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# Ultrastructural distribution of lectin-binding sites on gastric superficial mucus-secreting epithelial cells

## The role of Golgi apparatus in the initial glycosylation

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**Abstract.** Normal human gastric epithelial cells were examined by electron microscopy using each of five biotinylated lectins [*Ulex europaeus* agglutinin I (UEA-I), peanut agglutinin (PNA), wheat germ agglutinin (WGA), soybean agglutinin (SBA) and *Dolichos biflorus* agglutinin (DBA)] as a probe. We employed 35 gastric surgical specimens removed from complicated peptic disease. The lectin-binding sites were revealed with streptavidin-colloidal gold complex. All specimens were embedded in Spurr and LR White resins. In superficial foveolar epithelial cells, the lectins used were generally positive in all cell types (mainly UEA-1 and PNA) on the Golgi region and mucus cytoplasmic vacuoles, with many variations among cells in the same case. On the other hand, extracellular mucus was negative for WGA. Labelling with PNA revealed a biphasic pattern (peripheral positivity) on mucous droplets in surface and foveolar cells. The *cis* side of the Golgi apparatus was labelled with SBA and PNA and rough endoplasmic reticulum with SBA (only five cases). Lectin-binding variability could be related to heterogeneous composition of gastric mucus. Our results with SBA suggest initiation of *O*-glycosylation at the Golgi apparatus; however a role of the rough endoplasmic reticulum cannot be excluded (*N*-glycosylation). We propose the following sequence of sugar addition to the carbohydrate side-chains of gastric glycoproteins: (1) GaNAc (Golgi apparatus *cis*-side), (2) GlcNAc (Golgi apparatus intermediate face), (3) GalNac or Gal, α-L-fucose (Golgi apparatus *trans*-side).

### Introduction

One of the main functions of the gastric epithelial cells is the production of the protective and lubricant mucus. The gastric mucus, composed of high molecular weight glycoproteins (Fischer et al. 1984a), exhibits a heterogeneous composition with individual variation related to blood group specifities (McCartney 1986) and several diseases (Fischer et al. 1984a; Fortsner et al. 1982; Hounsell et al. 1982). The differences between normal and pathologic secreted mucus have been related to incompletely glycosylated oligosaccharide chains and/or other saccharide addition (Bur and Franklin 1985; Fischer et al. 1984b; MaCartney 1986; Smets and Van Beek 1984).

Ultrastructural investigation of lectin-binding sites on intracellular organelles, for the purpose of studying the sugar residues, has been limited (Eguchi et al. 1989; Pavelka and Ellinger 1991; Roth et al. 1988) probably because of the high molecular weight and asymmetry of lectin molecules (Sato and Spicer 1982a). Several technical procedures have been suggested for ultrastructural glycoprotein studies, including "traditional" chemical (Hayat 1989) and physical methods, such as cryofixation (Vanwinkle 1991). The majority of the studies have been carried out in animal tissues, including rat intestine, pancreas and kidney (Pavelka and Ellinger 1985; Roth 1983a, b, 1984; Sato and Spicer 1982a; Suzuki et al. 1982), and some in human normal gastric mucosa (Ito et al. 1985). Some similarities between oligomeric structures of human and rat gastric mucin have been described (Dekker et al. 1991).

Glycoproteins have been classified, according to the nature of the linkage between the oligosaccharide and polypeptide chains, into two main families; *N*- and *O*-glycosidically linked glycoproteins (Roth 1984, 1987). The biosynthesis of *N*-linked glycoproteins is considered to occur via a single pathway involving a reaction between an oligosaccharide from the lipid carrier and the nascent peptide chain, which seems to be located in the rough endoplasmic reticulum (Hanover and Lennarz 1981; Hubbard and Ivatt 1981). On the other hand, the initial glycosylation reaction of *O*-linked glycoproteins does not require a lipid carrier (Hanover and Lennarz 1981) as has been subsequently corroborated by the ab-

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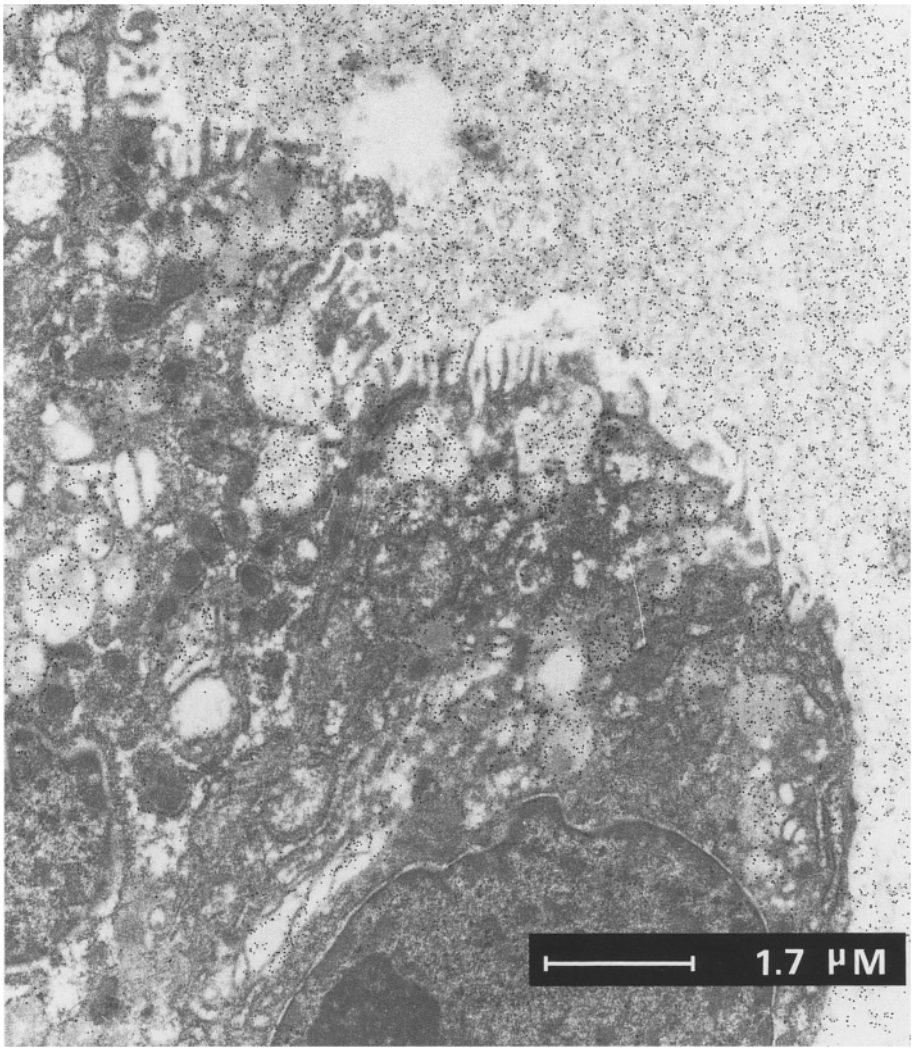


**Fig. 1.** Intense labelling with peanut agglutinin (PNA) is observed over the Golgi region, mucus droplets, and secreted mucins. ×4,000

sence of cytochemically detectable, terminal *N*-acetylgalactosamine (GalNAc) residues in the luminal space of the rough endoplasmic reticulum (Roth 1984, 1987). In the present work, we have made an ultrastructural, histochemical study of the qualitative carbohydrate composition of gastric mucus using lectins, with especial reference to the initiation of *O*-glycosylation and its subcellular distribution in gastric epithelium. In this study, the preparative procedure of tissue was performed following a routine non-physical method in acrylic resin.

## Materials and methods

Thirty-five gastric surgical specimens removed due to complicated peptic disease were selected. All of them were obtained from the Pathology Department of the Seville (Spain) University Medical School, during 1988–1991. Representative samples (1–2 $mm^3$ in volume) of mucosa, with no pathologic change at the light microscopic level, were used for routine (epoxy resin) and histochemical (hydrophilic resin) transmission electron microscopic studies. According to Fischer et al. (1984b), gastric epithelium was considered as normal when non-significant inflammatory infiltrate was present and the proportion and distribution of gastric epithelial cells retained their habitual architecture.

### *Tissue processing*

The specimens for histochemical study were fixed by immersion in 1% glutaraldehyde (Merck, Darmstadt, Germany) buffered to pH 7.2 in 0.1 *M* sodium cacodylate at 4° C for 2 h. After fixation, the samples were incubated in 0.05 *M* ammonium chloride in 1% phosphate-buffered saline (PBS) for 2 h at room temperature to block free aldehyde groups. Tissues then were dehydrated in ethanol (70, 80, 95 and 100%; 15 min each) and transferred directly into LR White resin (London Resin Co., Basingstoke, UK). After being stirred on a Rotamix for 4 h, the resin was renewed and left overnight at 4° C. The following morning, the resin was changed again, and then the specimens were placed into gelatin capsules. Polymerization occurred during 24 h in a 50° C oven using its accelerator (1 drop/10 ml of resin). Thick sections of the plastic-embedded material were stained with toluidine blue, and appropriate areas were selected for further ultrathin sectioning. Ultrathin sections, 800–1000 Å thick, were cut with glass knives and mounted without supporting filmon 800-mesh nickel grids, and were observed with an EM 900 Zeiss electron microscope. The specimens for routine ultrastructural study were post-fixed in 1% osmium tetroxide, embedded in Spurr resin, and counterstained with 2% methanolic uranyl acetate and 2% lead citrate.

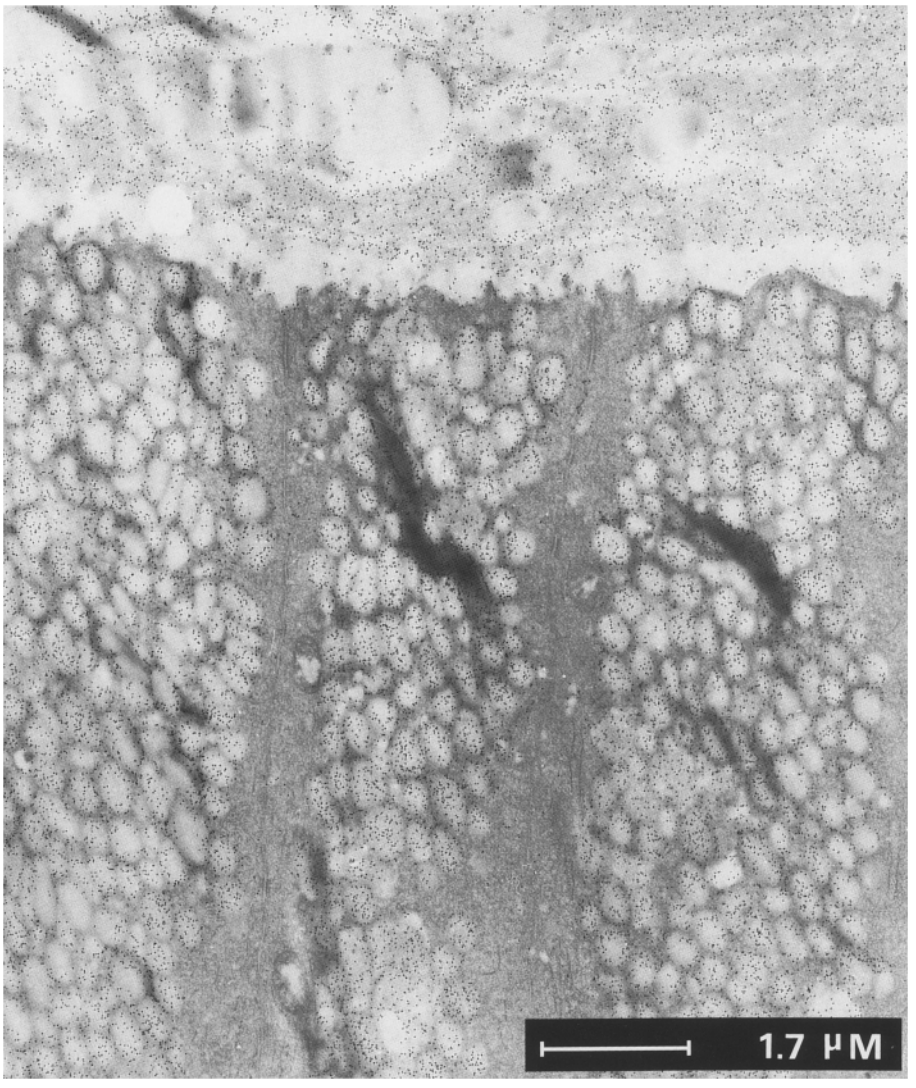


**Fig. 2.** The labelling with *Ulex europaeus* agglutinin I (UEA-I) is observed over intracellular and extracellular mucus. ×4,000

**Table 1.** List of lectins used with their specific and inhibitory carbohydrates

| Lectins | Specific carbohydrates | Inhibitory carbohydrates |
|---|---|---|
| Peanut agglutinin (PNA) | Gal β (1–3) GalNAc > β-D-Gal | β-D-Gal |
| Wheat germ agglutinin (WGA) | (β-D-GlcNAc)*n* > SAc | GlcNAc, SAc |
| Soybean agglutinin (SBA) | α and β GalNAc > α and β Gal | GalNAc |
| *Ulex europaeus* agglutinin I (UEA-I) | α-L-Fuc | α-L-Fuc |
| *Dolichus biflorus* agglutinin (DBA) | α-D-GalNAc | α-D-GalNAc |

Gal, Galactose; GalNAc, *N*-acetyl galactosamine; GlcNAc, *N*-acetyl glucosamine; SAc, sialic acid; Fuc, fucose

### *Reagents*

The biotinylated lectins used in this study were obtained from Vector, Burlingame, USA and are shown in Table 1. All of them were at a concentration of 100 μg/ml in 1% bovine serum albumin (BSA) in 1% PBS. The streptavidin-colloidal gold (15 nm) complex from Janssen (Olen, Belgium) was used at 1:20 dilution in 1% BSA-PBS.

### *Labelling procedure*

Labelling with lectins was performed as a two-step technique, without previous etching procedure. This indirect method is based on the biotin-streptavidin bridge affinity (Bonnard et al. 1984). All the steps were achieved in a moist chamber, at room temperature and the grids were placed on a drop of the different reagents. In the first step, the grids were placed for 10 min on a drop of 1% BSA in 1% PBS. The sections were then incubated with the biotinylated lectin for 30, 60 or 90 min followed by 1% BSA-PBS (three changes, 5 min each). In the second step, the grids were localized on a drop of streptavidin-colloidal gold complex for 30 min. Finally, the grids were washed with distilled water and counterstained with 2% methanolic uranyl acetate for 2 min.

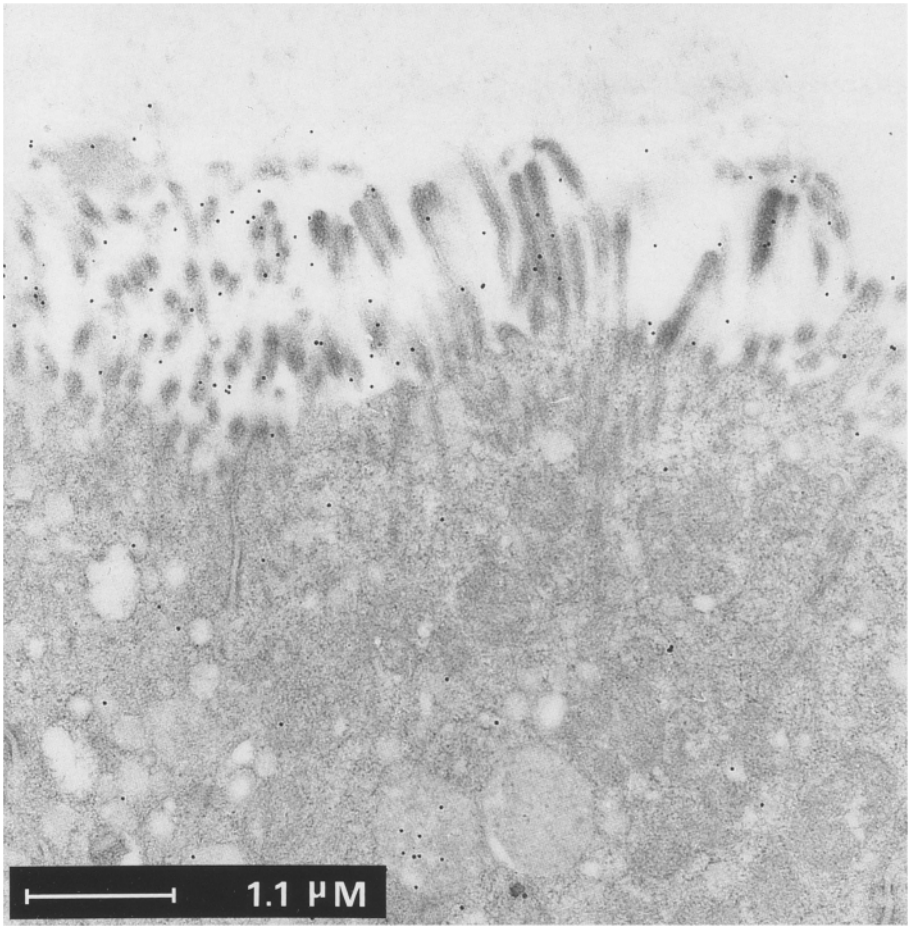


**Fig. 3.** Superficial microvilli are labelled with wheat germ agglutinin (WGA), whereas extracellular mucus is negative. ×7,000

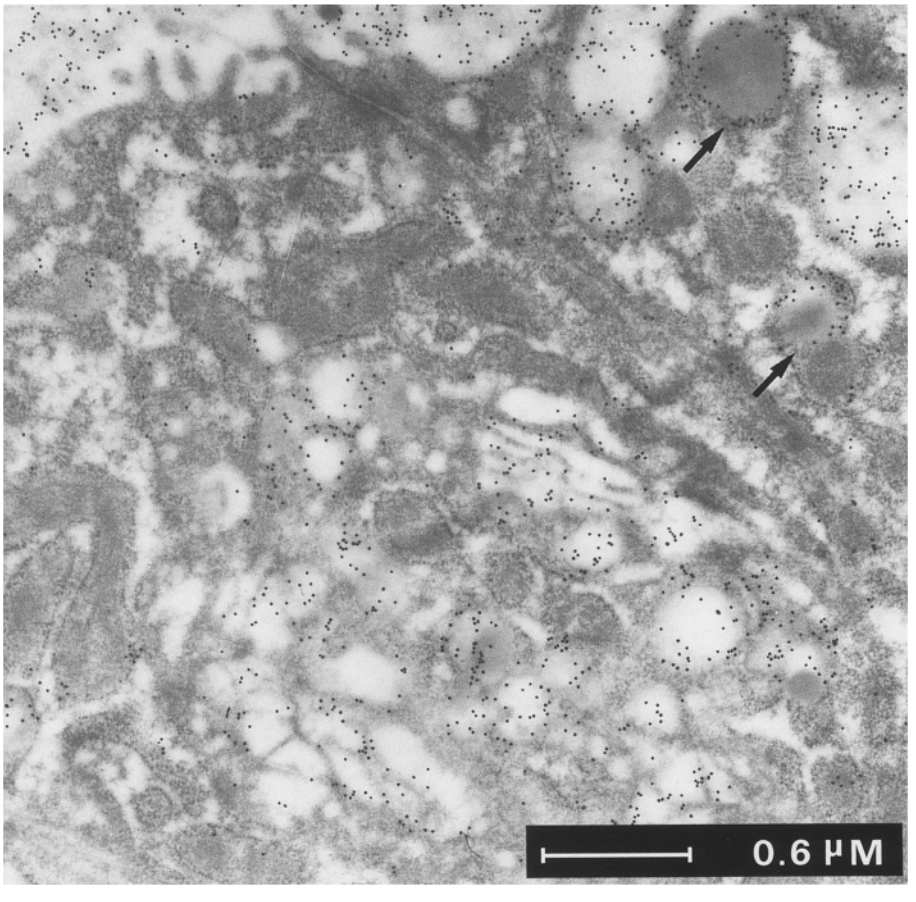


**Fig. 4.** Electron-dense mucous droplets with peripheral PNA reactivity (*arrows*); the cores remain unreactive. ×7,000

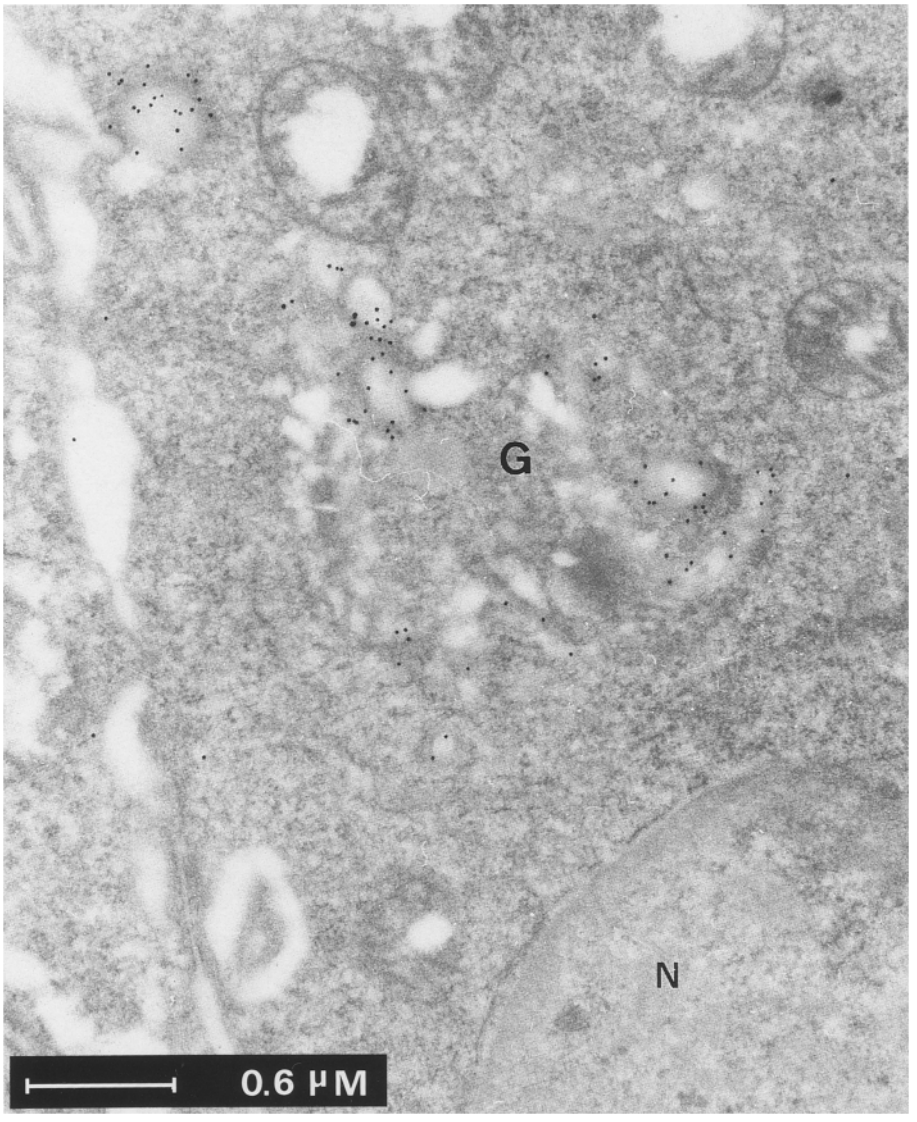


**Fig. 5.** Distal (*trans*) and slightly proximal (*cis*) faces of the Golgi apparatus (*G*) are labelled with PNA. ×12,000

*Controls for specificity*

The specificity of the lectin-binding sites was tested by adding 0.1 *M* of the corresponding inhibitor sugars (Table 1) to the lectin incubation medium for 30 min. Negative controls were performed with streptavidin-colloidal gold complex (1:20) without previous lectin incubation.

## Results

The technique used for specimen ultrastructural investigation resulted in good preservation of fine cellular structure, enabling adequate visualization of cytoplasmic membranes, mitochondria, mucus vacuoles, and endoplasmic reticulum (ER) and Golgi apparatus (GA) membranes. The incubation period of intra- and extracellular mucus glycoproteins, for which intensive lectin staining was required without background levels of reaction product, was 60 min. The results of the staining with biotinylated lectins and streptavidin-colloidal gold complex are shown in Tables 2 and 3. Lectin-binding sites of mucus glycoproteins in normal gastric epithelium revealed a great heterogeneity in the reaction intensity among the cells studied, even within the same case. An intense reaction with peanut agglutinin (PNA), wheat germ agglutinin (WGA), soybean agglutinin (SBA), and *Ulex europaens* agglutinin I (UEA-I) was viewed on corpora and antral surfaces and foveolar epithelial cells (Table 2; Figs. 1 and 2). These cells showed a lower and more focal staining intensity with DBA lectin (Table 2).

The extracellular mucus was labelled with UEA-I and PNA (Figs. 1 and 2); however histochemical staining for WGA was negative in this location (Fig. 3; Table 2). The rims of biphasic electron-dense mucous droplets in surface and foveolar cells in normal mucosae stained intensively with PNA but the cores were unreactive (Fig. 4). Distal (*trans*) and slightly proximal (*cis*) faces of the Golgi stack were stained with this lectin (Fig. 5). The three regions of the GA were labelled with SBA and showed more intensive reactivity over the *cis* and *trans* faces (Fig. 6). In only five cases could we obtain

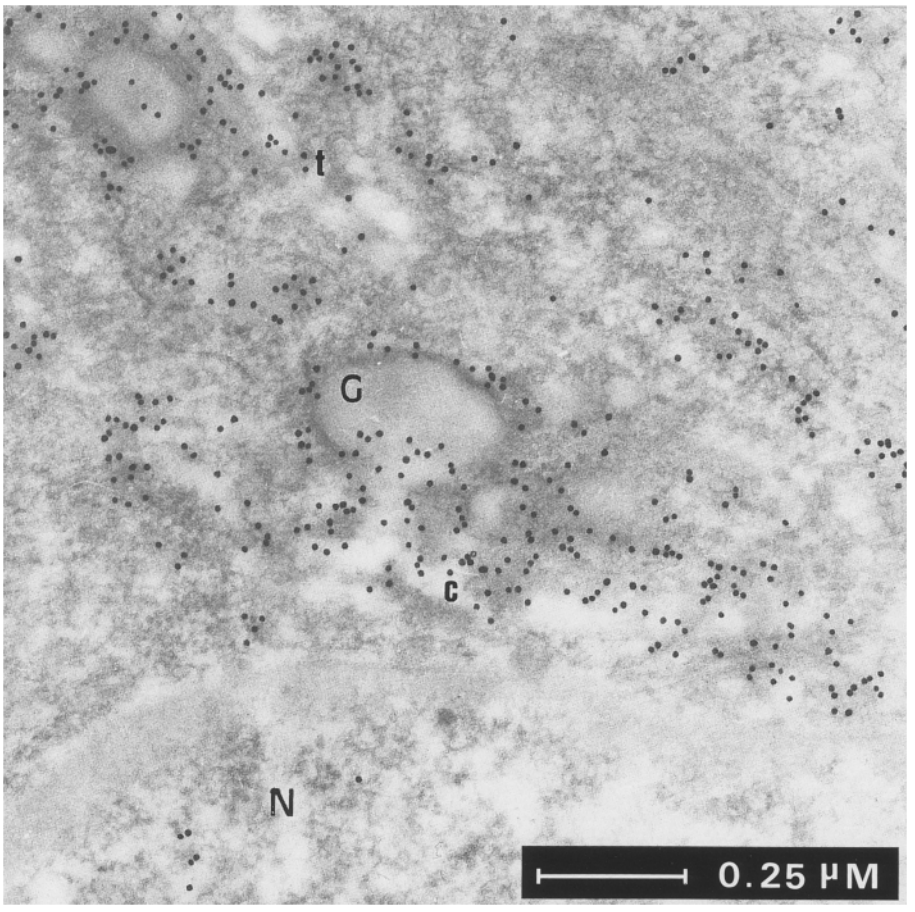


**Fig. 6.** Labelling with soybean agglutinin (SBA) is intense over *cis* (*c*) and *trans* (*t*) faces of the Golgi apparatus. ×12,000

**Table 2.** Staining reaction[a] with biotinylated lectins[b] and streptavidin-colloidal gold complex in 35 gastric mucosa specimens

| | PNA No. (%) | WGA No. (%) | SBA No. (%) | UEA-I No. (%) | DBA No. (%) |
|---|---|---|---|---|---|
| Intracellular mucus | ++ 34 (97) | ++ 30 (86) | ++ 33 (94) | ++ 34 (97) | + 20 (57) |
| Microvilli | + 25 (71) | ++ 32 (91) | + 19 (54) | ++ 30 (86) | + 17 (48) |
| Extracellular mucus | ++ 32 (91) | − 30 (86) | ++ 29 (83) | ++ 33 (94) | + 15 (43) |

[a] Staining quality was graded subjectively according to a scale ranging from: −, negative; +, focal/less positivity; and ++, intense labelling
[b] See Table 1 for explanation of lectin abbreviations

**Table 3.** Staining reaction[a] with biotinylated lectins[b] and streptavidin-colloidal gold complex in 35 gastric mucosa specimens, over the regions of the Golgi apparatus

| | PNA No. (%) | WGA No. (%) | SBA No. (%) | UEA-I No. (%) | DBA No. (%) |
|---|---|---|---|---|---|
| *Cis* face | + (27 (77) | − 29 (83) | ++ 30 (86) | − 34 (97) | − 35 (100) |
| Intermediate face | + (20 (57) | ++ 23 (66) | + 25 (71) | − 35 (100) | − 35 (100) |
| *Trans* face | ++ 32 (91) | ++ 25 (71) | ++ 27 (77) | ++ 25 (71) | − 32 (91) |

[a] see footnote to Table 2
[b] see Table 1

we could a focal reaction on rough ER membranes and inside luminal spaces (Fig. 7).

## Discussion

Consistent with previous reports (Ellinger and Pavelka 1985; Newman and Hobot 1987), our results (using an easy and "traditional" histochemical method) have permitted us to study lectin-reactive glycoproteins using LR White resin-embedded tissues, without previous etching procedures (Causton 1989; Ellinger and Pavelka 1985; Newman et al. 1983). Currently, the acrylic resins (Lowicryl KYM, LR White; Kellenberg et al. 1980; Newman et al. 1983) have permitted the application of lectins, applying post-embedding techniques without etching procedures, with adequate preservation of cell structure and retention of lectin-binding activity to intra- and extracellular glycoproteins (Ellinger and Pavelka 1985; Newman et al. 1983; Newman and Hobot 1987). Ellinger and Pavelka (1985) suggested the use of peroxidase-labelled reagents since colloidal-gold particles would re-

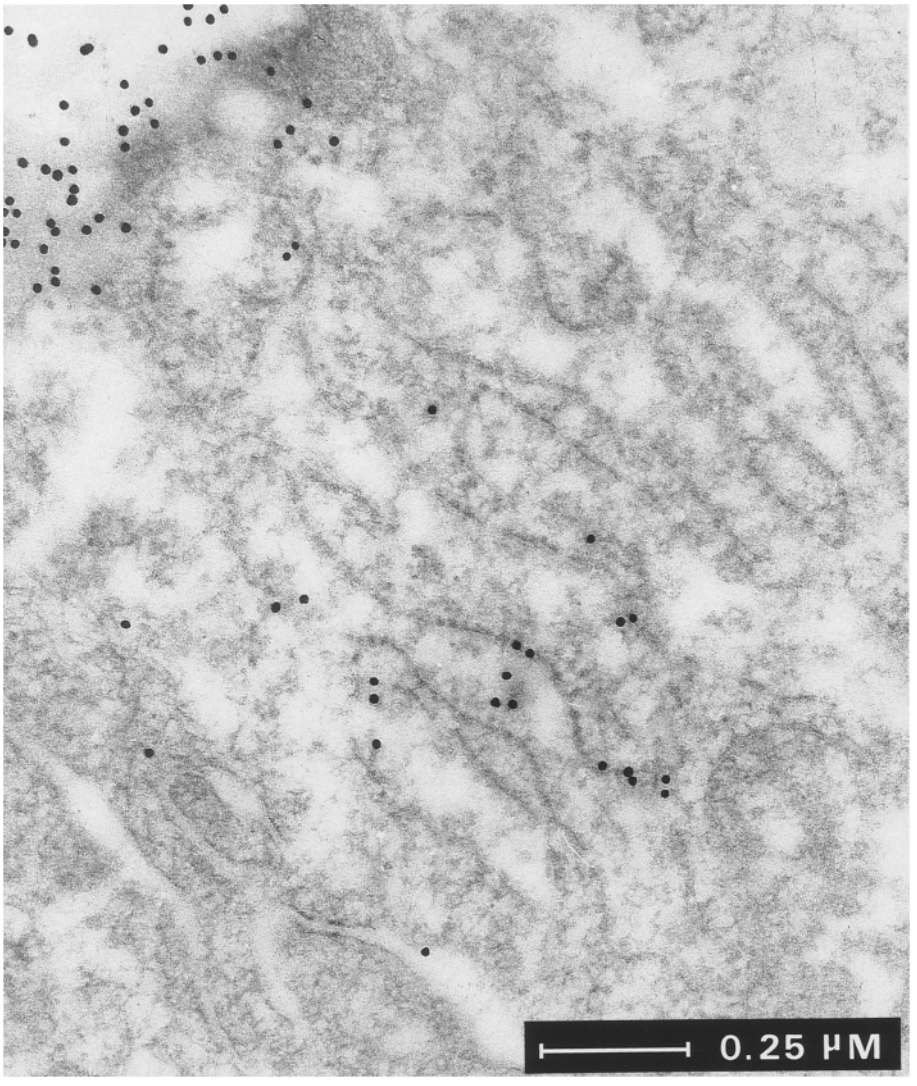


**Fig. 7.** Focal SBA labelling is shown over rough endoplasmic reticulum membranes and inside luminal spaces. ×12,000

duce the penetrating ability of the lectin probe. The use of the avidin-biotin indirect method can avoid this problem because, without colloidal-gold particles, the lectin molecule has a smaller size and easier penetration. In this study, the use of both negative and positive controls allowed us to eliminate possible sources of nonspecific labelling on thin sections. For controls lacking nonspecific binding between lectins and free aldehyde groups we use 0.05 *M* $NH_4Cl$ incubation of the tissues (Pavelka and Ellinger 1991; Roth et al. 1988). Incubation time, concentration, and temperature conditions are similar to those used by other investigators (Ellinger and Pavelka 1985; Herrera 1989). We have found some difficulties in visualization of lectin-binding sites when 70% ethanol was used as the only dehydration procedure, in opposition to Newman et al. (1983).

Our results are in accordance with previous qualitative biochemical data of gastric mucus composition (Fischer et al. 1984b; Hounsell et al. 1980; Schrager 1969; Schrager and Oates 1970, 1971, 1973). Thus the hexosamines, particularly *N*-acetyl-galactosamine (GalNAc) and *N*-acetyl-glucosamine (GlcNAc), were the main sugars found in the carbohydrate chain. Fischer et al. (1984b) have reported a higher lectin affinity to the hexosamine-rich components of mucus; however this finding should not be extrapolated as meaning that hexosamines are the major components of gastric mucus secretion. From our point of view, the most probably explanation for this finding is the sugar location, because SBA is an exolectin and therefore will bind extracellular mucus. However, we cannot exclude in this work the possibility of a quantitative hexosamine increase in some gastric mucosa specimens, which should be demonstrable biochemically.

By means of biochemical (Hanover and Lennarz 1981; Hounsell et al. 1980; Schrager 1969) and histochemical (Pavelka and Ellinger 1985; Suzuki et al. 1982) methods, it is known that *O*-glycosidically linked chains are characterized by a linkage between a GalNAc residue and the hydroxyl groups of serine or threonine of the polypeptide chain. The labelling with SBA and PNA lectins on the *cis* face of the GA indicates a predominance of terminal GalNAc residues and also suggests the beginning of *O*-linked glycosylation at this site, as

previously reported by other authors (Pavelka and Ellinger 1985; Suzuki et al. 1982). Results that were apparently contradictory to the present work have been obtained by Ito et al. (1985) using DBA lectin and were probably due to the high molecular weight of this lectin and its difficulty of penetrating into the cellular structure. The possibility cannot be excluded of the beginning of glycosylation processes at the level of rough ER in the gastric epithelium, based on our labelling with SBA lectin of these organellar membranes in five cases. This finding suggests the coexistence of *N*-glycosylation, although at lower frequency, in gastric glycoproteins (Dekker and Strous 1990; Hanover and Lennarz 1981; Kim et al. 1971; Strous 1979).

An increase in the labelling with all lectins, except for DBA, has been described through the GA, from the *cis* to the *trans* face. This feature can be explained by a progressive elongation of oligosaccharide chains (owing to the addition of different sugars like GalNAc, galactose, fucose) as the glycoprotein chains move from the *cis* towards the *trans* side of the GA (Pavelka and Ellinger 1991; Roth 1984, 1987; Sato and Spicer 1982b).

The mucous droplets (localized in the GA transport region) showed lectin-labelling patterns similar to those of the GA *trans* side. Moreover, biphasic mucous droplets have been identified with a peripheral distribution of PNA and, occasionally, of WGA lectin-binding sites at the periphery of the droplets, the droplet cores being unreactive. These biphasic electron-dense vacuoles have been described by several investigators using different histochemical techniques (Schrager and Oates 1973; Hanover and Lennarz 1981; Hounsell et al. 1980). Using pronase, Spicer et al. (1987) and Yeomans (1974) found a core composed of peptidic substance (pepsinogen), which explains the absence of a histochemical reaction for carbohydrates.

The extracellular mucus revealed a predominance of terminal α-L-fucose (Fuc) (high labelling with UEA-I), and a relative decrease of terminal GalNAc and galactose (Gal; mild labelling with PNA and SBA) on the membrane-bound glycoproteins. The explanation of this finding could be the dense framework of glycoproteins in the glycocalyx, as well as the high content of α-L-fucose of gastric mucus, which has been previously reported in light microscopic studies (Bur and Franklin 1985; McCartney 1986; Narita and Numao 1992; Rivera et al. 1990). Our most interesting finding on intraluminal mucus was the absence of WGA-labelling, which would suggest the absence of GlcNAc at this level. This fact has not been previously reported in the literature and is suggested here possibly to be due to the action of gastric acid and peptic secretion. Similarly, Schrager and Oates (1970) found Gal and GlcNAc, in equimolar amounts, on fragments of the carbohydrate side-chains of gastric glycoproteins after mild acid hydrolysis. This suggests a selective action of acid on links between GlcNAc and other sugars, thus leaving GalNAc as terminal sugar residue. Both points support our lectin-binding results: a negative reaction for WGA and a positive one with PNA and SBA. On the other hand, we also obtained high levels of terminal α-L-fucose at the intraluminal level (UEA-I positive), which could be explained by a non-enzymatic glycosylation of gastric glycoproteins, in relation to the abundance of this sugar in the gastric mucus.

The following sequence of sugar addition to the carbohydrate side-chains of gastric glycoproteins is proposed: (1) GalNAc (Golgi apparatus *cis* side), (2) GlcNAc (Golgi apparatus intermediate face), (3) GalNAc or Gal, α-L-fucose (Golgi apparatus *trans* side). The initiation of glycosylation is located in the Golgi complex, although an *N*-glycosylation on rough ER is probably required for an efficient polymerization of sugar chains. The subcellular heterogeneity demonstrated in the lectin-binding distribution is related to differences in the functional status of gastric epithelial cells.

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