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Sex-related expression of sialic acid acceptor sugars in the mouse submandibular gland. Simultaneous visualization by confocal laser scanning microscopy

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Summary. The novel combination of sialidase digestion with simultaneous PNA and DBA binding yielded marked differences on sialoglycoconjugate occurrence and distribution in the mouse submandibular gland acinar cells of the two sexes. Striking differences in the structure of terminal disaccharides within stored secretory sialoglycoconjugates were also found. High content of sialic acid, characterized by the terminal sequence sialic acid-α-N-acetylgalactosamine, was established to only occur in the male acini where secretory cells appeared to be differently stained; indeed, some cells exhibited codistribution of sialic acid-α-N-acetylgalactosamine and sialic acid-ß-galactose terminal disaccharides, whereas other ones exclusively contained one of the two kinds of terminal sequences. In the female acinar cells, the secretory products were found to be almost exclusively composed by glycoconjugates having sialic acid subtended to ß-galactose without appreciable differences between acinar cells. Our finding of such extensive differences in the acinar cells of male and female mice adds new insights into the submandibular gland sexual dimorphism, commonly attributed to the androgen responsiveness of the granular convoluted tubule portion of the gland.

Key words: Sialic acid, Sialidase, Lectins, Confocal microscopy, Mouse, Submandibular gland

Introduction

The rodent submandibular gland is generally considered to be an organ that has two functions. One is the exocrine function aimed to constitute the salivary fluid; the other is the endocrine function consisting in the release into the circulatory system of various growth factors and hormones. The exocrine secretion is chiefly performed by acinar cells, while the endocrine function is assigned to the convoluted granular tubules that secrete a large number of polypeptides, with biologically defined properties (Barka, 1980), in response to various hormonal stimuli, in particular to androgens (Gresik, 1980).

In the studies of mammalian salivary glands, a variety of methods has been used to differentiate cell types within the secretory cells. These have included morphological analysis and histochemical techniques (Pinkstaff, 1980; Garrett et al., 1996). Variability and sex-related dimorphism of the rodent submandibular gland regarding the histology and carbohydrate histochemistry has appeared to be mainly an expression of the tubular portion (Lacassagne, 1940; Caramia, 1966; Schulte and Spicer, 1983, 1984; Tsukitani and Mori, 1986; Schulte, 1987; Menghi et al., 1991; Akif et al., 1993; Menghi and Materazzi, 1994; Hirshberg et al., 1996). The rat submandibular gland was also found to secrete families of tissue-specific proteins that can express sexual dimorphism (Rosinski-Chupin et al., 1988, 1993; Rosinski-Chupin and Rougeon, 1990). Recently, lectin histochemistry revealed a more marked dimorphism in the rat rather than in the mouse submandibular gland (Menghi et al., 1997).

The aim of the present study was to investigate the mouse submandibular gland by confocal laser scanning microscope (CLSM) in order to give insight into the sialoglycoconjugate spatial distribution, that cannot be defined at conventional optic or electron microscopy level. To this purpose, we performed appropriate lectin labeling, with selected fluorescent conjugates, following sialic acid removal on Bioacryl-embedded submandibular glands of male and female adult mice. The sections were analyzed by confocal laser scanning microscope and results were compared with lectin binding patterns visualized with different probes at optic and electron microscopy level. Indeed, earlier studies indicated that, at optical level, sexual differences concerning the sialic acid location, acetylation degree, and linkage kind are present in the submandibular gland of rat and mouse (Accili et al., 1996). More recently, findings from lectin-gold probes, combined with sialidase digestion, revealed different occurrence and distribution of sialic acid acceptor sugars in the secretory granules of male and female mouse submandibular gland acinar cells (Menghi et al., 1998).

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Materials and methods

Tissue collection

Submandibular glands promptly removed from sexually mature white male (10) and female (10) mice (*Mus musculus*, Swiss strain) were used. Animals were sacrificed by CO_2 suffocation. Removal of the tissue was carried out according to recommendation of the Italian Ethical Committee and under the supervision of authorized investigators.

Tissue processing

On the basis of previous researches (Marchetti et al., 1997), small pieces of glands were immersed, at 4 °C, in a fixative solution containing 1% glutaraldehyde, 4% paraformaldehyde, and 0.2% picric acid dissolved in 0.1M phosphate buffer, pH 7.6, added with $CaCl_2$, for 3 h at 4 °C. After rinsing with 0.1M phosphate buffer containing 3.5% sucrose, and quenching of free aldehydes with 50 mM ammonium chloride in sucrose-phosphate buffer for 1 h at 4 °C, specimens were post-fixed with 2% uranyl acetate in sucrose-maleate buffer for 2 h at 4 °C. As embedding medium, the Bioacryl resin (Scala et al., 1992) was used after dehydration with acetone up to 90% as previously detailed (Menghi et al., 1997). Sections (3 μm thick) were collected on glass slides pretreated with 5% potassium chrom sulfate and 0.5% gelatin.

Lectin binding

Sections were immersed for 30 min in 0.05M phosphate-buffered saline (PBS), pH 7.4, containing 0.03% Triton X-100 and 1% bovine serum albumin (BSA), rinsed in PBS (three times, 5 min each), and processed for binding with PNA (from *Arachis hypogaea*, for visualizing terminal ß-galactose) and DBA (from *Dolichos biflorus* for visualizing terminal α-N-acetylgalactosamine) conjugated to fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) fluorochromes at a concentration of 2 μg/ml. Lectins were diluted 1:100 in PBS-Triton X-100-BSA and incubated for 90 min at room temperature. Specimens were then rinsed in PBS (three times, 10 min each) and mounted on Top-watermount.

Single labeling as well as double labeling with sequential and simultaneous binding were tested. In the case of double labeling experiments we performed one type of labeling and the reverse reaction, i.e. PNA-FITC and DBA-TRITC or PNA-TRITC and DBA-FITC to test possible steric hindrance of fluorochromes or manipulation artifacts.

To control lectin specificity, the appropriate inhibitory sugars at concentrations of 0.2-0.4M were added to the lectin solutions; unconjugated lectins were also tested. Additional controls included the evaluation of autofluorescence phenomena.

Sialidase digestion

This was carried out by incubating sections, before lectin staining, at 37 °C for 16 h in sialidase (neuraminidase, Type V, from *Clostridium perfringens*) at a concentration of 0.5 U/ml in 0.1M sodium acetate buffer, pH 5.5, containing 10 mM $CaCl_2$. In order to verify specificity and efficiency of the enzymatic degradation, control sections were incubated in the enzyme-free buffer solution under the conditions above.

Image acquisition and analysis by CLSM

The lectin reactive patterns were analyzed in an Ar/Kr Bio-Rad MRC-600 Confocal Laser Scanning Microscope (Bio-Rad, Hertfordshire, UK) attached to a Nikon Diaphot-TMD-EF inverted microscope equipped with a Plan Apo, oil-immersion, objective (x60, NA=1.4) in order to obtain optical sections with the improved separation of the two dye emission and reduced intrinsic statistical noise of images (Shotton, 1989; Pawley 1995). The standard BHS block (excitor filter 488 DF 10) and the YHS one (excitor filter 568 DF 10) were employed for FITC and TRITC, respectively. Microphotographs were taken using a polaroid Quick Print VI-350 Video Printer equipped with a 35 mm camera (Velvia Fujichrome film).

Chemicals

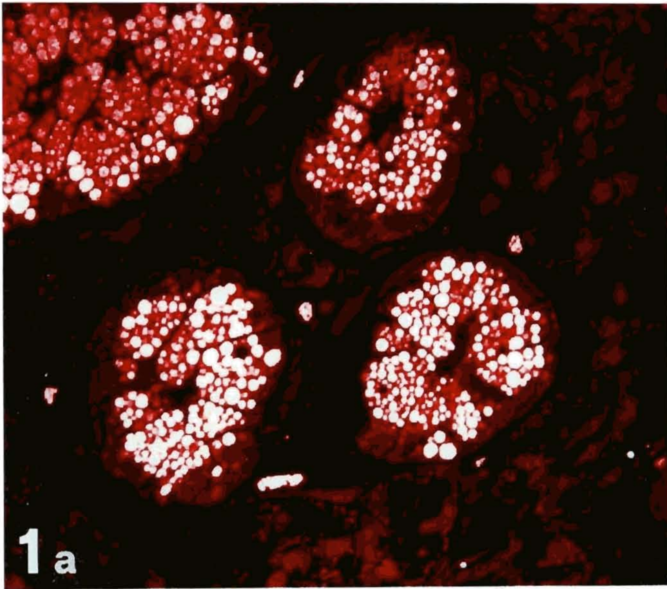
The reagents used for this experiment were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except for Bioacryl resin and Top-watermount obtained from Bio-Optica (Milano, Italy) and Pabish (Milano, Italy), respectively.

Results

The most reactive sites exhibited a white central core indicating the highest density of lectin labeling; in

Fig. 1. Male submandibular gland. Double PNA-TRITC and DBA-FITC staining. Optical section. Micrograph showing PNA-positive convoluted granular tubules fluorescing red **(a)** and DBA-positive convoluted granular tubules green **(b)** in the same area. The composite merged image showed complete overlapping of PNA and DBA lectin binding sites (yellow) **(c)**. Acinar cells (A) did not react. x 750

Fig. 2. Male submandibular gland. Double PNA-TRITC and DBA-FITC staining performed with prior sialidase digestion. Optical section. Removal of terminal sialic acid strongly increased PNA-TRITC affinity at acinus level **(a)**. Also DBA-FITC exhibited new binding sites after sialic acid cleavage **(b)**. The merged image revealed the heterogeneous sialidase induced reactivity at different acinar cell (A) level (c). Note the presence of cells containing the terminal disaccharide sialic acid-ß-galactose (red), cells exhibiting the disaccharide sialic-α-N-acetylgalactosamine (green), and cells having both disaccharides (yellow) in the combined image. x 750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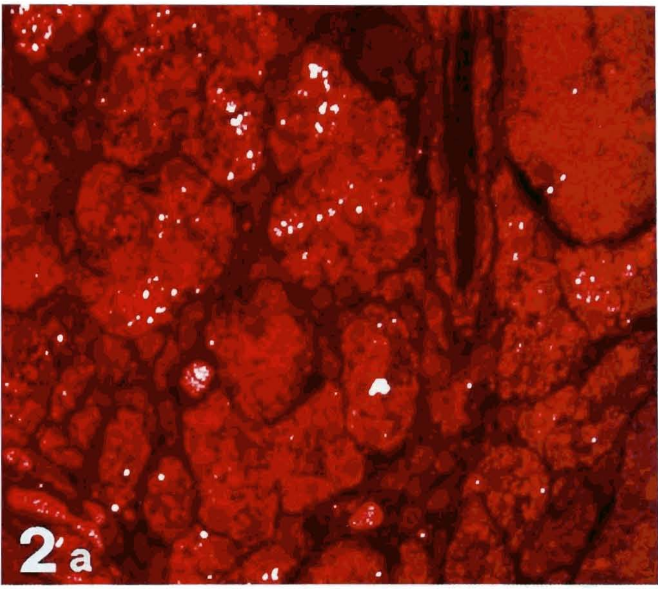
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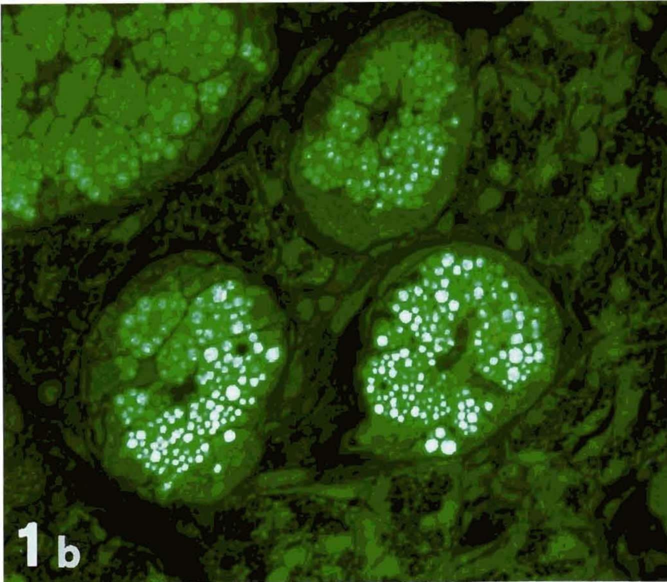
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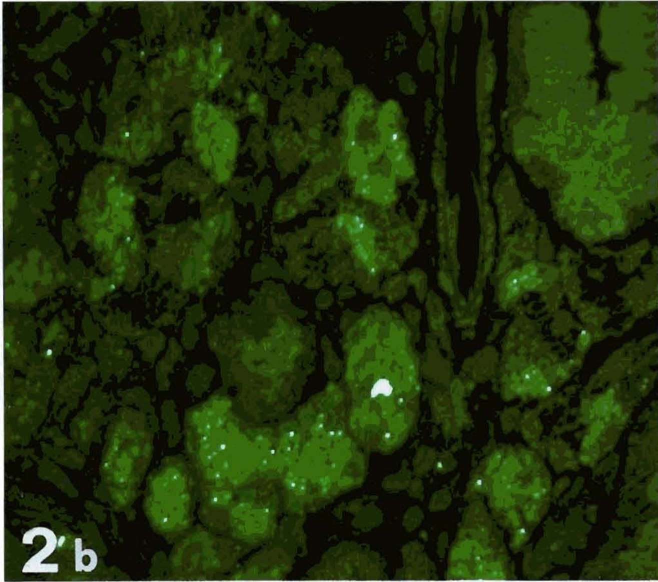
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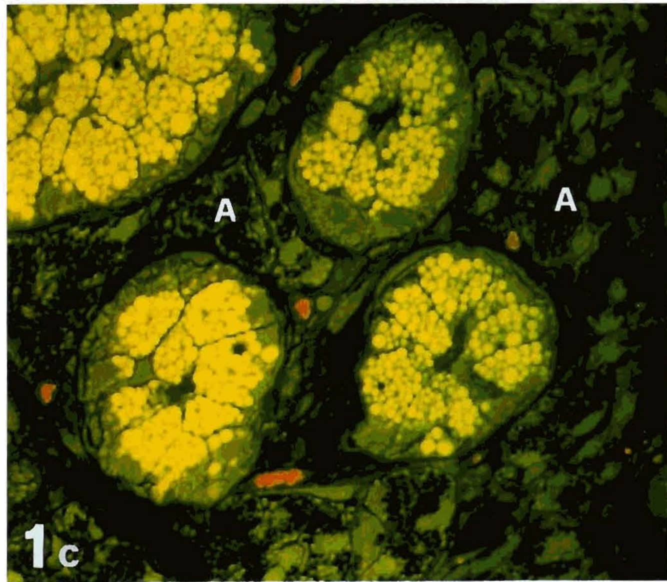
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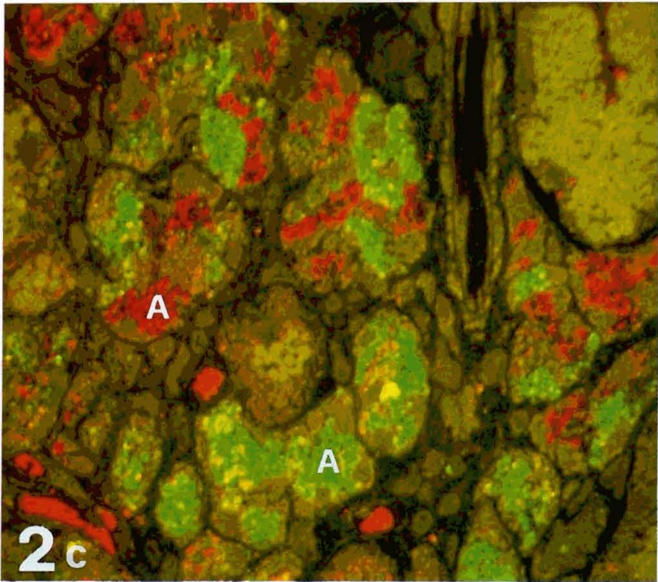
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particular, the central core was surrounded by a region shown in green (gradually darker green in the periphery indicating lesser densities of binding) for DBA-FITC, or in red to bright orange for PNA-TRITC. The colocalization of DBA-FITC and PNA-TRITC affinity sites was shown in yellow in the merged images.

Appreciable inter-sectional and inter-individual differences as well as autofluorescence phenomena were not observed at CLSM level.

Males

PNA (Fig. 1a) and DBA lectins (Fig. 1b) intensely stained the convoluted granular tubule secretory granules and exhibited codistribution of receptor sites (Fig. 1c).

Removal of sialic acid by neuraminidase digestion induced the visualization of a very strong PNA staining at acinar cell level (Fig. 2a). Also DBA affinity increased after sialidase digestion on acinar cells although with a minor intensity (Fig. 2b). The merged image showed that some cells presented codistribution of sialidase/PNA and sialidase/DBA induced stainings, while others showed differential binding patterns that expressed only PNA- or DBA-induced affinities (Fig. 2c).

Females

Native PNA (Fig. 3a) and DBA (Fig. 3b) lectin stainings were found to occur at convoluted granular tubule level and exhibited overlapped binding (Fig. 3c).

Pretreatment with sialidase before lectin incubation caused the occurrence of numerous PNA reactive sites on all acinar cells (Fig. 4a) in contrast to a negligible DBA binding (Fig. 4b) as also confirmed by the merged image (Fig. 4c).

Discussion

Information about the sialic acid acceptor sugars in the acinar cells of the mouse submandibular gland was here obtained through a combined analysis with lectins labeled to fluorochromes and sialidase digestion.

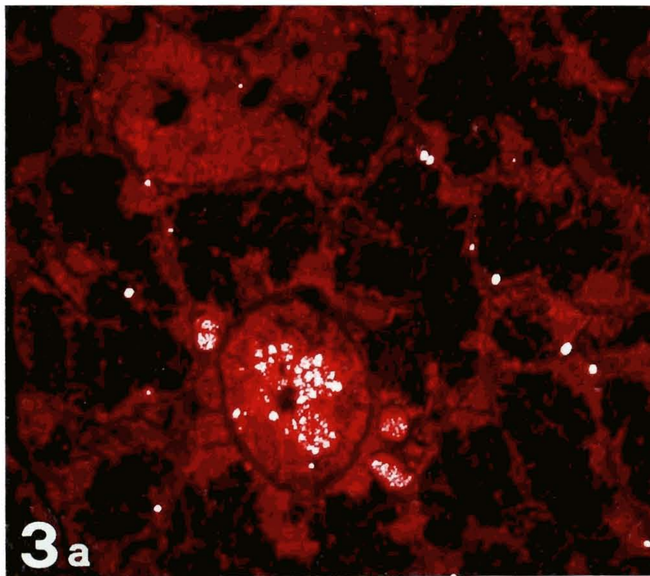
Because of their specific affinities for sugar moieties and on the basis of preliminary experiments, PNA and DBA lectins were selected to visualize ß-galactose and α-N-acetyl-D-galactosamine, respectively. Also the choice of fixation procedure with 1% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid followed by embedding in Bioacryl was based on previous experiments on undigested samples resulting in satisfactory retention of morphological details and lectin labeling resolution (Marchetti et al., 1997; Menghi et al., 1997).

Moreover, in order to evaluate artifacts or altered binding patterns consequent to the double labeling, we firstly verified single binding with PNA-FITC and -TRITC or DBA-FITC and -TRITC; secondly, we carried out sequential binding methods by combining suitably conjugated lectins with alternate fluorescent dyes; thirdly, we planned simultaneous binding approaches by checking also the reverse labeling with inverted fluorochromes. The results were not consistently changed by the different binding procedures; the combination of PNA-TRITC with DBA-FITC was selected to document the binding patterns.

Our data indicated that sialoglycoconjugates with the terminal disaccharide sialic acid-ß-galactose, as deduced by the sequence sialidase/PNA, are widely distributed on acinar cells of both sexes. Conversely, oligosaccharides containing the terminal sequence sialic acid-α-N-acetylgalactosamine proved to be restricted to the male acinar secretory products. Thus, despite the common acceptance of sexual dimorphism in rodent submandibular gland such as due to differences in morpho-functional development of convoluted granular tubules (Lacassagne, 1940; Caramia, 1966), our findings give further support to the occurrence of sex-related differences in the *Mus musculus* submandibular gland acinar cells, as indicated at light and electron microscopy level (Accili et al., 1996; Menghi et al., 1998). Previously, genetic and sex-related differences have also been found in the structure of *Mus hortolanus* and *Mus domesticus* submandibular gland acinar cell glycoconjugates where, however, the terminal disaccharide sialic acid-α-N-acetylgalactosamine was not detected (Schulte, 1987). A different expression of both androgen-regulated genes and their products, SMR_1 and SMR_2 polypeptides, has been previously demonstrated by in situ hybridization in male and female acini of rat submandibular gland (Rosinski-Chupin et al., 1993). Evidence for the presence of androgen receptors in male mouse and rat acinar cells has been documented (Morrell et al., 1987; Sar et al., 1990) and, more recently, Vcs_2 transcripts were found only in the acinar cells of the male mouse submandibular gland where they are expressed in response to androgens and show differential accumulation among cells (Senorale-Pose et al., 1998). This suggests that the convoluted granular tubule cells may be not the only target cells for androgens in the

Fig. 3. Female submandibular gland. Double PNA-TRITC and DBA-FITC staining. Optical section. Comparison of the distribution of PNA-TRITC **(a)** and DBA-FITC **(b)** revealed numerous reactive sites at CGT level. The merged image showed regions of overlap (yellow) supporting the colocalization of PNA and DBA receptor sites **(c)**. Acinar cells (A) were unstained. x 750

Fig. 4. Female submandibular gland. Sialidase digestion/double PNA-TRITC and DBA-FITC staining. Optical section. Removal of sialic acid unmasked the subterminal acceptor sugar and induced PNA affinity at acinus level **(a)**. In contrast no appreciable binding of DBA was found **(b)**. The superimposition of images confirmed the location of the terminal disaccharide sialic acid-ß-galactose (red), deduced by sialidase-PNA sequence, on all acinar cell (A) secretory granules **(c)**. x 750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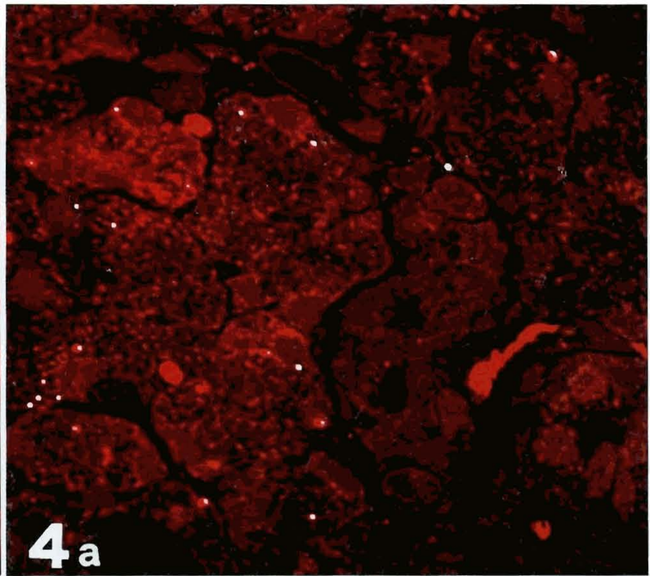
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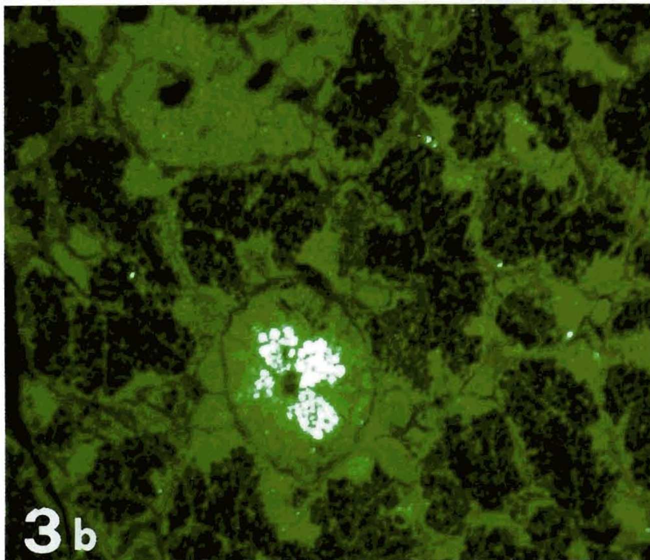
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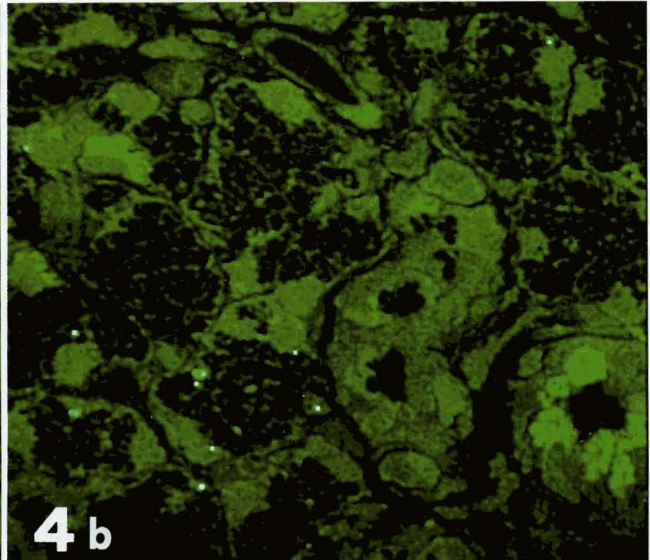
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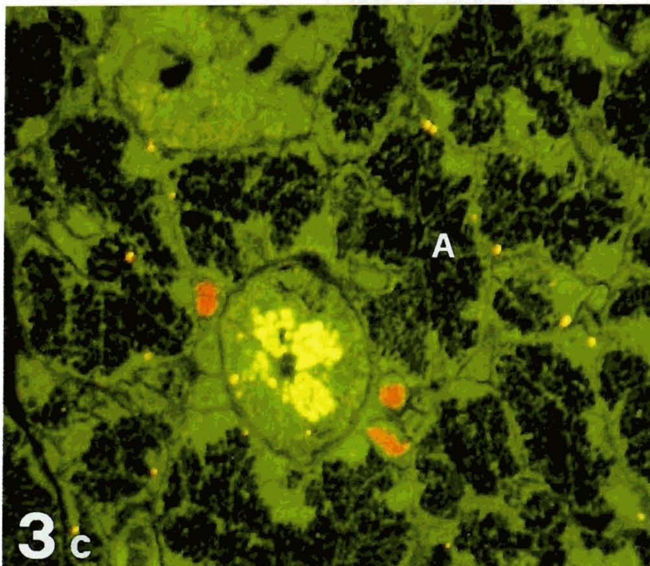
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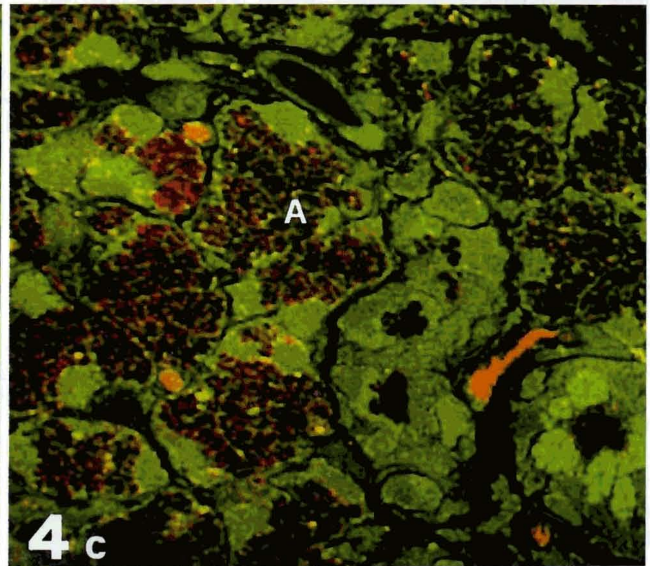
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submandibular glands. On the other hand, the endocrine status was found to affect the histochemical expression of sugar moieties of glycoconjugates as well as endolectins, thus modifying both sides of presumed protein-carbohydrate interactions (Akif et al., 1993).

The confocal laser analysis, by allowing the simultaneous visualization of the lectin distributional patterns as well as the evaluation of sialoglycoconjugate spatial arrangement, resulted in a useful approach for providing additional information. In particular, in the male acini we observed cells secreting glucidic material with the terminal sequence sialic acid-ß-galactose or sialic acid-α-N-acetylgalactosamine and cells containing both the types of terminal sequences. Such a different location did not occur in female submandibular gland in which the prominent terminal disaccharide sialic acid-ß-galactose was evidenced on all acinar cells.

Recently, PNA immunogold labeling, induced by sialidase digestion, evidenced the subcellular location of the terminal sequence sialic acid-ß-galactose: a homogeneous distribution of binding sites was found in male acinous granule matrix, while in female acinar cells an intragranular compartmentation was evidenced (Menghi et al., 1998). It would be derived that the sexual diversity in sialoglycoconjugate intragranular arrangement, i.e. the occurrence of compartmentation in the female acinar granules and the absence in the male ones, results in homogeneity and heterogeneity of acinar cell populations, respectively. It remains to clarify if the reported findings correspond to a "constitutive" cell specialization within acinar secretory elements or represent the visualization of a "dynamic" condition.

Biochemical analysis of mouse salivary glands has revealed a mixture of O-glycosylproteins, with sialic acid linked to either penultimate ß-D-galactose by an $\alpha2\rightarrow3$ glycosidic bond or N-acetyl-D-galactosamine by an $\alpha2\rightarrow6$ bond, and of N-glycosylproteins in which a great deal of sialic acid residues is linked to ß-D-galactose by an $\alpha2\rightarrow6$ glycosidic bond (Pigman, 1977; Herp et al., 1979; Montreuil, 1980; Denny and Denny, 1982; Nieuw Amerongen et al., 1983, 1987). Our previous histochemical data indicated the absence of $\alpha2\rightarrow3$-linked ß-D-galactose in the male acinar cells (Accili et al., 1996). It follows that the terminal sequence sialic acid-N-acetyl-D-galactosamine, here visualized, belongs to the O-glycosylproteins which appear to be differently expressed at the male acinar cell populations. The findings of glycoconjugates with the terminal sequence sialic acid-ß-D-galactose in the female acinar cells are fairly consistent with the presence of N-linked oligosaccharides biochemically identified in the adult female mouse submandibular gland mucin (Denny et al., 1995, 1996).

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