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Cellular compartmentation of lysozyme and α-amylase in the mouse salivary glands. Immunogold approaches at light and electron microscopy level

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Summary. The research was planned to study the subcellular distribution of enzymatic secretory products within the secretory structures of the mouse major salivary glands at light and electron microscopy level by immunogold silver stain (IGSS) technique and double-sided post-embedding immunogold binding and silver amplification in order to speculate about their compartmentation. In particular, we experimented the above immunogold labeling approaches to localize the lysozyme and to verify its distribution patterns in relation to another secretion enzyme, α-amylase.

Co-presence of lysozyme and α-amylase was observed in the convoluted granular tubule cells of the submandibular gland and in the demilunar cells of the sublingual gland as well as in the electron-dense regions of the mottled secretory granules in the parotid gland. Exclusive binding patterns of lysozyme were observed in the acinar cells of the submandibular and sublingual glands where α-amylase did not occur.

Key words: Lysozyme, α-amylase, IGSS, Double-sided binding, Salivary glands, Mouse

Introduction

Lysozyme is an enzyme broadly present and secreted from epithelial cells in many human and other vertebrate tissues such as those corresponding to digestive, respiratory and genital tracts, as well as lacrimal, mammary, sweat and salivary glands (Gad, 1969; Erlandsen, 1974; Mason and Taylor, 1975; Bowes and Corrin, 1977; Spicer et al., 1977; Montero and Erlandsen, 1978; Machino et al., 1984, 1986; Franken et al., 1989; Staneva-Dobrovski, 1997). Lysozyme is also elaborated and released from neutrophils, monocytes and histiocytes and it plays an important role as a functional marker in many kinds of diseases due to its serum level changing (Osserman and Lawlor, 1966; Miyauchi et al., 1985; Mutasa, 1989; Resnitzky et al., 1994). Lysozyme has a direct bacteriolytic action hydrolyzing glycosidic bond N-acetylmuramic acid-ß-(1,4)-N-acetyl-D-glucosamine of peptidoglycans commonly present in the bacterial cell walls (Jolles and Jolles, 1984). Furthermore, mammalian lysozymes, classed as c-type muramidases, are also capable of transglycosylation (Jolles et al., 1974; Jolles and Jolles, 1984). Mandel (1989) proposed an inhibition of bacterial growth, metabolism and dechaining by lysozyme, while other authors have shown even an *in vitro* dose- and time-dependent killing effect (Kamaya, 1970; Marquis et al., 1982; Samaranayake et al., 1993). Due to this characteristic, lysozyme also combines another property; indeed, lysozyme, a cationic protein, possesses the ability to form complexes with a variety of anions (Imoto et al., 1972) which could produce a non-enzymatic injury to cytomembranes following a direct cationic-protein binding (Marquis et al., 1982; Schenkels et al., 1995). Moreover, Jolles and Jolles (1984) have found important correlations between lysozyme and immunoglobulins, lactoferrin, macrophages, and granulocytes.

Lysozyme is also known to form complexes with a variety of acidic mucopolysaccharides (Imoto et al., 1972); however, other authors have referred the association of lysozyme with neutral mucosubstances present in demilunar serous cells of mixed salivary glands, or other glands of the digestive tract (Schackleford and Klapper, 1962; Gad, 1969). Sometimes its localization has been very difficult to find due to acidic components of mucous that could hinder the lysozyme antigenic determinants which are very important for the recognition by the specific antibody (Klockars and Reitamo, 1975).

The aim of the present study was to obtain insight into the subcellular distribution of lysozyme in the mouse major salivary glands. Indeed, the difficulty to preserve the salivary gland secretory products in the rat has been referred (Staneva-Dobrovski, 1997). For this purpose we experimented different fixation and embedding media, carried out at optic and electron microscopy level, as well as immunogold silver stain

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technique (IGSS) and double-sided binding on semithin or paraffin and ultrathin sections, respectively. These methods allowed us to locate lysozyme and verify its distribution patterns in relation to α-amylase, an enzyme present in the mouse salivary gland secretion products investigated in our previous research (Menghi et al., 1999).

Materials and methods

Tissue collection

Mouse submandibular, parotid and sublingual glands were taken from mature male and female Swiss strain mice (*Mus musculus*) (Stock Morini, S. Polo d'Enza, RE, Italy) weighing approximately 25 g. Animals, having free access to water and pellet food, were sacrificed in compliance with the recommendations of the Italian Ethical Committee under the supervision of authorized investigators. Tissues were immediately removed and differently processed as described below.

Fixative solutions and embedding procedures

1. After fixation in 1% glutaraldehyde-4% paraformaldehyde-0.2% picric acid in 0.1M phosphate buffer, pH 7.6, containing 0.5mM $CaCl_2$, at 4 °C for 2-3 h, and rinsing with several changes of cold 0.1M phosphate buffer, pH 7.6, containing 0.5mM$CaCl_2$ and 3.5% sucrose, free aldehydes were quenched with 50mM NH_4Cl in the same buffer-supplemented solution for 1 h at 4 °C (4 times, 15 min each). After removing phosphate groups with 0.1M maleate buffer, pH 6.5, containing 3.5% sucrose (4 changes, 15 min each at 4 °C), samples were post-fixed for 2 h at 4 °C with 2% uranyl acetate in 0.1M maleate buffer, pH 6.0, containing 3.5% sucrose. Dehydration and embedding were carried out with acetone (up to 90%) and Unicryl (British Bio Cell International, Cardiff, UK), respectively. The polymerization time was 72 h at 4 °C under UV lamp (Menghi et al., 1996a).

2. Tissues were fixed in 4% paraformaldehyde in 0.1M phosphate buffer, pH 6.8, at 4 °C for 2-3 h, then they were rinsed in the same buffer containing 3.5% sucrose for 1 h at 4 °C. Samples were subsequently dehydrated in acetone up to 90%, and finally infiltrated and embedded in Unicryl for 72 h at 4 °C under UV lamp.

3. Specimens were processed as described in protocol 1 but dehydration and embedding were carried out at progressively lowered temperatures (ranging from 4 to -20 °C). The tissue pieces were embedded in Lowicryl K4M (Polysciences, Warrington, PA, USA) for 72 h at -20 °C under UV lamp.

4. Tissue fragments were fixed in 0.2% glutaraldehyde and 2% paraformaldehyde in 0.1M cacodylate buffer, pH 7.4, at 4 °C for 2-3 h. Then, they were rinsed in the same buffer containing 7% sucrose (4 changes, 30 min each at 4 °C). Some samples were treated for the quenching of free aldehyde groups keeping them in 50mM NH_4Cl in 0.1M phosphate buffered saline solution (PBS), pH 7.4, for 3 h at 4 °C. Dehydration was carried out in ethanol from 50% up to absolute at 4 °C, then samples were embedded in Unicryl (at 4 °C) or in Lowicryl K4M (at -10 °C) under UV lamp for 72 h.

5. Samples were fixed for 24 h in Carnoy's fluid and post-fixed for 3 h in a 2% calcium acetate-4% paraformaldehyde mixture (1:1) at room temperature. Tissue samples were then dehydrated through graded alcohols, cleared in xylene, and embedded in paraffin block.

Sectioning

Semithin (about 2 μm thick) and ultrathin (about 60 nm thick) sections from plastic embeddings were cut with glass knives by means of an LKB Ultrotome V. Semithin sections were collected on SuperFrost/Plus slides (Bio-Optica, Milano, Italy), while ultrathin ones were collected on uncoated, 400 mesh, nickel grids. Paraffin sections (about 5 μm thick) were cut by means of a LEICA 2040 Autocut microtome and collected on SuperFrost/Plus slides; before labeling these sections were dewaxed and rehydrated.

Single and double-sided immunogold labeling technique

Floating ultrathin sections, without prior etching, were rehydrated with 0.05M Tris-buffered saline solution (TBS) (Sigma Chemical Co., St. Louis, MO, USA), pH 7.6, and pre-incubated with 1% bovine serum albumin (BSA), fatty acid free, (Sigma Chemical Co., St. Louis, MO, USA) and 1% instant milk in 0.05M TBS, pH 7.6, for 30 min at room temperature. Grids were then incubated with rabbit polyclonal anti-human lysozyme EC 3.2.1.17 (Dako, Glostrup, Denmark) diluted 1:15 in 1% BSA-5% normal goat serum (NGS) (Sigma Chemical Co., St. Louis, MO, USA) in 0.05M TBS, pH 7.6, overnight at 4 °C in a humid chamber. After rinsing in 0.05M TBS, pH 7.6, for 20 min at room temperature, sections were floated on drops of 1% BSA in 0.05M TBS, pH 7.6, for 30 min at room temperature. Then, grids were incubated with Auroprobe EM goat anti-rabbit G-10 (10 nm gold labelled IgG) (Amersham Life Science, Buckinghamshire, England) diluted 1:10 in 0.05 M TBS, pH 7.6, containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO, USA), for 90 min at room temperature in a humid chamber. Sections were then washed several times in 0.05M TBS, pH 7.6, and distilled water.

In the double-sided staining, sections were lysozyme labeled as above and then enhanced for 5 min at room temperature by means of a silver enhancing kit (British Bio Cell International, Cardiff, UK). After rinsing in distilled water, the reacted grid face (face A) was covered by a Formvar film (0.15% in 1,2-Dichloroethane) or carbon film of about 15 nm (MED010, Balzers Union). Then, grid was turned out and the

opposite face (face B) was incubated with anti-human α-amylase raised in rabbit (Sigma Chemical Co. St Louis, MO, USA) diluted 1:100 in 0.05M TBS, pH 7.6, added with 1% BSA and 5% NGS for 40 min at room temperature in a humid chamber. After rinsing in TBS, sections were treated with 1% BSA in 0.05M TBS, pH 7.6, for 30 min at room temperature and incubated with the above described goat anti-rabbit IgG conjugated with 10 nm colloidal gold diluted 1:10 in 0.05M TBS, pH 7.6, containing 0.05% Tween 20 for 60 min at room temperature in a humid chamber. Then, grids were rinsed with TBS and distilled water and, finally, counterstained with uranyl acetate (1 min) and lead citrate (1 min) at room temperature. Specimens were analyzed by means of a Philips EM 201C electron microscope at an accelerating voltage of 60 kV.

In order to confirm the presence or absence of colocalized sites, the double-sided method was also performed by reversing the sequence of labeling alternatively on the two faces.

Immunogold silver stain (IGSS) technique

Semithin and paraffin sections mounted on slides were covered with NGS diluted 1:30 in 0.05M PBS, pH 7.4, containing 0.4% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) and 1% BSA for 30 min at room temperature. After rinsing, sections were incubated with the rabbit polyclonal anti-human lysozyme diluted 1:20-1:40 in 0.05M PBS, pH 7.4, containing 0.4% Triton X-100 and 1% BSA for 1 h at room temperature in a humid chamber. Then, slides were rinsed in 0.05M PBS, pH 7.4, and left in the same buffer containing 0.4% Triton X-100 and 1% BSA for 30 min at room temperature. Subsequently, sections were incubated with Auroprobe LM goat anti-rabbit IgG (H+L) (Amersham Life Science, Buckinghamshire, England) or with Protein A-10 nm colloidal gold labeled (Sigma Chemical Co., St. Louis, MO, USA) diluted 1:15 in 0.05M PBS, pH 7.4, for 60-90 min at room temperature in a humid chamber, and finally they were washed in PBS and distilled water. Before mounting coverslips with Eukitt (Bio-Optica, Milano, Italy), immunogold labeling was enhanced twice (5 min each) with a silver enhancing kit. Development was stopped by washing in tap water for several min and finally in distilled water.

Controls

In order to test lysozyme specificity, the antibody was adsorbed, before incubation, with an excess of lysozyme from hen egg white (Boehringer, Mannheim, Germany).

Additional controls were carried out by omitting the primary antibodies.

To confirm the validity of double-sided reactions, controls were performed as follows: a) binding with primary and secondary antibodies, silver enhancement, Formvar or carbon film protection on face A and incubation with Auroprobe EM goat anti-rabbit G-10 without primary antibodies on face B; and b) binding with primary antibodies followed by Formvar or carbon film protection on face A and incubation with Auroprobe EM goat anti-rabbit G-10 without primary antibodies on face B.

Results

The analysis of immunolabeled samples for the localization of lysozyme showed that satisfactory results were obtained with both the fixative solutions and embedding media of protocols 1 and 4, even if the morphology at electron microscopy level was better preserved using protocol 1. At optical level Unicryl sections were found to be less reactive when compared with paraffin sections, maybe due to the thickness. Moreover, protein A-gold and Auroprobe LM goat anti-rabbit IgG gave similar results although the latter showed more specific binding. The fixation and embedding procedures of protocol 1 were found to be optimal also for α-amylase detection.

Single binding for lysozyme

Submandibular gland

The secretory granules of acinar and convoluted granular tubule cells exhibited lysozyme immunostaining in both sexes (Figs. 1-6). In the acinar cells, some differences occurred in the intragranular distribution of gold signals that were scattered within the matrix of pale granules in males (Fig. 1), whereas they appeared to be restricted to the weakly electron-dense areas of the pale granules in females (Fig. 2). Also the intensity of convoluted granular tubule binding was found to depend on the animal sex; indeed, we found more numerous lysozyme immunoreactive sites in males than in females (Figs. 3, 4). The control reaction gave lack of staining (Fig. 5). The IGSS technique (Fig. 6) confirmed the results obtained at electron microscopy level.

Parotid gland

A few lysozyme molecules were found in bizonal, target and mottled granules where the enzyme was preferentially located in the electron-dense areas (Figs. 7, 8). Differences between sexes did not occur. The IGSS method, at light microscopy level, also produced a modest staining (Fig. 9).

Sublingual gland

Lysozyme determinants, identified by gold particles, were found to occur on the acinar cells where they were mainly located at cytoplasmic level (Fig. 10) as well as on the electron-dense granules of demilunar cells (Fig. 11). Sex-related binding patterns were not found. The

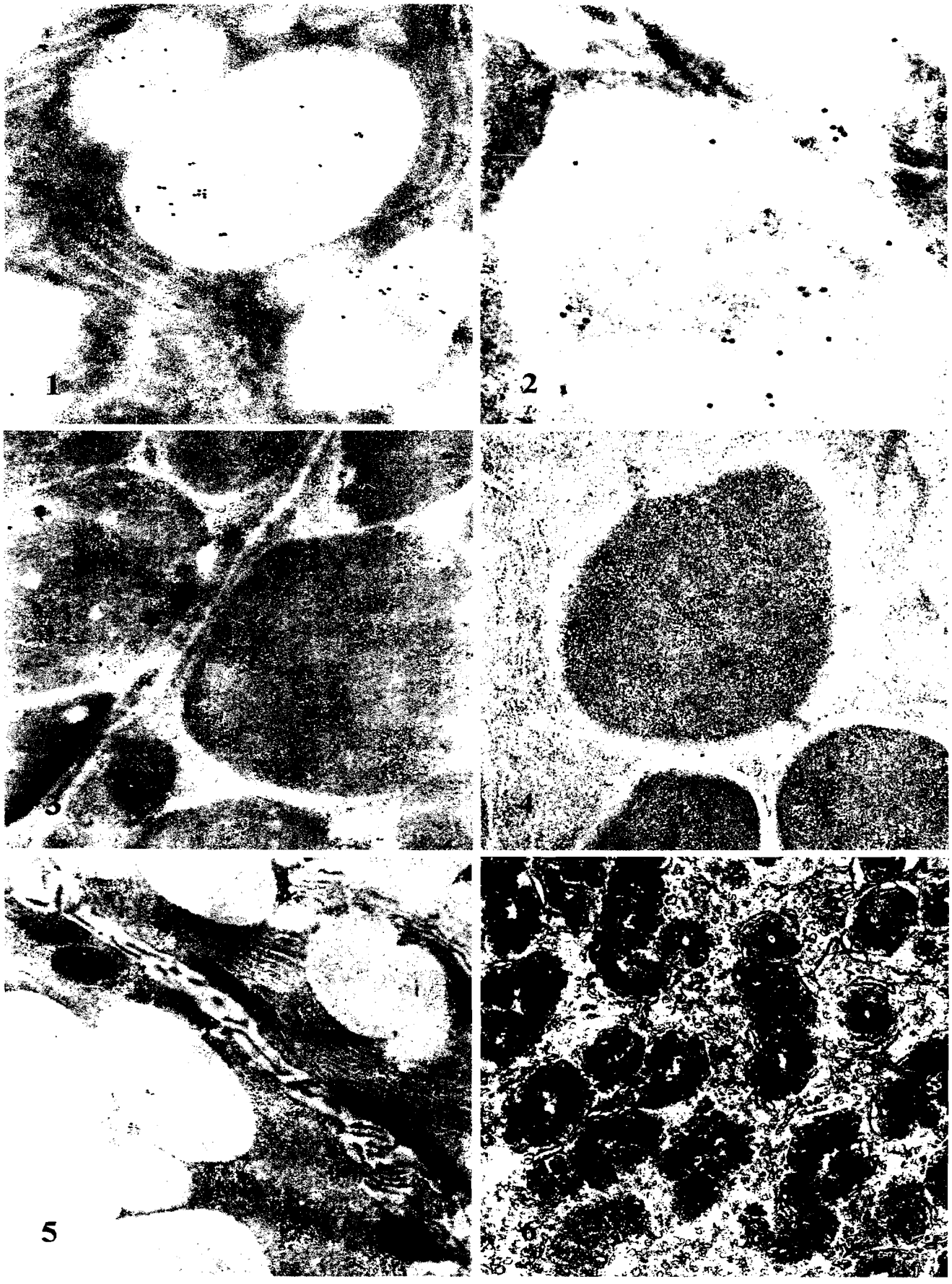
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Fig. 1. Male submandibular gland. Single labeling to evidence lysozyme. Pale secretory granules of acinar cells exhibit gold particles heterogeneously located in the matrix. x 48,800

Fig. 2. Female submandibular gland. Single labeling to evidence lysozyme. The gold signals are mainly located on the weakly electron-dense region of the pale secretory granules of acinar cells. x 54,400

Fig. 3. Male submandibular gland. Single labeling to evidence lysozyme. The dark granules of convoluted granular tubules present gold signals. x 23,800

Fig. 4. Female submandibular gland. Single labeling to evidence lysozyme. In females the dark granules show less intense immunostaining when compared with males. x 35,600

Fig. 5. Female submandibular gland. Control section showing findings from preadsorbtion test. Note the lack of immunostaining at acinar cell level. x 17,800

Fig. 6. Male submandibular gland. Paraffin section. IGSS to evidence lysozyme. Note the abundance of immunopositive sites in the convoluted granular tubules and the moderate binding of acinar cells. x 225

IGSS sequence confirmed these data (Fig. 12).

Double-sided binding for lysozyme and α-amylase

Submandibular gland

The double-sided binding revealed that both enzymes were located in the convoluted granular tubule cell secretory granules (Fig. 13) and confirmed that only lysozyme occurs at acinar cell level (Fig. 14) where gold signals were heterogeneously distributed within the granule matrix of acinar cells in females (Fig. 15). The reverse technique of binding gave similar results and confirmed the validity of the method (Fig. 16).

Parotid gland

The double labeling indicated that both enzymes were located in the bizonal, target, and mottled granules; in particular, within the mottled granules, α-amylase reactivity was more intense and randomly distributed over the matrix, while lysozyme was almost completely confined to the electron-dense zones (Fig. 17).

Sublingual gland

There was perfect overlapping of results between single and double labeling techniques that substantiated the cytoplasmic binding of only lysozyme at acinar cells (Fig. 18) and confirmed the co-presence of both enzymes within the demilunar cell dark granules (Fig. 18-inset).

Controls

Control experiments supported the specificity of the antibodies tested.

In addition, it was ascertained that both Formvar and carbon films are able to prevent contamination of reagents between the opposite faces of the uncoated grid.

Discussion

In this study we examined the subcellular distribution of lysozyme in the mouse major salivary glands and attempted to investigate the intragranular compartmentation of this secretion enzyme and its relationship with α-amylase.

Lysozyme synthesis pathway and intracellular distribution are still not well understood in salivary glands and their localization in the mouse is still object of study, probably owing to the difficult preservation of the salivary secretion products. In particular, Korsrud and Brandtzaeg (1982) noticed the influence of the fixative mixture in the staining activity for lysozyme.

We have previously described a double-sided method of labeling and experimented a methodological approach in order to prevent reaction interferences between steps carried out on the grid face A and face B (Menghi et al., 1997). In this study we tested both

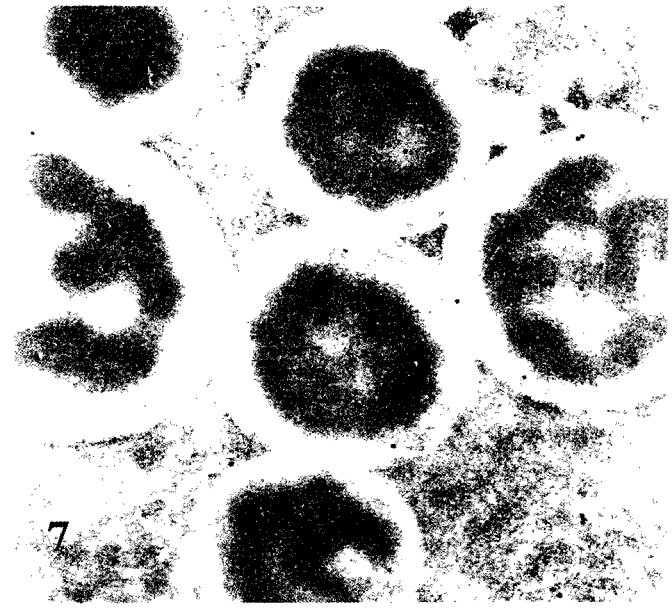
Figs. 7 and 8. Parotid gland. Single labeling to evidence lysozyme. Note the occurrence of immunostaining almost exclusively located on the electron-dense zones of mottled granules. x 35,600

Fig. 9. Parotid gland. Paraffin section. IGSS to evidence lysozyme confirmed the faint staining observed at electron microscopy level. x 225

Fig. 10. Sublingual gland. Lysozyme immunostaining. Only a few gold particles are located on the electron lucent secretory granules, whereas numerous gold signals are found in the cytoplasm of acinar cells. x 17,800

Fig. 11. Sublingual gland. Lysozyme immunostaining. The electron dense granules of demilunar cells contain numerous reactive sites. x 24,000

Fig. 12. Sublingual gland. Paraffin section. IGSS technique to evidence lysozyme at light microscopy level confirmed the main reaction at demilunar cells. x 430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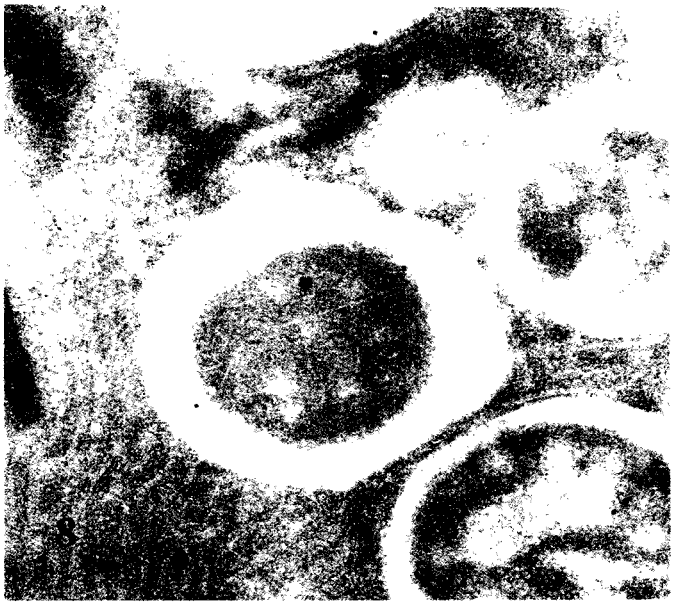
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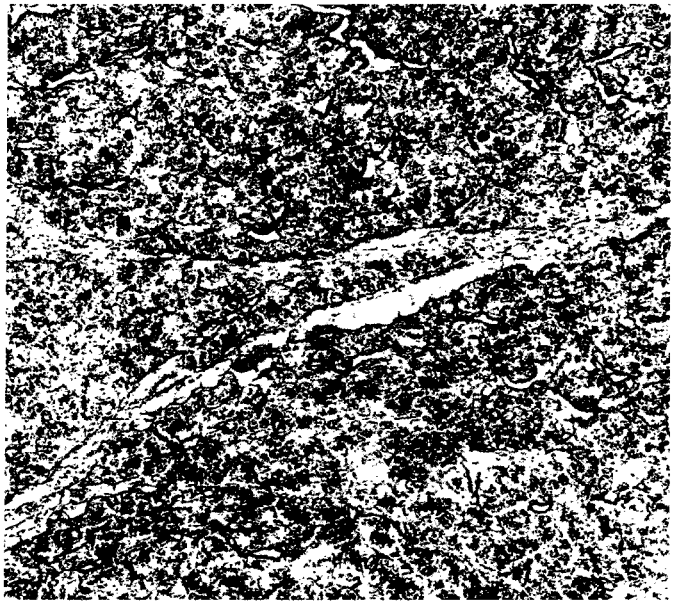

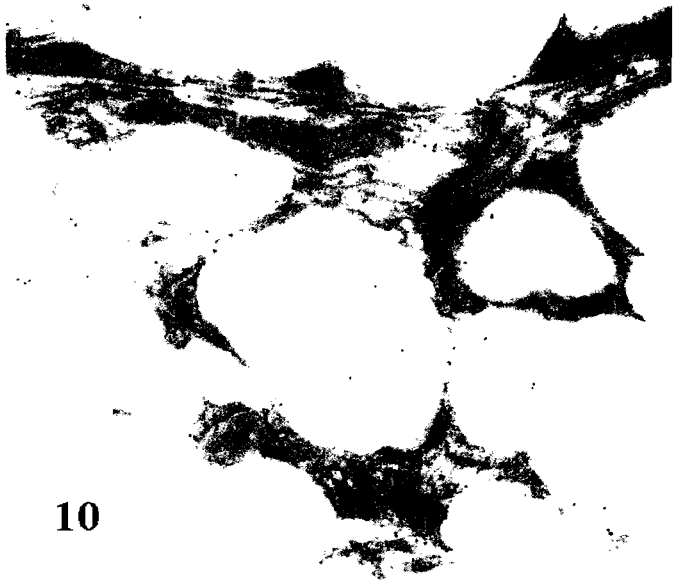
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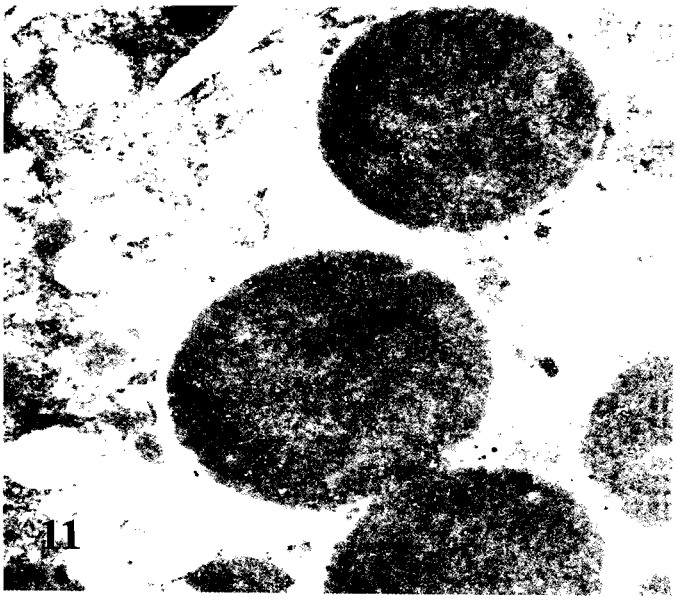
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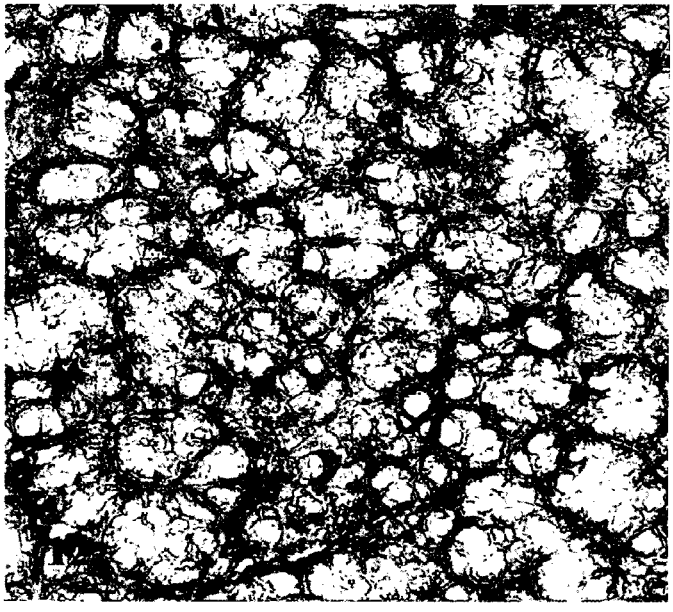

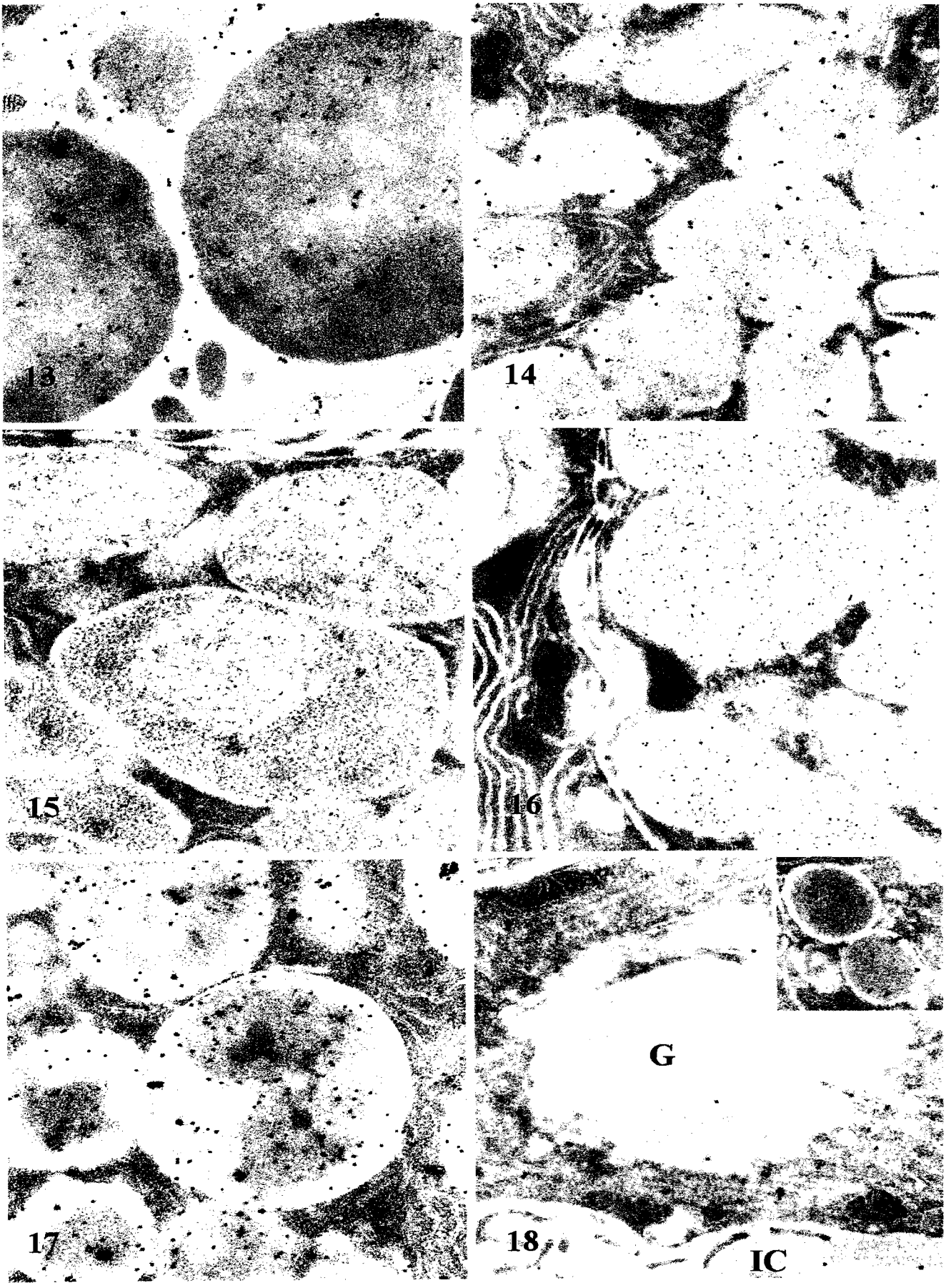
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Fig. 13. Male mouse submandibular gland. Double-sided labeling of lysozyme (large particles) and α-amylase (small particles). Note that co-presence of secretory enzymes occurs at dark granules of convoluted granular tubules. x 23,800

Fig. 14. Male mouse submandibular gland. Double-sided labeling of lysozyme (large particles) and α-amylase (small particles). Acinar cell. Small particles reflecting the location of α-amylase are not found at this level. x 23,800

Fig. 15. Female mouse submandibular gland. Double-sided labeling of α-amylase (large particles) and lysozyme (small particles) illustrates that only lysozyme is present in the acinar cells. Note the lack of reaction in the more electron-lucent zones of secretory granules. x 38,000

Fig. 16. Male mouse submandibular gland. Double-sided labeling. The reverse reaction, with respect to Fig. 14, with lysozyme (small particles) and α-amylase (large particles) confirms that the first labeling step and the Formvar film did not appreciably influence the labeling. Also the lack of α-amylase in the acinar cells is confirmed. x 23,800

Fig. 17. Parotid gland. Double-sided binding of lysozyme (large particles) and α-amylase (small particles) evidences the strong staining of α-amylase in contrast to the modest staining of lysozyme chiefly sited in the electron-dense regions of mottled granules. x 35,600

Fig. 18. Sublingual gland. Double-sided staining of lysozyme (large particles) and α-amylase (small particles). Acinar cell. Note the only occurrence of lysozyme in the marginal region of merged electron-lucent granules (G); gold particles are present in the rough endoplasmic reticulum as well as in the intercellular canaliculi (IC). Inset: Note the occurrence of lysozyme and α-amylase at demilunar cell secretory granules. x 23,800

carbon and Formvar films, after the enhancement of the first immunogold labeling, and found comparable results although here we used the same secondary antibody, differently from previous researches (Menghi et al., 1999).

Lysozyme has been chiefly studied in the human salivary glands but Korsrud and Brandtzaeg (1982) have hypothesized that the presence of lysozyme in the striated duct cells (Kraus and Mestecky, 1971) and in the intralobular duct cells (Klockars and Reitamo, 1975) of human parotid gland could be attributed to false-positive reaction. Other authors (Keinanen et al., 1989; Konttinen et al., 1990) explained the possible location of lysozyme in those tracts as a result of reabsorption from the saliva or filtration from the blood or surrounding tissues. Indeed, by in situ hybridization techniques, they found lysozyme mRNA only in acinar serous cells.

Lee and co-workers (1990) have described the presence of lysozyme in developing human salivary glands. They never found muramidase from 23 to 40 weeks of gestation in striated and excretory ducts of human major salivary glands. These authors have suggested a possible role of lysozyme and other enzymes during the development of human major salivary glands, linked to protection and defence mechanisms, particularly in serous cells and ductal segments. A protective role of the non-specific defence factor lysozyme at the entrance of the acinar units was also supported by Staneva-Dobrovski and Zanger (1995). Klockars and Osserman (1974) have found absence of lysozyme reactivity in the rat parotid gland, whereas Staneva-Dobrovski (1997) has shown, by immunogold techniques, its localization in this gland.

Many authors maintain the importance of the fixative mixture, and the subsequent processing steps in lysozyme preservation for immunohistochemical (Klockars and Osserman, 1974; Reitamo, 1978) and immunogold studies (Machino et al., 1986; Staneva-Dobrovski, 1997). Reitamo (1978) described lysozyme as a stable low-molecular-weight molecule that is resistant to acid and heat and indicated many factors for lysozyme antigenicity preservation; the author also asserted that the optimal fixative solution for lysozyme staining was an aldehyde aqueous mixture with pH near to neutrality and that the best results could be obtained by fixing for 2 to 6 hours at 4 °C. Keeping this in mind, we fixed our tissue samples with respect to these parameters.

On the basis of actual data it clearly emerged that, among the mouse salivary glands, the digestive role is mainly supported by the parotid gland; in fact, α-amylase was abundantly present on the electron-lucent and electron-dense areas of mottled granules in addition to bizonal and target ones, while lysozyme occurred in a modest amount and was preferentially located on the electron-dense zones of mottled granules. Conversely, both the digestive and defensive roles seem to be played by the submandibular gland which produces α-amylase at convoluted granular tubule secretory granules and lysozyme at both convoluted granular tubules and acinar cells. The sublingual gland appeared to be mainly involved in the protective function owing to the secretion of a modest amount of α-amylase at demilunar cells and a more relevant amount of lysozyme at both acinar and demilunar cell level.

As far as the association of lysozyme with acidic (Imoto et al., 1972) or neutral (Shackleford and Klapper, 1962; Gad, 1969) mucosubstances, this cannot be clarified by actual data. Indeed, the present data show that in the submandibular gland the association of lysozyme with acidic as well as neutral glycoconjugates can occur. We found that only in the female submandibular gland lysozyme, confined to the lightly electron-dense regions of pale granules, was not associated with the sialoglycoconjugates which were previously visualized in the electron-lucent areas of the same granules (Menghi et al., 1998a). Also, findings from the sublingual gland seem to validate the hypothesis that lysozyme is not preferentially associated with acidic or neutral glycoconjugates previously

detected at electron and confocal microscopy level (Menghi et al., 1998b). With respect to the parotid gland, further studies are required to define the association of lysozyme with glycomolecules within target and bizonal granules, in which heterogeneous sialoglycoconjugates were located at dark regions of target granules and pale regions of bizonal ones (Menghi et al., 1996b).

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