e–i). Sometimes, the apical surface of the SMCs was stained with different intensity (white arrows).

In some cases, the interspersed parietal cells were labeled by the lectins (asterisks). Abbreviations: ah: acid hydrolysis; ms: methylation-saponification; pF, incubation with PNGase F; β1d, β-elimination for 1 day. Scale bars, 20 μm.

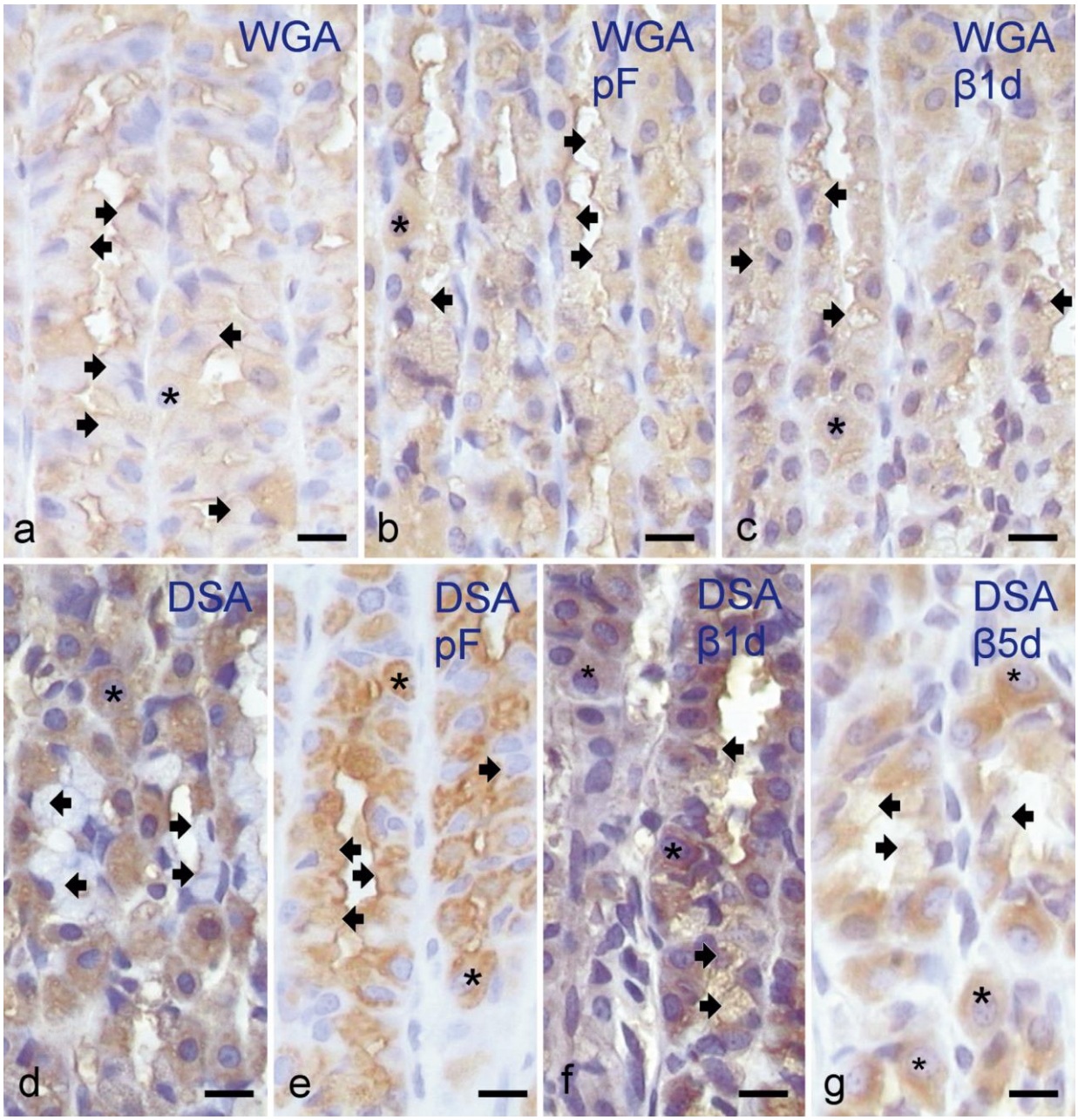

Figure. 10. Histochemical staining of MNCs with GlcNAc-binding lectins. The lectins are abbreviated as indicated in Table 1. WGA labeling of MNCs slightly increased after deglycosylation pretreatments (a–c). These cells were negative for DSA alone, but all the pretreatments turned the cells positive to the lectin with different intensities (d–g). Some MNCs are marked by arrows, and some of the interspersed parietal cells are tagged with asterisks.

Abbreviations: pF, incubation with PNGase F; β1d, β-elimination for 1 day; β5d, β-elimination for 5 days. Scale bars, 20 μm.

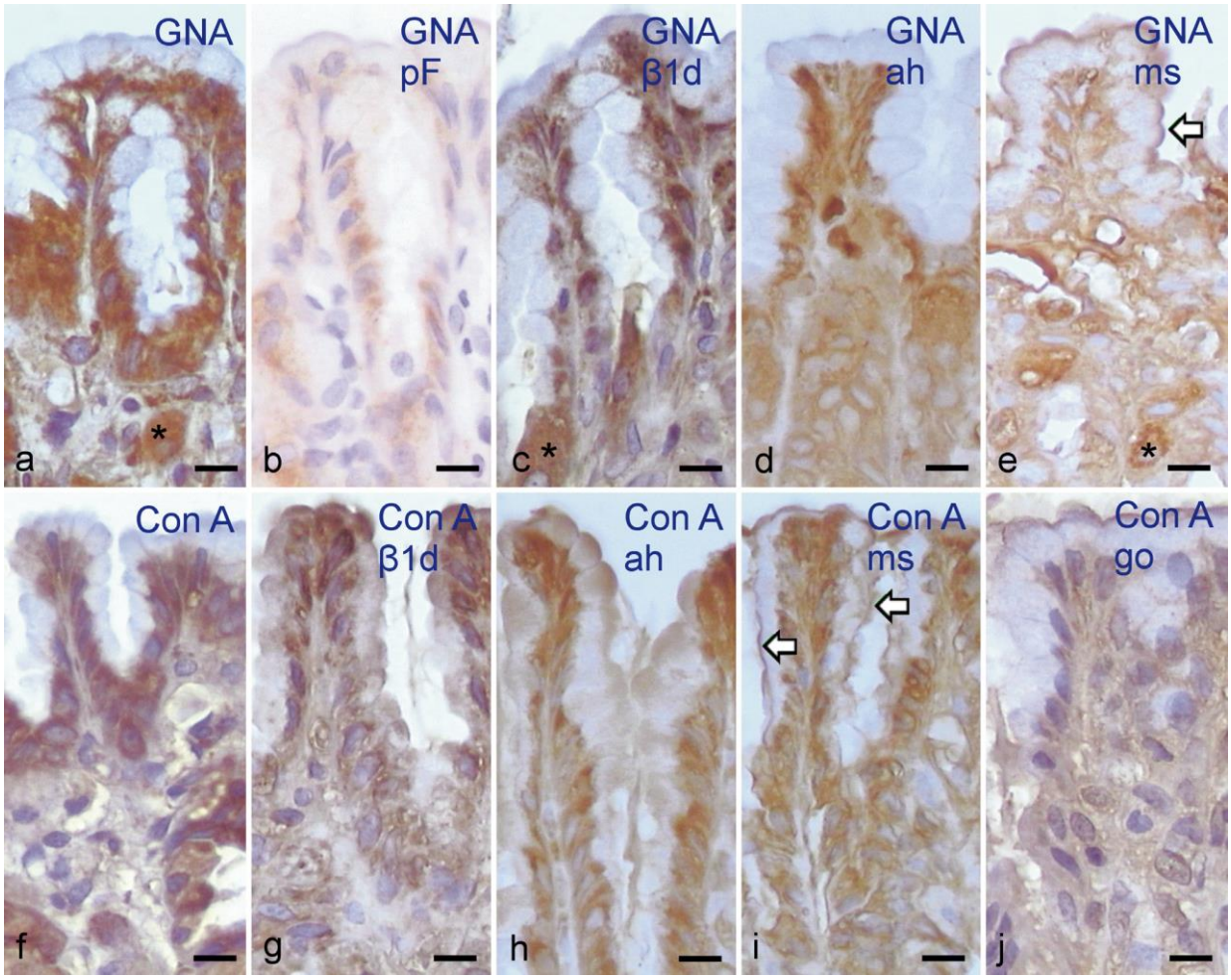

Figure. 11. Histochemical staining of SMCs with Man-binding lectins. The lectins are abbreviated as indicated in Table 1. GNA labeled the perinuclear region of the SMCs with different intensities with all the procedures employed (a–e), only after incubation with PNGase F a decreased labeling was observed (b). The pattern of Con A labeling was similar to that of GNA, but the apical cytoplasm was labeled after β-elimination or acid hydrolysis (f–j). Sometimes, the apical surface of the SMCs was stained with different intensity (white arrows). In some cases, the interspersed parietal cells were labeled by the lectins (asterisks).

Abbreviations: ah: acid hydrolysis; go: incubation with glucose-oxidase, ms:

methylationsaponification; pF, incubation with PNGase F; β1d, β-elimination for 1 day. Scale bars, 20 μm.

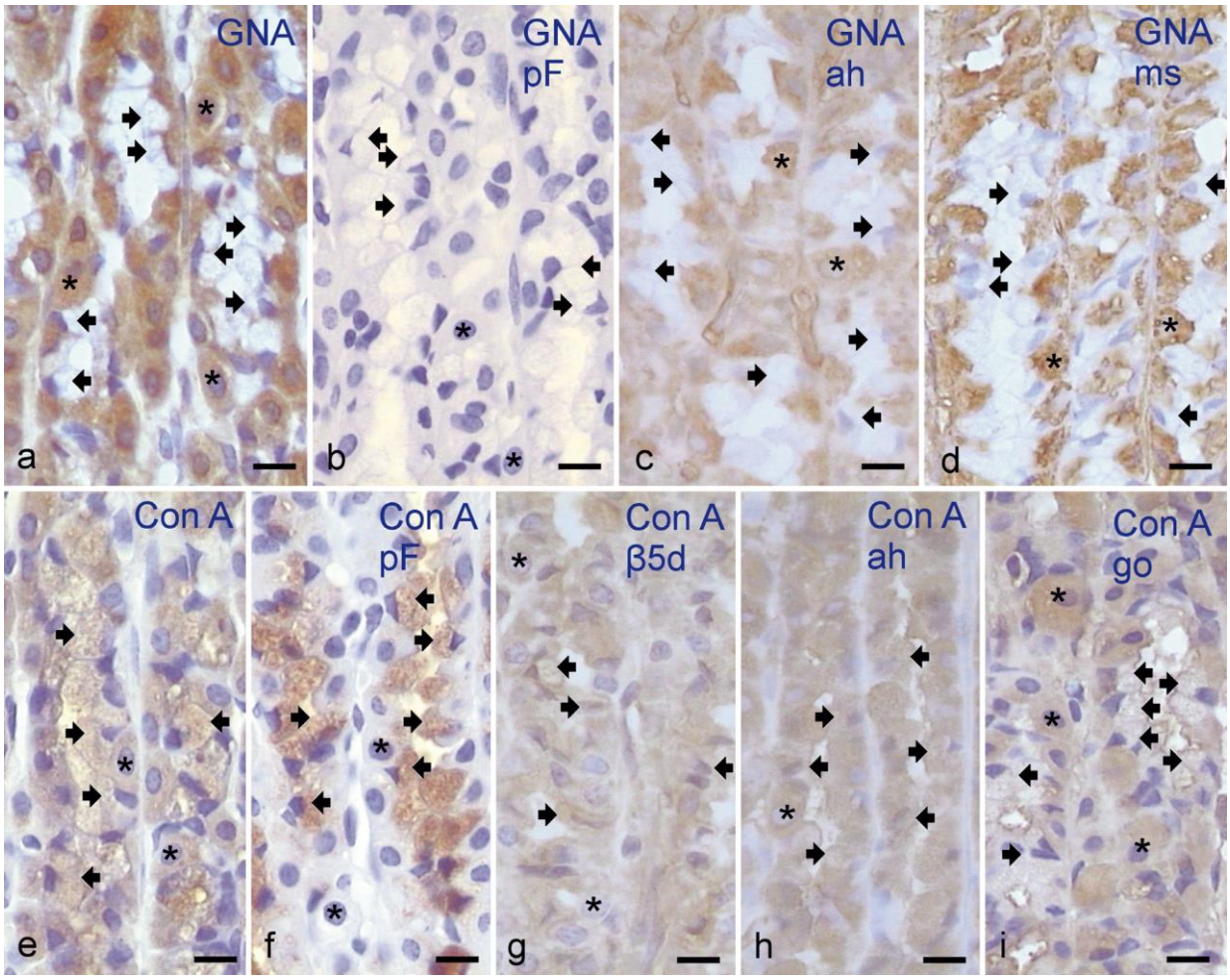

Figure. 12. Histochemical staining of MNCs with Man-binding lectins. The lectins are abbreviated as indicated in Table 1. The MNCs were always negative for GNA (a–d). These cells were always labeled by Con A, but the intensity of labeling was slighter after glucose-oxidase incubation (e–i). Some MNCs are marked by arrows, and some of the interspersed parietal cells are tagged with asterisks. Abbreviations: ah: acid hydrolysis; go: incubation with glucose-oxidase, ms: methylation saponification; pF, incubation with PNGase F; β5d, β-elimination for 5 days. Scale bars, 20 μm.