Sialoglycoconjugate dimorphism of the mouse submandibular gland acinar cells. Ultrastructural evidence by lectin-protein A-gold probes and sialidase digestion

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Summary. An ultrastructural analysis of lectin receptors on the submandibular glands from mice of both sexes was performed utilizing horseradish peroxidase-labelled lectins in conjunction with antiperoxidase antibody and protein A-gold. Both qualitative and quantitative sex-related differences in terminal sugar expression within secretory granules were detected. Following sialidase digestion, also subterminal acceptor sugars for terminal sialic acids, proved to be differentially expressed in the submandibular glands of males and females. Heterogeneous distribution of sialoglycoconjugates characterized by the terminal disaccharide sialic acid-ß-galactose was found to occur in female acinar cells. Also DBA reactive sites indicating the presence of terminal α-N-acetylgalactosamine discriminated between male and female acinar secretory glycoconjugates. This difference was emphasized by sialidase pretreatment that evidenced a marked occurrence of sialic acid subtended to α-N-acetylgalactosamine in males in contrast to a modest presence in females. The different sialylation patterns of acinar cell secretory products, probably related to a different expression of O- and N-linked sialoglycoconjugates, give insight into the sexual dimorphism of the mouse submandibular gland known until recently for the convoluted granular tubules.

Key words: Lectin-gold techniques, Sialidase, Submandibular gland, Male and female mouse

Introduction

Lectins have been extensively experimented with as biochemical tools to isolate glycoproteins, glycolipids and polysaccharides and have especially been used to investigate the quality, distribution, assembly, and turnover of glycoconjugates in normal and pathological tissues as well as during embryonic differentiation (Lis and Sharon, 1986a,b; Sharon and Lis, 1993).

When tagged with appropriate markers, lectins may be used as histochemical probes to characterize and discriminate between oligosaccharide chain residues (Roth, 1983; Spicer et al., 1983; Damjanov, 1987; Thomopulos et al., 1987; Spicer and Schulte, 1992; Danguy and Gabius, 1993; Danguy et al., 1994; Menghi and Materazzi, 1994; Danguy, 1995; Garrett et al., 1996). The choice of an adequate marker is very important at light and electron microscopy level, so this research field attracted numerous authors and, in the last decade, the staging of methodologies employing colloidal gold particles has been emphasized (Roth, 1984; Skutelsky et al., 1987; Fujimori et al., 1988; Beesly, 1989; Horisberger, 1992; Roth et al., 1992). Lectins were among the first substances to be conjugated with colloidal gold and their receptor sites have been investigated in various organ tissues by transmission (Horisberger, 1979; Roth, 1983; Vorbrodt et al., 1986; Quatacker, 1989; Herken et al., 1990; Ueno and Lim, 1991; Castells et al., 1992; Martínez-Menárguez et al., 1992) and scanning electron microscopy (Horisberger et al., 1975; Horisberger, 1981; Hodges et al., 1987). By applying the indirect technique of binding, lectins linked to horseradish peroxidase can also be used and then labelled to gold particles (Peruzzo and Rodriguez, 1989). In contrast to the preembedding methods (Gabius and Gabius, 1993), the postembedding lectin-gold binding allows a wide range of lectins to be tested on the same block of fixed tissue.

Recently, we employed both direct and indirect techniques of lectin-gold labelling to investigate the distribution of individual glycosidic residues and specific carbohydrate sequences in the secretory granules of the mouse parotid acinar cells (Marchetti et al., 1995; Menghi et al., 1996). The present research was undertaken to study, at electron microscope level, the subcellular distribution of sugars biochemically

determined in the submandibular gland of mice of both sexes (Roukema et al., 1976; Denny et al., 1980; Denny and Denny, 1982a; Niew Amerongen et al., 1982, 1983; Menghi et al., 1986) and to get insight into the sexual dimorphism of the submandibular gland of this species. The glycosidic residues were visualized by applying the indirect technique of binding based on lectin-horseradish peroxidase conjugates, antiperoxidase antibody, and protein A-gold. The terminal sialoglycoconjugate sequences were characterized by employing sialidase digestion combined with lectin histochemistry.

Materials and methods

Animals

Sexually mature male (10) and female (10) mice (*Mus musculus*), Swiss strain (Stock Morini, S. Polo d'Enza, RE, Italy) were used. Submandibular glands were removed immediately after sacrifice and processed for lectin histochemistry. All manipulations were carried out in accordance with the recommendations of the Italian Ethical Committee under the supervision of authorized investigators.

Chemicals

All horseradish peroxidase (HRP)-labelled lectins (DBA from *Dolichos biflorus*, PNA from *Arachis hypogaea*, WGA from *Triticum vulgaris*, Con A from *Canavalia ensiformis*, LTA from *Tetragonolobus purpureas*), anti-horseradish peroxidase antibody (raised in rabbit) and reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA); Bioacryl was purchased from Bio-optica (Milano, Italy).

Lectin histochemistry

The fixation step was carried out at 4 °C to better preserve the glucidic components packaged in secretory granules. Preliminary experiments proved that the optimal fixative was a mixture containing 4% paraformaldehyde, 1% glutaraldehyde, and 0.2% picric acid in 0.1M phosphate buffer, pH 7.6, in presence of 0.5mM $CaCl_2$ for 3 h as suggested by Berryman and Rodewald (1990). After quenching of free aldehydes with 50mM ammonium chloride in sucrose-phosphate buffer for 1 h at 4 °C, samples were treated with 0.1M maleate buffer, pH 6.5, added with 3.5% sucrose 4 times, 15 min each at 4 °C and fixed in 1% uranyl acetate in 0.1M maleate buffer, final pH 6.0, containing 3.5% sucrose for 2 h at 4 °C.

The dehydration step was performed twice, 5 min each time, with acetone at increasing concentrations (50%, 70%, 90%), at 4 °C and under gentle stirring.

The embedding step was carried out in Bioacryl resin (Scala et al., 1992). The specimens were infiltrated in a solution of 100% Bioacryl (twice, 2 h each time) at 4 °C under gentle stirring and subsequently immersed overnight in fresh pure resin at 4 °C; the polymerization was performed by UV light for 72 h at 4 °C in carefully closed gelatine embedding capsules filled up with resin and degassed for 30 min at 4 °C.

The indirect lectin binding was applied to freshly cut thin sections (about 60 nm thick) mounted on uncoated, 400 mesh nickel grids. HRP-labelled lectins were used under the following conditions: DBA, PNA, WGA, Con A (2 μg/ml) and LTA (10 μg/ml) were diluted in 0.1M TBS (Tris buffered saline), pH 7.4, plus 1% BSA (bovine serum albumin) and with or without 3mM $MgCl_2$, 3mM $MnCl_2$, 3mM $CaCl_2$. After lectin incubation, prolonged for 3 h at room temperature, sections were rinsed with 0.05M TBS containing 1% BSA and reacted with anti-HRP antibody (diluted 1:100 in TBS-1% BSA) for 60 min. Finally, sections were washed with 0.05M TBS-1% BSA and treated with protein A-gold (10 nm) diluted 1:50 in 0.1M TBS, pH 7.4, plus 1% BSA and 0.05% Tween 20, for 60 min at room temperature. After counterstaining, with uranyl acetate (10 min at 30 °C) and lead citrate (1 min at 20 °C) by an LKB Ultrostainer, samples were carbon-coated and analyzed in a Philips 201C TEM.

Sialidase digestion

Sections were incubated with sialidase (neuraminidase type V, from *Clostridium perfringens*) at a concentration of 0.5U/ml in acetate buffer, pH 5.5, containing 10mM $CaCl_2$ for 16 h at 37 °C (Menghi et al., 1989a).

Deacetylation

This was carried out by pretreating sections with 0.5% KOH in 70% ethanol for 30 min at room temperature.

Controls

The control for the specificity of the lectin binding was run with sections prepared either by incubating the lectin solutions with their specific competing sugars (N-acetyl-D-galactosamine for DBA, D-galactose for PNA, N-acetyl-D-glucosamine for WGA, D-mannose for Con A, α-L-fucose for LTA) at a concentration of 0.2-0.4M or by omitting the antiperoxidase antibody or lectin HRP conjugates. Controls for sialidase aimed to investigate both the action of the enzyme-free buffer and the specificity of the enzymatic treatment.

Results

The fine structure of the mouse submandibular gland has been reported elsewhere (Caramia, 1966; Menghi and Bondi, 1987) and the description of the secretory tracts will not be detailed here. Briefly, the secretory endpieces are composed of terminal acini filled with electron-lucent granules and preterminal convoluted
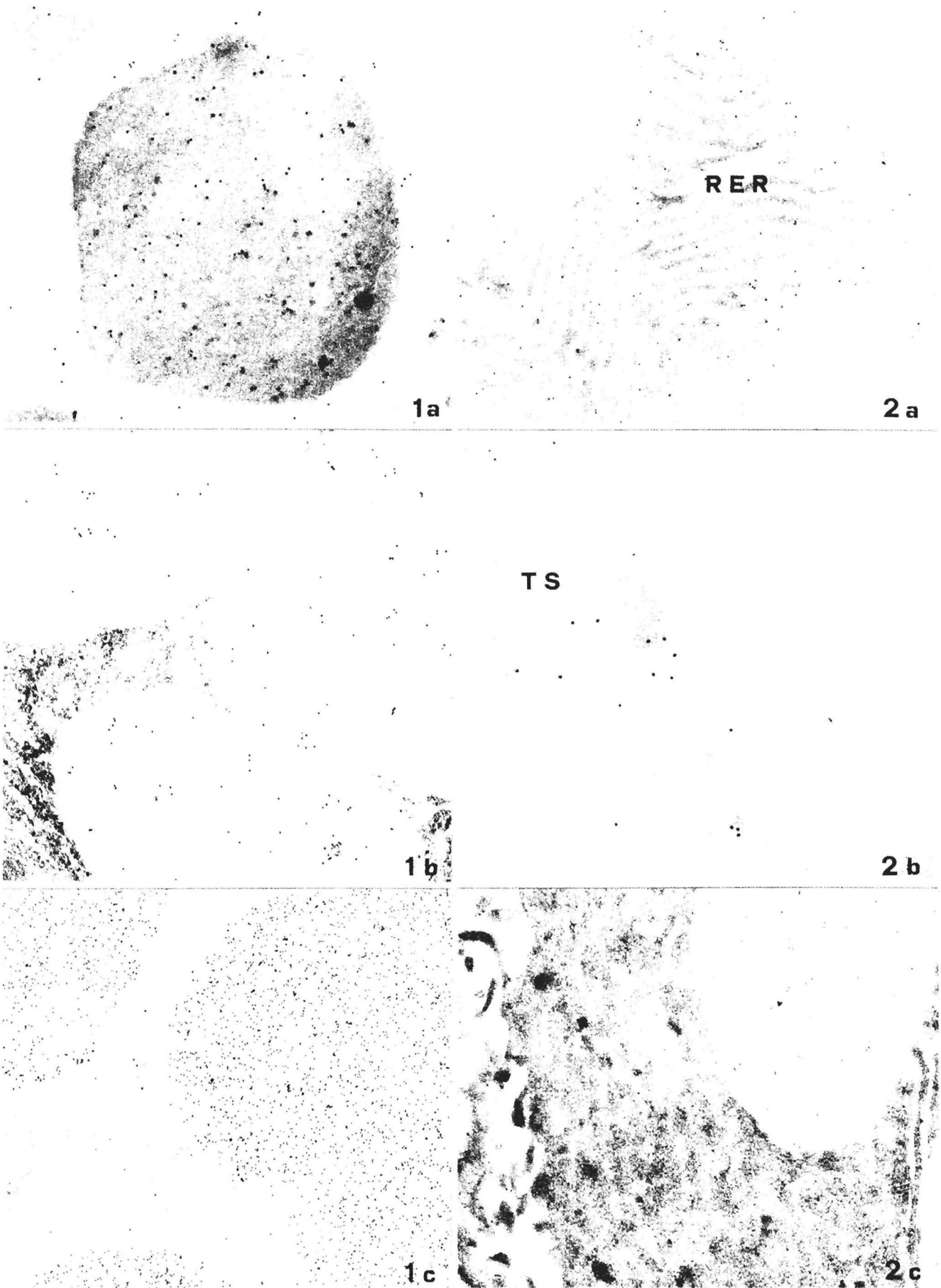
1a

2a

RER

1b

2b

TS

1c

2c

Mouse submandibular gland

Fig. 1. DBA staining. Male submandibular gland. **a.** Convoluted granular tubule. Electron-dense granule exhibiting affinity sites. **b.** Acinar cell. Note the moderate binding on electron-lucent granules. **c.** A very strong increase of gold particles occurred after sialidase digestion that removed terminal sialic acids with consequent accessibility of DBA to the α-galactosamine which acts as receptor sugar. a, x 41,600; b, x 39,600; c, x 31,400

Fig.2. DBA staining. Female submandibular gland. Acinar cell. **a.** Some labelling was evident over the rough endoplasmic reticulum (RER). **b.** Also intercellular tissue spaces (TS) shared positive sites located above all on membrane protrusions. **c.** The removal of sialic acid induced modest binding to appear over the electron-lucent granules. a, x 29,700; b. x 59,400; c, x 33,000.

granular tubules containing electron-dense granules. The fixation and embedding methods here used let us evidence paler areas within the electron-lucent granule matrix in both sexes.

DBA

Positive sites were observed on both convoluted granular tubule (CGT) (Fig. 1a) and acinus (Fig. 1b) secretory granules of male subjects. The moderate affinity of the electron-lucent granules of acinar cells was greatly enhanced by sialidase digestion (Fig. 1c). KOH deacetylation did not affect the sialidase/DBA binding. In females, DBA binding was evident on the electron-dense granules of CGT, whereas the negative electron-lucent secretory granules of acinar cells (Fig. 2a,b) were found to exhibit scarce binding sites only after removal of sialic acid (Fig. 2c). Affinity sites for DBA before sialidase digestion were restricted to the rough endoplasmic reticulum (Fig. 2a) and intercellular tissue space membranes (Fig. 2b).

PNA

No great differences were evidenced between male and female PNA staining. Indeed, secretory products of both sexes (Figs. 3a, 4a) showed moderate labelling on CGT electron-dense granules, whereas only few binding sites were discovered on the electron-lucent granules of acinar cells and intercellular tissue spaces (Fig. 3b). Differences between sexes emerged, instead, after sialidase digestion since this treatment produced a homogeneously distributed PNA binding on the electron-lucent granule matrix of males (Fig. 3c), whereas it induced new positive sites to occur mainly in the paler areas of acinar secretory granules in females (Fig. 4b). Alcoholic deacetylation was ineffective. Control sections did not exhibit appreciable staining (Fig. 4c).

Con A

Affinity sites were observed with different intensity

in both CGT (Fig. 5a) and acini (Fig. 5b). A large variability of staining was found at CGT level. Some labelling was also found on the rough endoplasmic reticulum. Control samples failed to stain (Fig. 5c). Appreciable differences between sexes were not observed.

WGA

WGA binding showed modest differences related to the sex genotype. Although the electron-dense as well as the electron-lucent granules were found to contain WGA reactive sites in both sexes (Fig. 6a,b), females exhibited more numerous affinity sites located on the paler zones of the acinar electron-lucent granules.

LTA

CGT electron-dense secretory granules strongly reacted (Fig. 7a) in contrast to acinar cell granules that did not show staining. Reactive patterns were restricted to intercellular tissue spaces and material contained inside the lumen (Fig. 7b). Differences were not found between sexes.

Controls

Control experiments substantiated the nominal lectin specificity and sialidase efficacy. The enzyme-free buffer did not seem to affect binding.

Discussion

In the organ examined, background labelling with the lectin gold probes was very low over the tissue except for the convoluted granular tubules that constantly exhibited staining between secretory granules, regardless of the lectin tested, maybe due to the intricate assembly of these secretory cells that can prevent a satisfactory fixation. Control experiments resulted in no labelling.

It has been experimented and emphasized by

Fig. 3. PNA staining. Male submandibular gland. **a.** Convoluted granular tubule. Electron-dense granules show gold particles reflecting the presence of terminal ß-galactose. **b.** Acinar cell exhibits very few gold particles distributed over the rough endoplasmic reticulum (RER), intercellular tissue spaces (TS) and electron-lucent granules. **c.** The sialidase digestion promoted an increased PNA staining of the electron-lucent secretory granules. a, b. x 33,000; c, x 19,800

Fig. 4. PNA staining. Female submandibular gland. **a.** The binding of electron-dense granules was scattered over the matrix. **b.** The cleavage of sialic acid produced a PNA staining that appeared heavier over the paler regions of the matrix of the acinar cell granules. **c.** Control section showing the heterogeneous matrix of the acinar cell secretory granules. a, b, x 39,600; c, x 24,750

3 a

4 a

RER

TS

3 b

4 b

3 c

5 a

T S

5 b

5 c
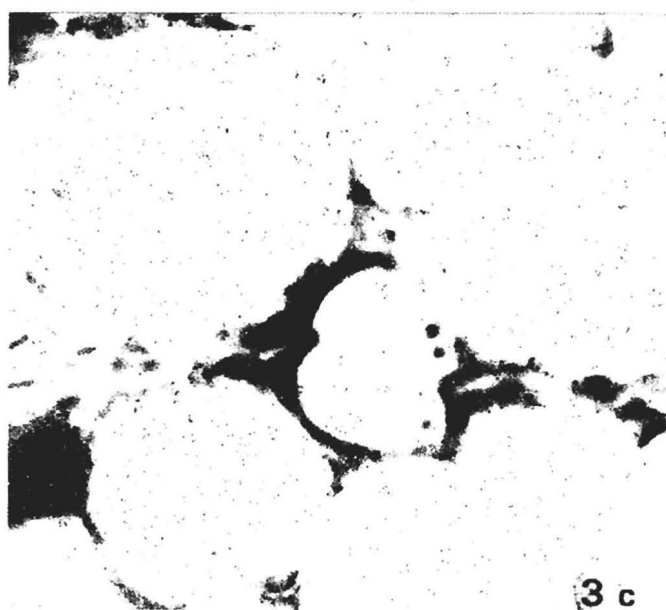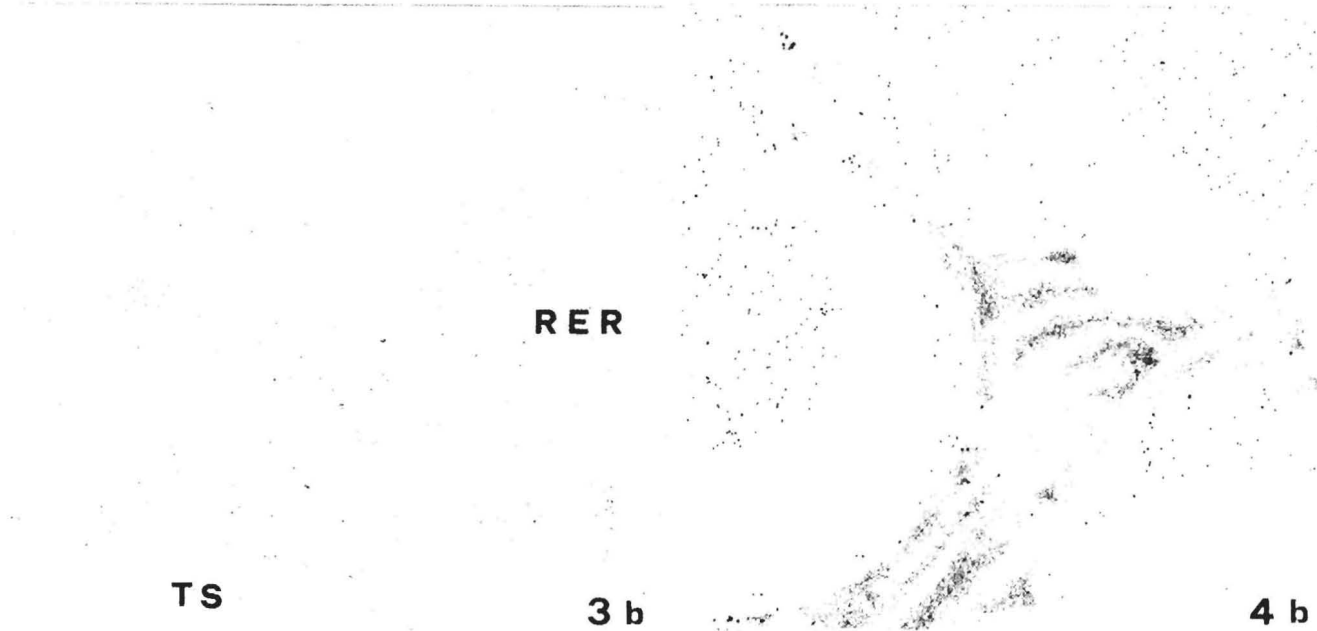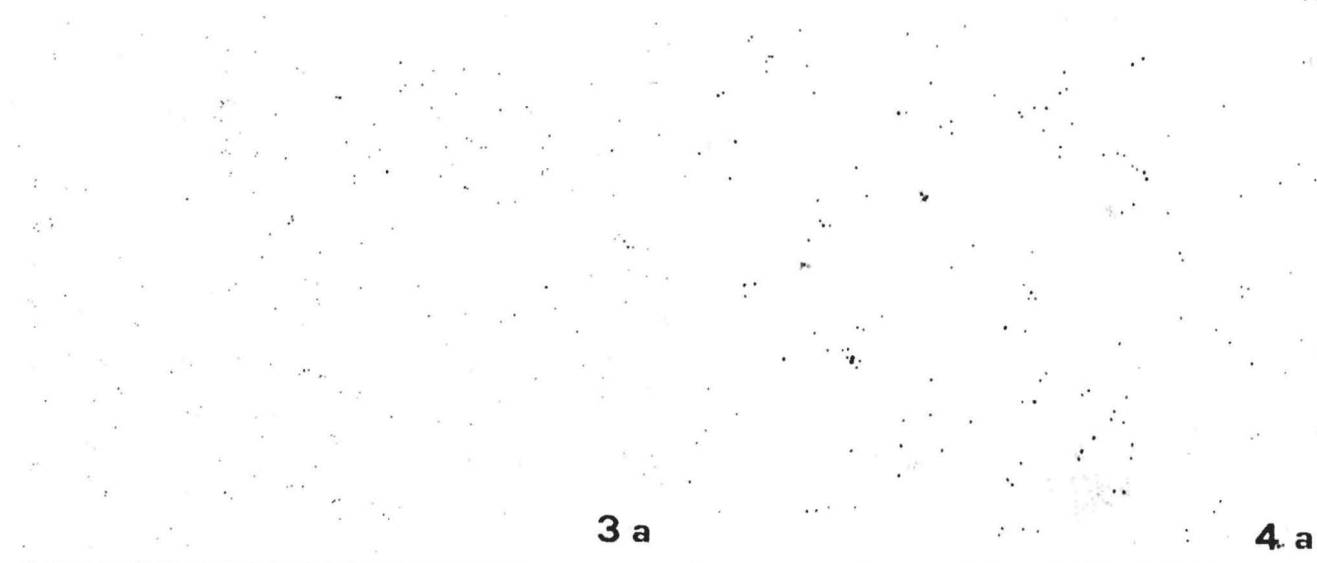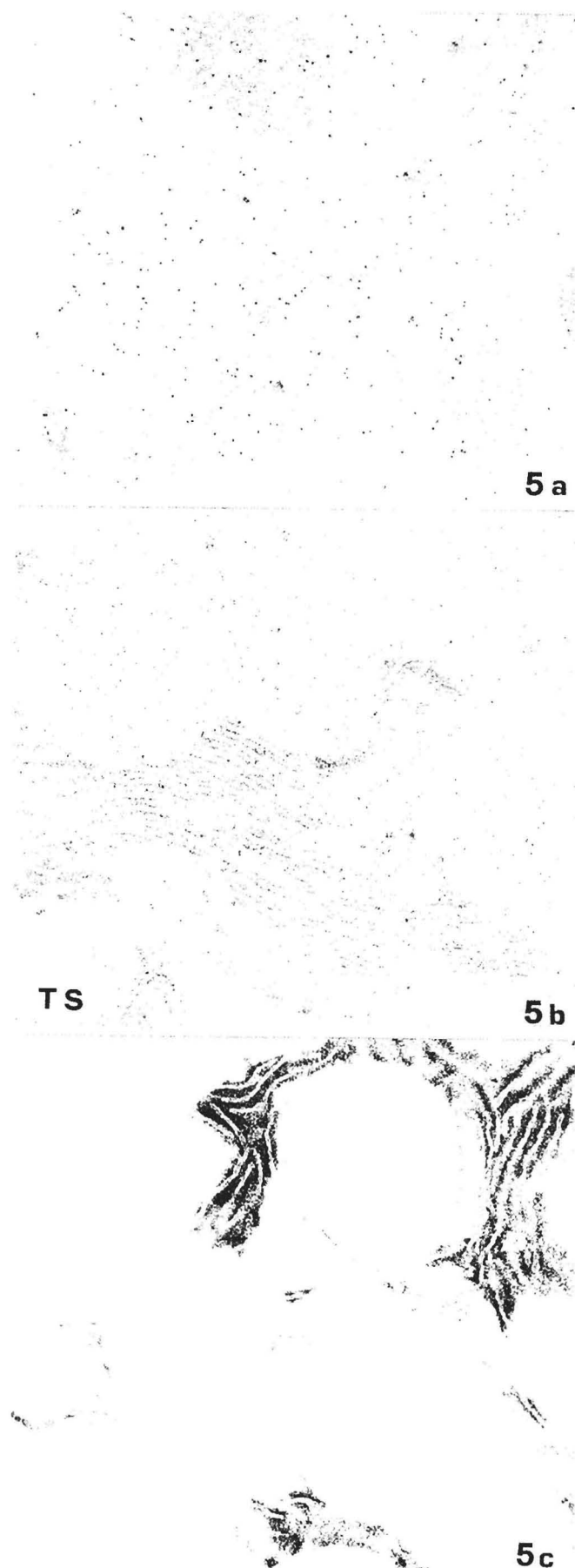
Bendayan et al. (1987) that always, for each class of antigens and binding molecules, conditions for optimal labelling must be worked out, since there is no single procedure that can be recommended as the best approach for colloidal gold cytochemistry. We encountered many problems in searching for appropriate fixation and dehydration agents for the submandibular gland, also probably on account of the very different degree of condensation and chemical nature of the granule content. For the morphological and ultracytochemical analysis, generally, the ideal conditions of fixation foresee the use of aldehydes at high concentrations and a post-fixation with osmium tetroxide. For immunogold techniques, it is recommended to employ formaldehyde and low concentrations of glutaraldehyde to reduce molecular modifications and, accordingly, minimize the loss of antigenicity without compromising the cytoarchitecture. The addition of picric acid to the fixation agents has been advocated to improve antigen retention (Dae et al., 1982; Somogi and Takagi, 1982). Many components generally stand the above fixations without undergoing relevant structural alterations but do not resist osmium treatment (Berryman and Rodewald, 1990; Stirling, 1990; Bendayan, 1995).

Compared to other acrylic resins, tested in preliminary studies, showing some disadvantages related to the laborious staging of monomeric mixtures or to the difficult sectioning, we noted that Bioacryl resin (also termed Unicryl) offered advantages consisting in easy sectioning, stability under the electron beam, and resistance to enzymatic treatment at low pH for long time with minimal background staining or sample damage. Analogously to Lowicryl K4M (Roth, 1983), also Bioacryl embedding permits the detection of various lectin binding sites in different subcellular structures as well as satisfactory preservation of surface and cytoplasmic glycoconjugates.

The acinar cells of salivary glands have served as useful models for studying various aspects of the secretory process. The appearance of secretory granules strictly depends upon their chemical composition, but the biochemical basis for their complex structural arrangement is poorly understood. Previous researches demonstrated that the mouse submandibular gland contains large quantities of hexosamines, hexoses, and sialic acid, whereas fucose and uronic acids are present in modest amounts (Roukema et al., 1976; Denny et al., 1980; Denny and Denny, 1982a; Menghi et al., 1986, 1989b). The present research indicated that N-acetylglucosamine, N-acetylgalactosamine, ß-galactose, D-mannose, L-fucose and sialic acids are located on

Fig. 5. Con A staining. Male submandibular gland. **a.** Numerous binding sites were distributed on electron-dense granules. **b.** Electron-lucent granules shared a conspicuous reactivity. Gold particles were also located inside intercellular tissue spaces (TS) and rough endoplasmic reticulum. **c.** In control section, the Con A staining performed in the presence of 0.2M mannose was not appreciable. a, b, x 27,700; c, x 19,800

convoluted granular tubule or acinar cell secretory granules. Sialic acids linked to ß-galactose and α-N-acetylgalactosamine were exclusively found on the
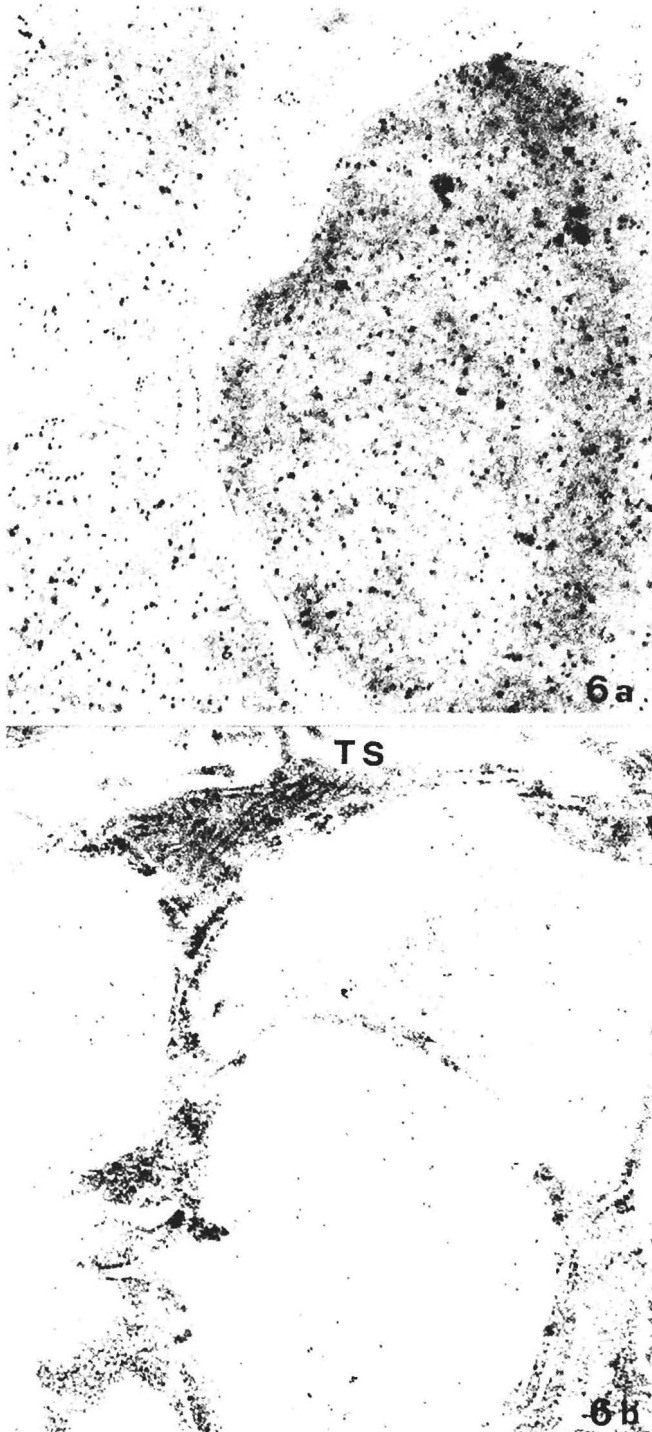

Fig. 6. WGA staining. Male submandibular gland. Sites of labelling were more evident in electron-dense granules of CGT (**a**) than in electron-lucent granules of acini (**b**). Sparse gold particles are also located at intercellular tissue space (TS) level. a, x 33,000; b, x 31,000
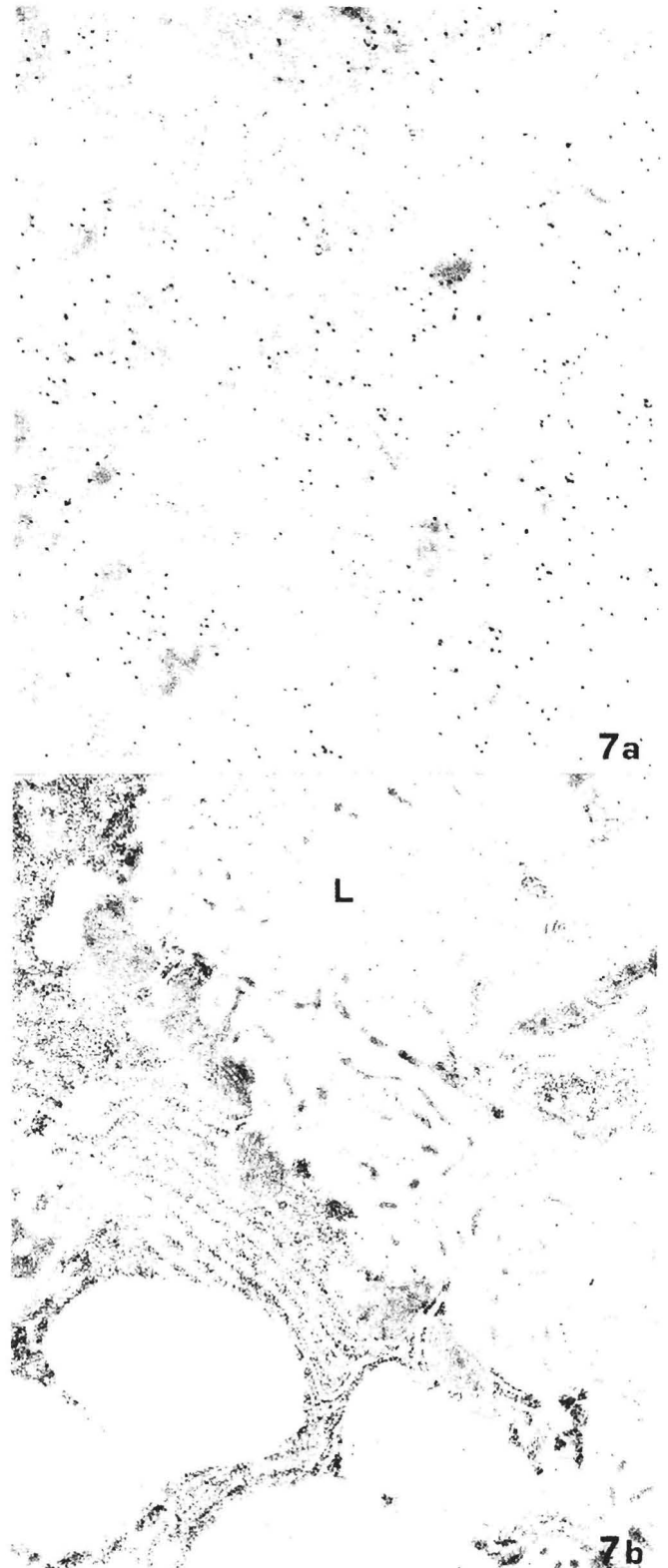

Fig. 7. LTA staining. Male submandibular gland. Electron-dense granules of CGT were heavily stained **a**, whereas electron-lucent secretory granules failed to react **b**. Only material contained inside the acinar lumen (L) strongly reacted. a, x 26,400; b, x 19,800

electron-lucent granules of acini. Only rare gold particles specified the presence of ß-galactose on the electron-lucent granules of acinar cells of both sexes, since at this site these residues seem to act as acceptor sugars for terminal sialic acids. Differences between sexes were not evidenced on the basis of the amount of acinar sialo-glycoconjugates characterized by the terminal di-saccharide sialic acid-ß-galactose but a different location inside the granule matrix was pointed out. Indeed, the disaccharide sialic acid-ß-galactose was found to be almost homogeneously distributed on the acinar secretory products of males, while it was restricted to the paler areas of granule matrix in females. Conversely, the sialoglycoconjugates having α-N-acetylgalactosamine as subterminal sugar residue for sialic acid showed quantitative rather than distributional differences between sexes and such dimorphism resulted in a prominent occurrence of these sialoglycoconjugates in acinar secretory products of males and in a modest occurrence in females. The present findings are confirmatory of and supplementary to previous data concerning the sialic acid distribution and characteri-zation at light microscopy level (Accili et al., 1996), and add new parameters to the sexual dimorphism of the mouse submandibular gland (Lacassagne, 1940; Caramia, 1966; Tsukitani and Mori, 1986; Schulte, 1987; Menghi et al., 1991; Akif et al., 1993). Indeed, while the differences known until now in the male and female glands appear to be an expression of the cytosolic differentiation of the tubular portion of the gland, in this report we demonstrated that striking differences also concern the acinar cell sialoglycoconjugates that exhibited the most pronounced sex related dimorphism.

There are valid indications that sialic acids influence or even determine the recognition of low and high molecular weight compounds, the action of certain hormones, physicochemical and catalytic properties of enzymes, hemostasis, cellular adhesiveness, antigenicity, transport processes and synaptic transmission. In particular, sialic acids are involved in protection of glycoconjugates and cells from recognition and degradation (Schauer, 1985; Reuter et al., 1988; Niew Amerongen et al., 1995). It is difficult to explain the sex-related expression of sialoglycoconjugates in males and females since only few data are available on the hormonal influence on both biosynthesis and function of sialic acid containing glycoconjugates. It is known that oestrogens lead to a fluctuation of protein-bound sialic acid in serum (Reutter et al., 1982) and protein- and lipid-bound sialic acid in the rat salivary glands (L. Vitaioli, personal communication), but this is the first report showing both quantitative and distributional differences of sialoglycoconjugates with different penultimate acceptor sugars. Biological processes such as saliva lubricating and supporting functions can involve the participation of carbohydrate-rich macro-molecules probably containing sialic acids. This correlates well with the present sialic acid distributional patterns, differently expressed on the acinar cell

secretory granules of females, likely linked to the reproductive cycle, and of males, probably in relation to distinct functions such as the production of territorial markers. Thus, diverse arrangements and composition of secretory sialoglycoconjugates might be attributable to different behaviour trends related to the animal's sex. However, most of these functions and their mechanisms have not yet been elucidated. Many different theories have been advanced concerning the biological roles of the oligosaccharide units of individual classes of glyco-conjugates and it is also possible that the same oligo-saccharide sequence may mediate different functions at different locations (Varki, 1993).

The mucous producing cells are widely distributed in the alimentary tract and secrete a large amount of mucin. It has been found that oligosaccharide chains of salivary glycoproteins are linked O-glycosidically to the core polypeptide, but the moderate to strong affinity of acini and convoluted granular tubules for Con A and WGA emphasizes their rich supply of N-linked oligo-saccharides in accordance with previous findings (Denny and Denny, 1982a; Niew Amerongen et al., 1983; Denny et al., 1995, 1996). In addition, the failure to detect fucose at electron-lucent granules, where sialic acids are present, is fairly consistent with the immunohisto-chemical demonstration of the fucose-less sialomucin in the mouse acinar cells and their putative progenitors (Denny and Denny, 1982b; Denny et al., 1988). A distribution of fucose residues as detected with *Ulex europaeus* lectin, virtually the opposite of that of sialic acid, was previously found also on the rat small intestinal epithelial cells during post natal development (Taatjes and Roth, 1990).

In conclusion, our study indicated that the terminal sialylated sequences can serve as markers of sex-related dimorphism in the mouse submandibular gland acinar cells. A determination of biochemical and physiological significance of the sugar moieties identified by actual lectin binding patterns will require further study. Preliminary biochemical data, obtained by means of fluorimetric high performance liquid chromatography, indicated that fluctuations of N-acetylneuraminic acid and N-glycolylneuraminic acid, as well as their O-acyl derivatives, occur between male and female mouse submandibular glands.

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