ISSN- 0102-9010

ULTRASTRUCTURE OF THE RAT SUBLINGUAL GLAND DURING PERIOD OF HIGH PROLIFERATIVE ACTIVITY IN POSTNATAL DEVELOPMENT

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ABSTRACT

The postnatal development of the rat sublingual gland was studied using autoradiography and electron microscopy. The labeling indices of parenchymal and stromal cells were determined in autoradiographs from rats injected with ^{3}H-thymidine. The labeling index of parenchymal cells remained high from the second to the twentieth day after birth, but declined significantly thereafter. Ultrastructural analysis showed that during the period of high proliferative activity the secretory cells had an ultrastructure that was qualitatively similar to that of cells from the glands of old rats, but were still immature. Only after day 20 did these cells show large amounts of rough endoplasmic reticulum and secretory granules that reached the adult pattern 30 days after birth. Maturation of the myoepithelial cells occurred during the same period.

Key words: Development, sublingual gland, ultrastructure, proliferation, rat

INTRODUCTION

The rat sublingual gland consists of mucous tubuloacinar units, serous demilunes, intercalated ducts, striated ducts, excretory ducts and a main excretory duct [2,6,8,11]. This gland begins to develop in the embryo on day 14, alongside the mesenchymal capsule of the submandibular gland. Cytodifferentiation of the sublingual gland secretory cells is complete by the time of birth [10,12,14,21]. Although at this point all of the intraglandular structures are well defined, they are still morphologically immature [8]. During the first month of postnatal development, the gland undergoes marked exponential growth [18] with the secretory units and ducts showing clear maturation.

Analysis of total DNA content [1] and morphometric evaluation of the gland cell number [18] indicates that this growth occurs mainly through an increase in the number of cells as a result of mitotic activity. Assessment of the cellular incorporation of ^{3}H-thymidine [18], shows high values for most cell types during the first 15-20 days of postnatal life, with a subsequent marked decrease in labeling. Thus, an appreciable amount of the growth in the rat sublingual gland during postnatal development involves the proliferation of cells with complex cytoarchiteture, such as mucous cells, demilune cells and striated duct cells.

In this study, we examined the ultrastructure of the various cell types in postnatally developing rat sublingual gland during the period of high proliferative activity following labeling of the tissues with ^{3}H-thymidine.

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MATERIAL AND METHODS

Electron microscopy and autoradiography

Wistar albino rats (*Rattus norvegicus*) were used. The pregnant females and dams with litters were provided with water and pelleted Purina chow *ad libitum.* The litters remained with their mothers until post-partum day 20.

Groups of four rats of both sexes 2, 5, 10, 15, 20, 30 and 40 days old were injected intraperitoneally with a single dose (4 µCi/g body weight) of ^{3}H-thymidine (specific activity 20 Ci/mM, New England Nuclear, Boston, USA) and killed 1.5 h later by ether inhalation. The glands were always collected between 8:00 and 10:00 a.m. to avoid circadian variations in the S-phase of the cell cycle. The glands were cut into small fragments, fixed in cold 2% glutaraldehyde in 0.09 M phosphate buffer, pH 7.3 for 1 h and post-fixed in cold phosphate-buffered 1% osmium tetroxide containing 106 mg of sucrose/ml for 2 h; before staining *en bloc* with 0.5% uranyl acetate plus 106 mg of sucrose/ml for 18 h. Following dehydration in ethanol and propylene oxide, the tissues were embedded in Araldite resin. Thin sections obtained using a

Porter-Blum ultramicrotome were contrasted with 2% uranyl acetate and lead citrate and examined with a Zeiss EM9S-2 or Philips EM-301 electron microscope.

Eight araldite-embedded blocks from each animal were randomly selected for the autoradiographic study. Sections 0.5 µm thick were obtained from each block and placed on a glass slide. The sections were coated with Ilford K5 autoradiographic emulsion by the dipping technique. After an exposure time of 30 days, the autoradiographs were developed in Kodak D19B solution and stained with methylene blue-azure II.

For each animal, about 2000 transected nuclei of parenchymal and stromal cells were randomly counted and scored for labeling. The nuclei were counted with a Zeiss light microscope using an immersion oil objective (x100) and a Zeiss Kpl x8 eyepiece containing a Zeiss II integration grid.

Statistical analysis

The labeling index or proportion of parenchymal and stromal cells labeled during a given period, expressed as a percentage of the total number of each cell type counted was analyzed by one way analysis of variance (ANOVA) using version 1.0 of Sigma Stat software (Jadel Scientific) for Windows. Pairwise multiple comparisons were done using the Student-Newman-Keuls test. The labeling indeces were submitted to arcsin transformation before statistical analysis.

RESULTS

Autoradiographic results

As shown in Figure 1, the labeling indices of the parenchymal cells were relatively high from day 2 to day 20 of postnatal life, with a peak of 14.2 on day 5, after which there was a progressive decline up to day 40. The percentage of stromal cells that incorporated ^{3}H-thymidine declined from day 2 to day 15 and remained stable thereafter.

Morphological observations

In the early days of postnatal development, the sublingual glands already showed all of the epithelial structures of adult rats, i.e., mucous acini, serous demilunes, intercalated ducts, striated ducts and excretory ducts. Nevertheless, the glands were very small and immature but grew markedly during the first month of development. The results for the different structures are described separately below.

Mucous acinar and serous demilune cells

Between 2 and 5 days of age, the immature acini were small and exhibited centrally located mucous cells surrounded by serous demilune cells. The ultrastructure of both immature cell types was qualitatively similar to that of adult rats. The mucous cells had a flattened nucleus occupying the basal region of the cytoplasm and a well-organized cisternal rough endoplasmic reticulum (RER) arranged in parallel arrays and containing flocculent material (Figs. 2A,B). The Golgi complex was prominent and contained a significant number of smooth transport vesicles budding from the transitional ER (arrowheads in Figure 2B), as well as coated vesicles formed from membranes of the Golgi saccules. Electron-lucent granules with a delimiting membrane were observed in the trans surface of the Golgi saccules (Fig. 2B). The mucous secretory granules were large and occupied a large part of the supranuclear cytoplasm. These granules contained thread-like or flocculent material and, in some cases, a small electron-dense core (Figs. 2A,B). In contrast, the serous demilune cells contained a large amount of cisternal RER around the nucleus, small electron-dense secretory granules located close to the apical region and a Golgi complex that was less conspicuous than that of the mucous acinar cells (Figs. 2A and 3A).

From day 5 to day 20, the gland increased markedly in size, mainly through an increase in the number of acinar and demilune cells. At 20 days of age, the ultrastructure of the secretory cells was not morphologically different from that at earlier ages (compare Figures 2A and 6A), except for an increase in the area of the Golgi complex in serous demilune cells (Fig. 3B). Between 20 and 40 days of age, the mucous, but not serous cells increased markedly in volume.

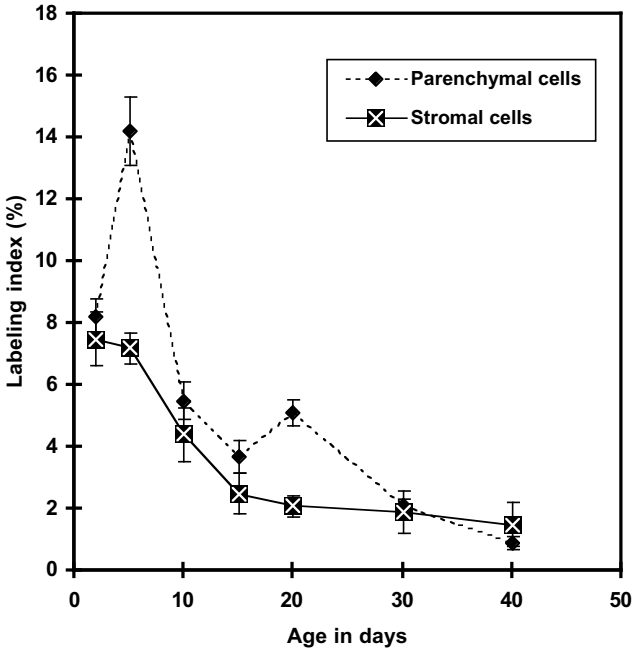

Figure 1. Labeling indices of the parenchymal and stromal cells of rat sublingual glands during postnatal development. Each point is the mean of four rats with the bars indicating the SEM.

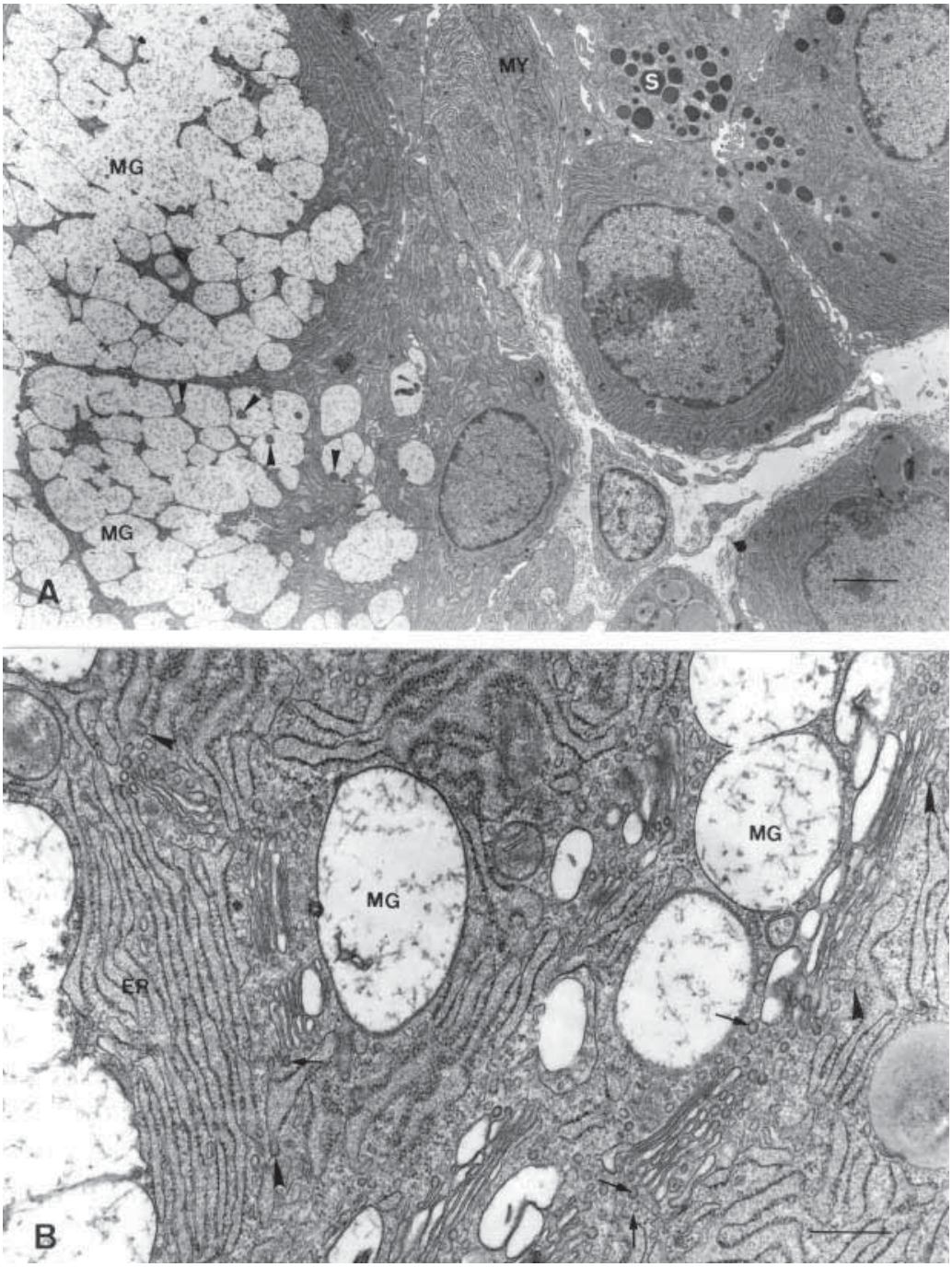

Figure 2. (A): Mucous acinar and serous demilune cells of a 2-day old rat. Note the mucous secretory granules (MG) with a small electron-dense core (arrowheads), the serous electron-dense secretory granules (S) and the myoepithelial cell prolongation (MY). Bar = 2 µm. **(B):** Golgi region of a mucous acinar cell of a 2-day-old rat. Note the rough endoplasmatic reticulum (ER), the smooth transitional microvesicles and transitional ER zone (arrowheads), the coated vesicles (arrows) and electron-lucent granules on the trans surface of the Golgi. Bar = 0.5 µm.

Intercalated duct cells

From day 2 to day 5 of postnatal development the intercalated ducts, already showed a definitive morphology. Throughout the period studied, i.e., from day 2 to day 20, the ducts were lined by low cuboid cells with a very simple ultrastructure (see Figure 4A).

Striated duct cells

During early development (2 to 5 days of age) the striated duct cells had already acquired the morphology of adult ductal cells.

Cells of the transition zone between intercalated and striated ducts

In the elongated transition zone between the intercalated and striated ducts seen from day 2 to day 15, the cells gradually acquired the morphological characteristics of striated duct cells, i.e., an increase in cell height, an accumulation of clear vesicles and a network of fine and intermediate filaments in the apical cytoplasm (compare the typical intercalated duct cells in Figure 4A with those from the transition zone in Figure 4B).

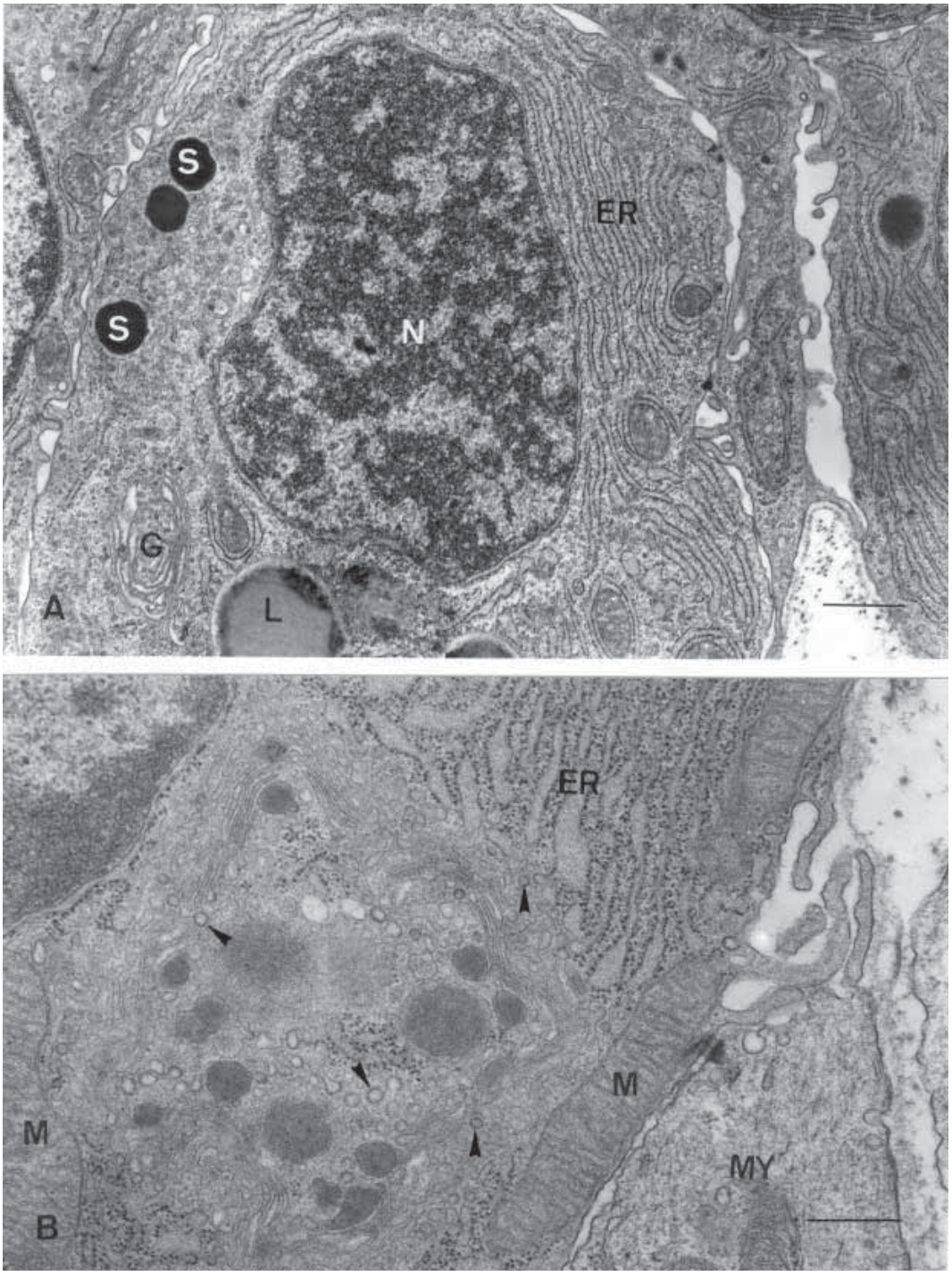

Figure 3. (A): Post-telophasic serous demilune cell of a 2-day-old rat. The cell structures shown include the nucleus (N), endoplasmic reticulum (ER), secretory granules (S), lipid dropets(L) and Golgi complex (G). Bar = 1 µm. **(B).** Serous demilune cell of a 15-day-old rat. Note the rough endoplasmatic reticulum, nucleus, mitochondria (M) and Golgi zone with coated vesicles (arrowheads) and condensing vacuoles. A myoepithelial cell (MY) also can be seen. Bar = 0.5 µm.

Myoepithelial cells

From day 2 to 5 of postnatal development, elongated myoepithelial cells were frequently seen around the acini and/or intercalated ducts. In contrast to abundance of myofilaments in cell prolongation, the perinuclear cytoplasm contained a well-developed RER consisting of flattened and/or dilated cisternae, a large amount of polyribosomes and a conspicuous Golgi complex always facing the acinar or intercalated duct cells (Fig. 5A). In the cell perikarion, the myofilaments formed a subcortical layer immediately below the cell plasmalemma.

From day 5 to day 20, there was a progressive accumulation of myofilaments in the myoepithelial cell prolongations and in the perinuclear cytoplasm (Figs. 5B and 6A), which then stabilized between the

20th and 40th day of postnatal life. The conspicuous cisternal RER seen in the perinuclear cytoplasm during early postnatal period decreased substantially with development.

Glandular stroma

At 2-5 days of age, the interparenchymatous connective tissue of the sublingual gland was greater than that of adult rats. From day 5 to day 20, there was a gradual decrease in the stromal spaces accompanied by intense collagen deposition in the intra- and interlobular connective tissue. Occasionally, an electron-dense band formed by non-collagen fibrils (with diameter of 11-13 nm) was observed in some developing glands close to the basement membrane of the excretory ducts (Fig. 6B).

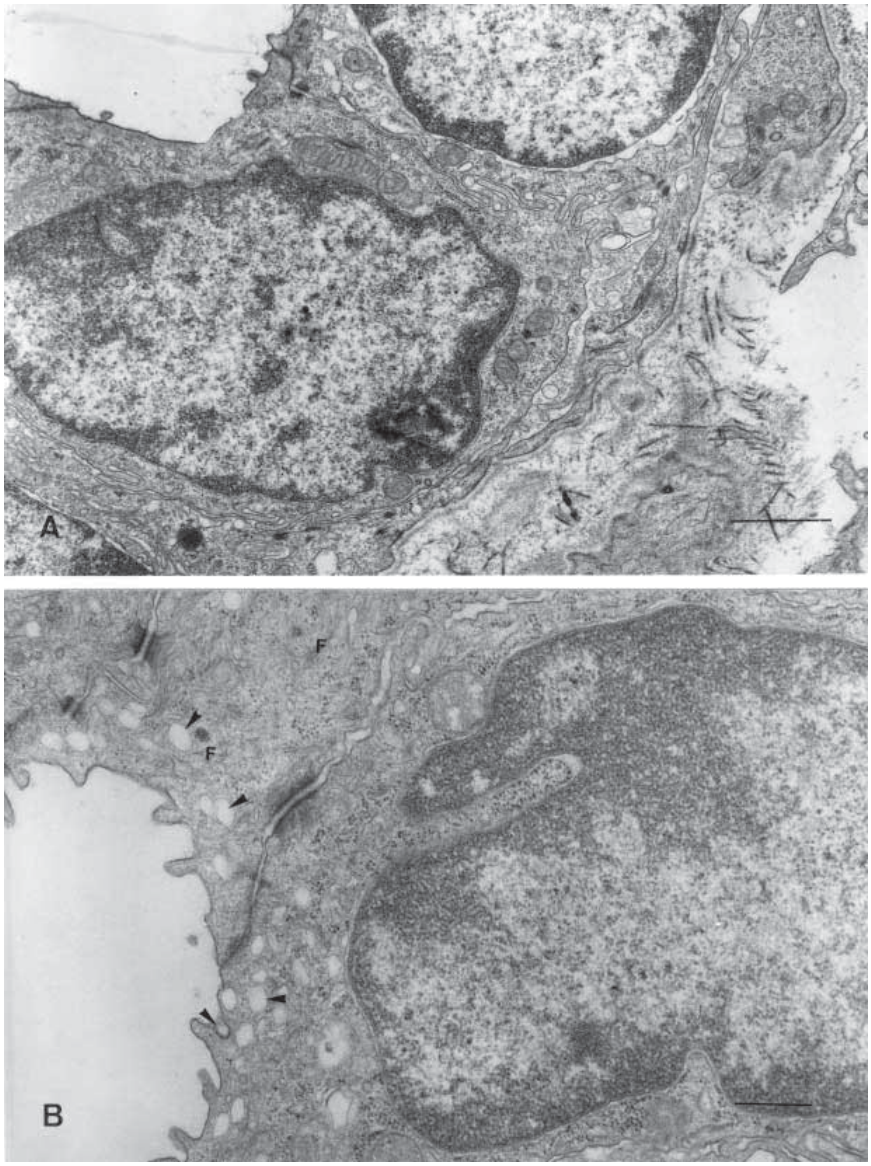

Figure 4. (A): Intercalated duct cells of a 15-day-old rat. Note the very simple cell morphology and the collagen fibrils in the connective tissue. Bar = 1 μm. **(B):** Cells of the transition zone between the intercalated and striated ducts showing apical vesicles (arrowheads) and the network of microfilaments (F). Bar = 0.5 μm.

DISCUSSION

Although the sublingual gland of neonatal rats in the first days after birth is structurally similar to that of adult rats, the cells are still morphologically immature [8,18].

However, during the first month of postnatal life, the sublingual gland increases in mass by more than 1000% [18]. This marked growth results from an increase in the volume of all of the morphological compartments of the gland, i.e., mucous acini and tubules, serous demilunes, intercalated ducts, striated ducts, excretory ducts and stroma [5].

Biochemical, morphometric and autoradiographic studies done in our laboratory have shown that an appreciable part of this gland growth is produced by intense mitotic activity of mainly mucous and serous secretory cells [1,6,18,19].

The autoradiographic results described here showed that the proliferation rate of parenchymal cells during postnatal development was higher from day 2 to day 20, but decreased significantly thereafter. The decline in mitotic activity coincided with weaning, when solid food was introduced in the diet of the developing rats. Girard *et al.* [4] suggested that the dietary changes associated with weaning causes important adaptative metabolic modifications in developing rats.

The electron microscopy results confirmed those of Leeson and Both [8] who reported that at the beginning of postnatal development the mucous and serous acinar cells were already ultrastructurally similar to those of glands from adult rats, as also observed by others [2,7,11,16].

A marked increase in the volume of mucous acinar cells was observed after day 20 and coincided with the decline in the proliferative activity. This gain in cell volume resulted from the accumulation of RER and Golgi complex membranes and secretory material. Two types of secretory granules were observed in immature mucous acinar cells from day 2 to day 20. One type contained flocculent or thread-like material characteristic of mucous secretions, while the other was similar to the first type but also contained a small electron-dense core.

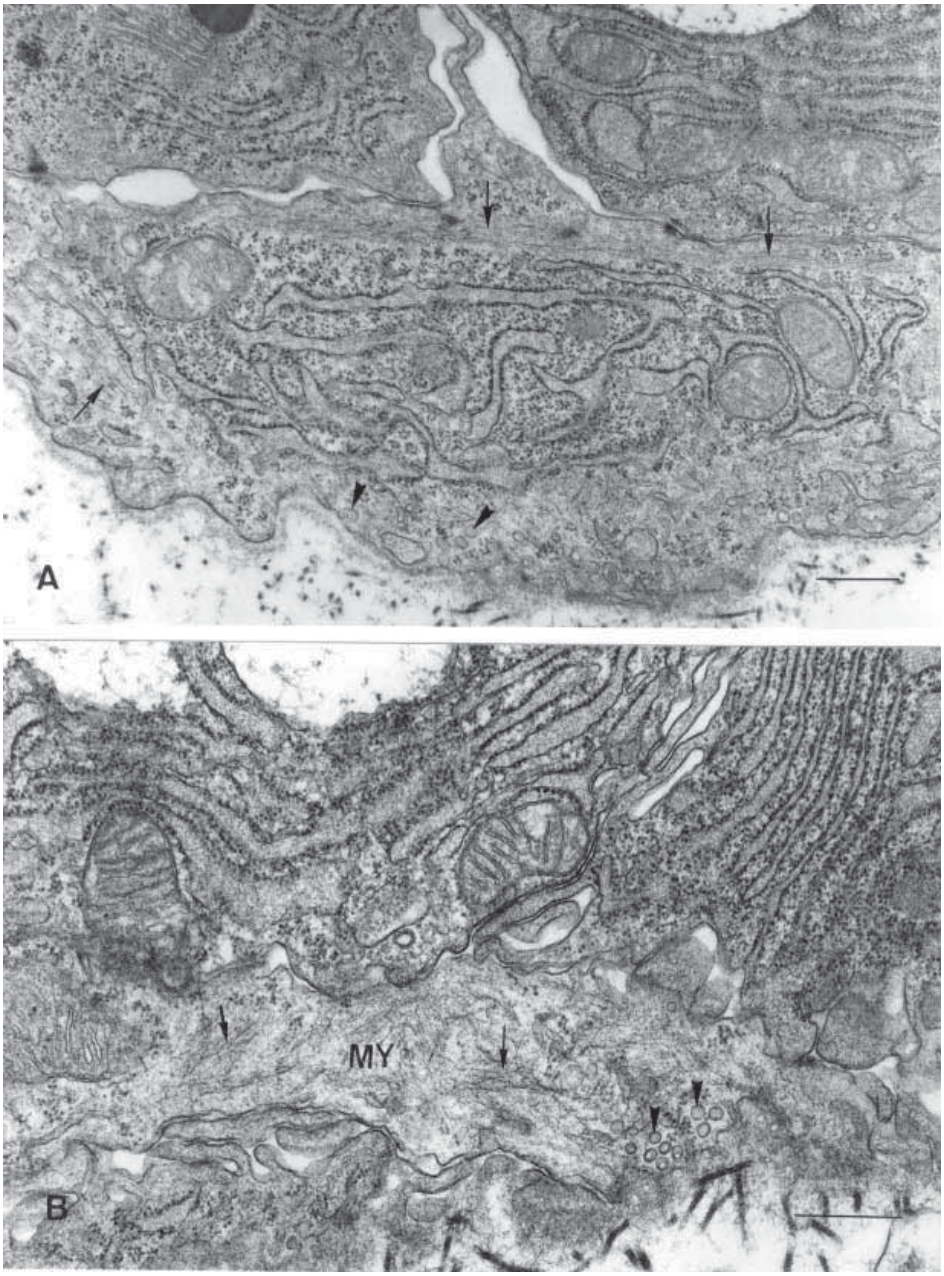

Figure 5. (A): Myoepithelium around mucous and serous acinar cells of a 5-day-old rat. Note the large number of polyribosomes and RER cisternae, the bundles of myofilaments (arrows) and micropinocytosis vesicles (arrowheads). Bar = 0.5 µm. **(B):** Myoepithelial cell prolongations (MY) of a 15-day-old rat. Note the bundles of myofilaments (arrows) and micropinocytotic vesicles (arrowheads). Bar = 0.5 µm.

Redman and Ball [12] reported three types of granules during differentiation of the secretory cells in the fetal rat sublingual gland. These included dense serous granules, empty-looking mucous granules and mucous-like mixed granules with a dense core of variable size. A more recent immunocytochemical study by Wolff *et al.* [21] using antibodies for various secretory proteins and for sublingual mucin showed that the cells containing serous granules and mucous granules were the precursors for adult serous demilune and mucous acinar cells, respectively. The mixed granules with a dense core showed anti-mucin antibody labeling and reacted with some anti-secretory protein antibodies, but never with antibodies protein markers of serous granules, suggesting that cells with mixed granules are actually developing mucous cells.

The ultrastructural results indicated that during early postnatal development some mucous acinar cells were still able to produce mucous-like secretory granules with dense core. This type of granule was also observed in transitory mucous acinar cells present in the mouse parotid gland in the early postnatal days [20].

Morphometric data obtained by Barbosa *et al*. [1] for developing rat sublingual glands showed no increase in the volume of serous demilune cells in the first month of postnatal life. However, our electron

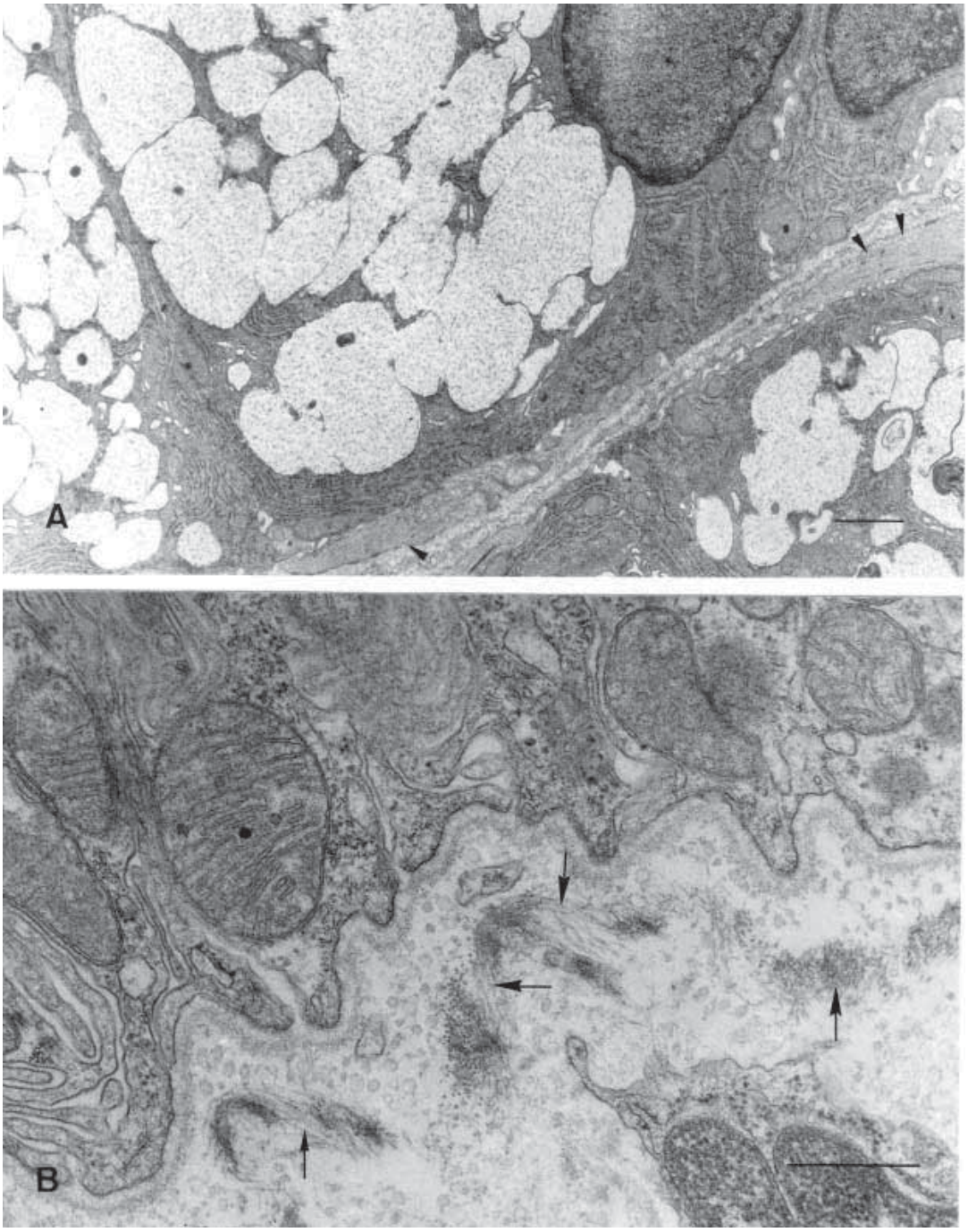

Figure 6. (A): Myoepithelial cell process (arrows) around mucous acinar cells in a 20 day-old-rat. Bar = 1 µm. **(B):** Basal region of excretory duct cells in a 15-day-old rat. Note in the connective tissue around the duct an electron-dense band formed by non-collagen fibrils (arrows). Bar = 0.5 µm.

microscopy results indicated that these cells underwent morphological improvement during postnatal development, with an accumulation of RER and Golgi complex membranes.

The presence of mitotic figures in the secretory cells from day 2 to day 20 confirmed that the main event responsible for the conspicuous gland growth seen in this period was mitotic activity and that the cell complexity was not a limiting factor for mitotic division.

The maturation of myoepithelial cells was also followed by electron microscopy. During early postnatal life, the perikaryon of immature myoepithelial cells showed a large amount of dilated RER cisternae containing delicate filamentous material associated with fine bundles of myofilaments

in the cytoplasm and a conspicuous Golgi zone. The observation that maturing cells in sublingual, submandibular and parotid glands have larger amount of swollen RER cisternae associated with the appearance of the myofilaments in the cytoplasm [3,13,15,17], suggests the rough-ER participation in myofilament protein synthesis.

The structure formed by non-collagen fibrils observed near the basement membrane of excretory ducts, may be pre-elastic fibers. This conclusion would agree with the findings of Lorber [9] who observed a large amount of elastic fibers at the same topographic location in the extralobular excretory ducts of adult rat submandibular glands.

The results described here show that during the period of high proliferative activity the acinar cells of the rat sublingual gland were already morphologically similar to the cells of adult rats, but were still immature. After day 20, the mitotic activity of parenchymal cells declined significantly coinciding with weaning, whereas the secretory cells, especially mucous cells, rose in volume as the result of an increase in the amount of RER and Golgi complex membranes and in the number of secretory granules in the cytoplasm. The latter also showed an increase in their capacity to synthesize and secrete mucous substance.

ACKNOWLEDGMENTS

The authors thank to Hélio Correa and Tânia Mary Cestari for technical assistance and Beonildes Teresinha Ruiz Correia for typing the manuscript. This work was supported by grants from FAPESP (84/1747-8 and 99/11597-9) and CNPq (98/11744-9).

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Received: May 9, 2002
Accepted: July 11, 2002