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Quantitation and localization of acinar cell-specific mucin in submandibular glands of mice during postnatal development

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Summary. In this study, antiserum to acinar cell-specific mucin was utilized to determine whether mucin could be detected in the mouse submandibular gland prior to cytodifferentiation of acinar cells. Results from radioimmunoassay indicated that mucin occurs in submandibular glands from newborn mice, i.e., before the appearance of mature acinar cells. Additionally, mucin quantitated in various stages of development was found to be antigenically identical to adult mucin. After sections of glands were treated with immunohistochemical reagents, we observed that the mature acinar cell-specific mucin was present in secretory terminal-tubule cells and in proacinar cells of newborn animals. The present findings suggest that in young animals, the proacinar cells are an immediate precursor of acinar cells and that the secretory terminal-tubule cells may represent an earlier stage in development of acinar cells. In adult female glands, mucin was also detected in the granular intercalating-duct cells. This latter observation is consistent with the hypothesis that these cells are an intermediate in the acinar cell replacement process.

Key words: Submandibular gland (mouse) – Development, ontogenetic – Acinar cells – Granular intercalating-duct cells – Mucin – Swiss-Webster mice

Submandibular glands of rodents undergo a protracted development, attaining full maturity at 3 to 4 months of age (Jacoby and Leeson 1959). Generally, postnatal development of the submandibular gland occurs more rapidly in the mouse than in the rat (Jacoby 1959). During development of the acinar cell in the rat, five secretory granule types have been identified; the mature acinar cell type appears between two and three weeks after birth (Cutler and Chaudhry 1974). Two secretory cell types co-exist in submandibular glands of the newborn mouse and rat: secretory terminal-tubule cells and proacinar cells (Yamashina and Barka 1972; Gresik and MacRae 1975). The granules of the secretory terminal-tubule cells, which are relatively small, stain intensely with toluidine blue (Srinivasan and Chang 1979). The proacinar cell granules generally stain less intensely with toluidine blue and are larger (Dvorak 1969; Yamashina and Barka 1973).

Some investigators postulate that the two cell types are on separate courses of development: the proacinar cells are the precursor to acinar cells and the terminal-tubule cells give rise to non-granular intercalated-duct cells (Yamashina and Barka 1973; Gresik and MacRae 1975). On the other hand, based upon histological similarities, Dvorak (1969) and Yohro (1970) suggest that proacinar cells represent an intermediate stage between the secretory terminal-tubule cells and acinar cells.

The presence of relatively high levels of peroxidase activity in the granules of proacinar cells has been used to distinguish this cell type cytochemically from secretory terminal-tubule cells, as well as from mature acinar cells (Strum 1971; Yamashina and Barka 1972). As development proceeds, the number of cells that contain peroxidase-positive granules is reduced, while the number of mature acinar cells increases. Whereas secretory granules of the mature acinar cells do not stain cytochemically for peroxidase (Strum 1971), they do contain low levels of peroxidase associated with the nuclear envelope and rough endoplasmic reticulum (Strum and Karnovsky 1970; Yamashina and Barka 1974). However, the absence of detectable peroxidase activity in secretory granules of developing acinar cells weakens biochemical support for the hypothesis that proacinar cells, which contain peroxidase-positive granules, are the precursors of mature acinar cells (Strum 1971).

A sialomucin has been localized by immunocytochemistry in the acinar cells of the submandibular gland of adult mice (Denny and Denny 1982; Vreugdenhil et al. 1982). The antiserum to this mucin exhibits no detectable cross-reactivity with the granular convoluted tubules of the submandibular gland or with other murine salivary glands (Denny and Denny 1982, 1984). Utilizing this antiserum against submandibular acinar cell-specific mucin, we sought to determine if mucin was present in the gland prior to the developmental appearance of mature acinar cells and if so, if it is present in granules of both proacinar and secretory terminal-tubule cells. The present study correlates biochemical data with cytochemical observations.

Materials and methods

Materials

Swiss-Webster mice from Simonson Labs (Gilroy, California) were housed under controlled conditions of lighting and temperature and maintained on an ad libitum diet of Purina Lab Chow and water until sacrifice by chloroform

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anesthesia. Postnatal animals were classified as newborn on the day of birth. Only female animals were chosen for ages 9 days and older.

Electrophoretically pure mouse-submandibular sialomucin was prepared from females, 80–90 days old (Denny et al. 1980). Anti-mucin sera were raised in female New Zealand white-rabbits by intradermal injections of purified mucin (Denny and Denny 1982).

Assays

Submandibular glands were dissected and homogenized in 0.01 M Tris (pH 7.4 at 25° C), 0.25 M NaCl. After a portion of the homogenate was removed for DNA assays and protein assays, 0.1 volumes of 10% NP-40 (Bethesda Research Labs, Gaithersburg, Maryland) were added. After further homogenization of the mixture, the homogenate was centrifuged 1 h at $50000 \times g$. The pellet was rinsed with 0.15 volumes of buffer and recentrifuged for 30 min. The combined supernatants were dialyzed overnight against buffer without NP-40. Radioimmunoassays (RIA) were performed for quantitation of mucin in the homogenate (Denny and Denny 1984). Sialic acid was quantitated according to the method of Denny et al. (1983). The protein content of the homogenates was determined by the method of Hartree (1972) using bovine serum albumin as the standard, while DNA was quantitated fluorometrically by using the fluorochrome Hoechst 33258 (LaBarca and Paigen 1980).

Tissue processing

Submandibular glands were excised, rinsed in 0.1 M sodium cacodylate buffer for 30 s and rapidly minced into approximately 1-mm^3 pieces. Subsequently, the tissues were fixed by immersion in 4% formaldehyde (freshly prepared immediately before use) and 0.5% glutaraldehyde, buffered to pH 7.2, in 0.1 M sodium cacodylate at 4° C for 30 min. After fixation, the specimens were rinsed in 0.1 M sodium cacodylate overnight at 4° C (Ellinger and Pavelka 1985). All subsequent steps were performed at −25° C. Tissues were transferred directly into 70% ethanol for 30 min, after which they were infiltrated by transferring into 1 part LR-Gold (Polysciences, Warrington, Pennsylvania) and 1 part 70% EtOH for 45 min, and then into 2 parts LR-Gold and 1 part 70% EtOH for 45 min. Finally, the tissues were transferred into LR-Gold resin for 1 h followed by LR-Gold with the addition of U.V. initiator Benzil, 0.1% w/v for 1 h, and then transferred into fresh LR-Gold with 0.1% w/v Benzil overnight. The tissues were then embedded in a final resin portion (LR-Gold with 0.1% w/v Benzil), placed into gelatin capsules and polymerized under an ultraviolet lamp for 24 h. Blocks were cut at 1 μm using a Reichert-Jung Ultracut E ultramicrotome. Serial sections were transferred onto separate microscope slides precoated in a hot 1% gelatin solution, and air-dried. So that various assessments could be made on the same cell, four adjacent sections were used. The 1st section was stained with toluidine blue; the 2nd and 3rd were used for immunofluorescence; and the 4th was tested for peroxidase activity or stained with alcian blue.

Immunofluorescence

Adjacent sections were treated overnight with non-diluted normal goat serum (Cooper Biomedical, Inc., Malvern, Pennsylvania) in a moist chamber at 4° C. The normal goat serum was then removed by carefully blotting off the excess serum. The experimental section was then incubated with non-diluted rabbit anti-mouse submandibular gland mucin serum while the control was incubated with normal rabbit serum, within a moist chamber at 4° C for 2–3 days. The sections were washed twice for 20 min with phosphate-buffered saline (PBS), and then stained with fluorescein-conjugated goat $F(ab')_2$ fragment prepared against rabbit IgG $F(ab')_2$ (Cooper Biomedical, Malvern, Pennsylvania), diluted 1:20 in PBS, for 1 h in the dark. Following this incubation, the sections were twice washed in the dark with PBS, 20 min per wash, rinsed with distilled water, air-dried, and mounted in non-fluorescing medium (Aqua-Mount, Lerner Laboratories, New Haven, Connecticut) prior to observation.

Staining procedure

Sections to be stained with alcian blue were rinsed with 3% acetic acid for 3 min, incubated in 1% alcian blue in water for at least 4 h, dipped in running tap water, and placed in 3% acetic acid for 3 to 5 min. They were then rinsed in running tap water for 3 min, briefly in distilled water, and air-dried. Alcian blue-stained sections were counterstained with 0.25% eosin for 1–2 min. Finally, the sections were dehydrated in 95% EtOH twice, 100% EtOH twice, cleared in terpineol-xylene for 5 s, then into xylene, air-dried, and mounted in Permount under glass coverslips. Other sections were stained with 0.5% toluidine blue in 0.1M phosphate buffer (pH 7.4) for 2–5 min, rinsed with distilled water and air-dried. Sections tested for peroxidase activity were reacted with 3,3′-diaminobenzidine (0.05%) with 0.002% H_2O_2, and 0.05 M Tris buffer, pH 7.6, at room temperature in the dark for at least 1 h (Yamashina and Barka 1972). Controls for peroxidase activity were incubated without H_2O_2.

Morphometry

Sections from 9- and 25-day-old mice were stained with toluidine blue, and 20 fields from each age were photographed and reproduced at 1600×. The acini in each photomicrograph were circumscribed, and all included nuclei were tabulated. The cross-sectional areas of the acini were determined using a Jandel digitizing tablet which was connected to an IBM PC-XT utilizing Sigma Scan software (Jandel Scientific, Sausalito, California). Resolution was ≥ 0.02 mm.

Results

Mucin quantitated by RIA in various age groups of mice (Table 1) was detected in newborn animals, although on a per gland basis, the amount was very low relative to other ages. Mucin content per gland for the 20-day group was the highest for the age groups tested. When concentrations were compared in terms of total DNA or protein, mucin was still lowest in the newborn group and highest in the 20-day animals. The concentration of mucin per cell increased 10-fold from newborn to 20 days. Concentrations of protein related to total DNA also showed an increase with increasing age; the largest gain occurring between the 10-day and 20-day groups. Table 2 indicates that there is a significant increase in the ratio of the combined surface

Table 1. Quantitation of mucin, DNA and protein in submandibular glands from mice of various age groups. All values are means ± standard errors of the mean of assay error

	Glands	Mucin (μg)/gland	Mucin/DNA (w/w)	Mucin/protein (w/w × 100)	Protein/DNA (w/w)
Newborn	16	2.34 ± 0.05	0.043	1.2%	3.7
10-day	24	46 ± 1	0.241	6.1%	4.0
20-day	10	227 ± 15	0.430	6.5%	6.6
30-day	6	83 ± 3	0.177	2.4%	7.6
Adult	4	173 ± 2	0.267	3.3%	8.0

Table 2. Comparison of cross-sectional area of acinar cell to nucleus ratio in submandibular glands from 9-day and 25-day-old mice

	Mean (μm^2)*	SEM (μm^2)	cv
9-day	45.156	3.008	29.8%
25-day	82.617**	5.664	30.7%

* values have been reduced to actual size; ** $p = < 0.00005$

The cross sectional area of each field was determined three times. For every field, the SD of the 3 measurements was less than 2% of the mean. The significance of the difference between the means of the 9-day and 25-day-old animals was tested by an ordinary *t*-statistic

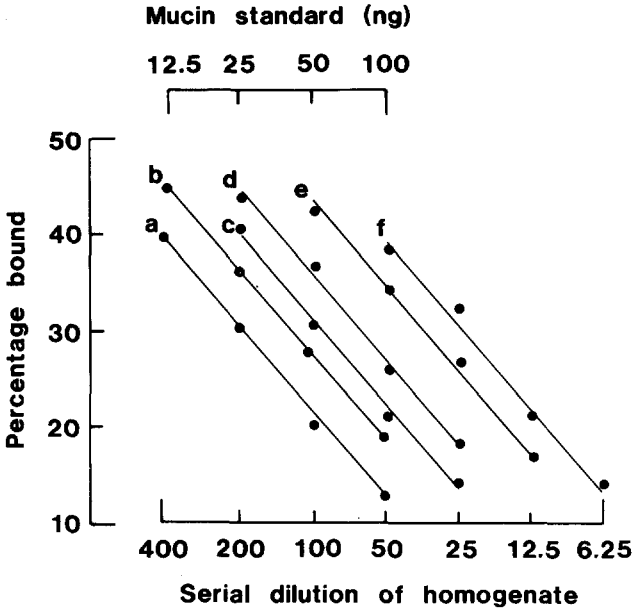

Fig. 1. Comparison by RIA of purified mucin with homogenates from submandibular glands from mice of different ages. *a* adult, *b* standard purified mucin, *c* 20-day, *d* 30-day, *e* 10-day, *f* newborn. *Abscissa* represents dilutions tested in the RIA for the homogenate from each age group; ordinate, data obtained from the RIA. Each homogenate was tested at 4 appropriate serial dilutions. Each serial dilution point represents the mean of quadruplicate assays. The standard error of the four values never exceeded ±3% of the mean. The *insert* indicates the quantity of mucin standard *b* used for the RIA

area of acinar cells to included nuclei, suggesting that acinar cells increase in size during this period.

It was important to determine if these mucins from various stages of development were antigenically similar. Production of parallel concentration curves by a standard and a sample is one means of demonstrating antigenic identity of reactants in a RIA system (Hunter 1978). Comparison of slopes produced by the homogenate dilution series in the RIA indicated that mucin present in all age-groups (newborn to adult) was antigenically identical to the mucin purified from adult females (Fig. 1).

A toluidine blue-stained section through a submandibular gland from a newborn mouse showed two types of secretory cells (Fig. 2a). The secretory terminal-tubule cells contained small, monochromatic granules that stained an intense magenta. These relatively uniform granules were loosely packed in some cells but tightly packed in others. The nuclei stained darkly with toluidine blue, were usually basal and often surrounded by a thin layer of intensely staining cytoplasm. The granules of secretory terminal-tubule cells stained only faintly with alcian blue (not shown). Occasionally, cells were observed among the secretory terminal-tubule cells which had granules that stained the intense color of terminal-tubule cell granules but that showed a range in sizes approaching those observed in adult acinar cells. The nuclei of the proacinar cells tended to be more compact and intensely stained than those of secretory terminal-tubule cells but were also basally located. The secretory granules were larger and stained only weakly with toluidine blue (Fig. 2a). These granules usually stained a light pink to light purple. Often, very little cytoplasm was observed to separate granules, giving a frothy appearance to the apical region of the cell. Some granules showed a segregation of pale blue material concentrated in the center, surrounded by material which stained pale pink. Alcian blue staining was strongly positive in the proacinar cell granules.

In sections of glands from 9-day animals (Fig. 2b) stained with toluidine blue, there appeared to be an overall decrease in the number of secretory terminal-tubule cells. Their appearance was similar to those of the newborn mouse. Cells with proacinar-type granules were rare. The majority of cells from 9-day animals now had the appearance of mature acinar cells except that the staining resembled that of secretory terminal-tubule cell granules and there was considerable variation in the intensity of granule staining.

Secretory terminal-tubule cells were very rare in 25-day animals (Fig. 2c). Granules in acinar cells appeared identical with those of adult acinar cells: relatively uniform in size and containing pale violet-staining flocculent material.

Four serial sections were employed to localize mucin in the newborn submandibular gland (Fig. 3). The toluidine blue-stained section portrays a tubule with lumen surrounded by secretory terminal-tubule cells (Fig. 3a). Characteristically, there was no evidence of peroxidase activity in the granules of these cells (Fig. 3d). Analysis of the four-section sequence indicated that the nuclei of three proacinar

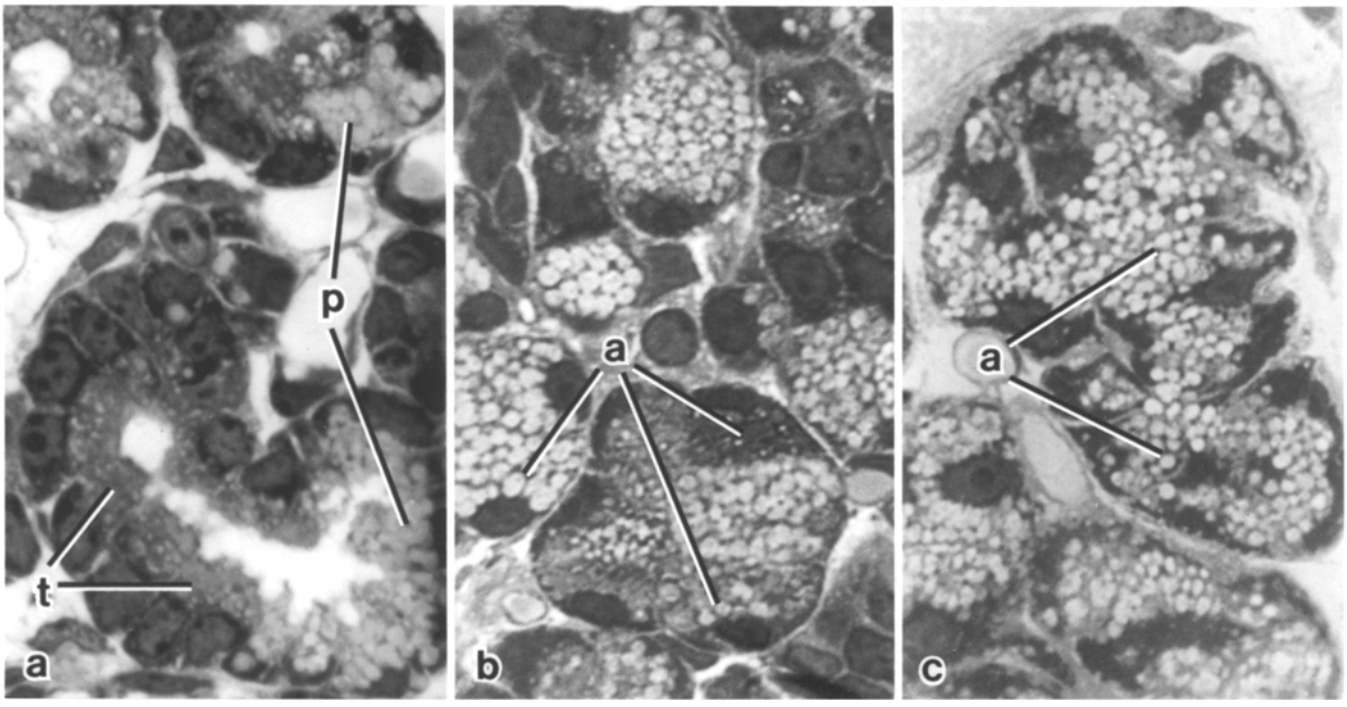

Fig. 2a–c. Comparison of sections (1 μm) of submandibular glands from three ages of mice. **a** Newborn, **b** 9-day-old, **c** 25-day-old. Acinar cells (*a*), proacinar cells (*p*) and secretory terminal-tubule cells (*t*). Toluidine blue. × 1600

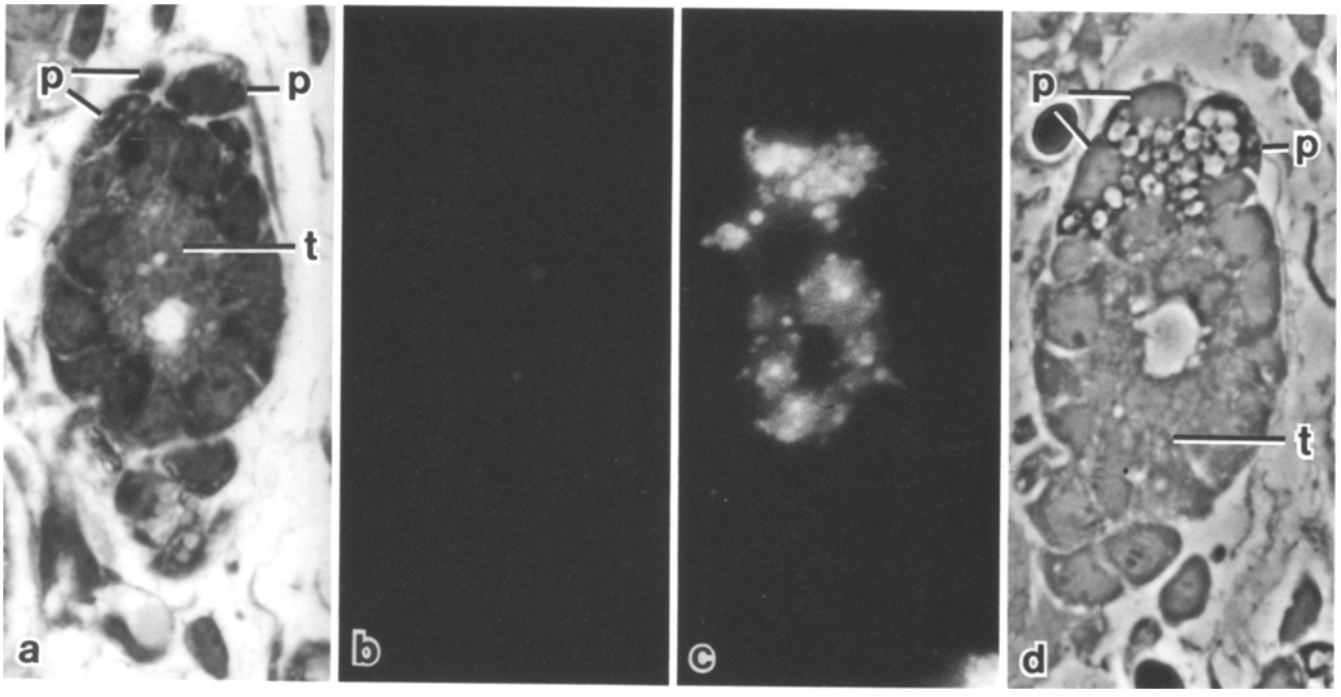

Fig. 3a–d. Adjacent sections (1 μm) through a submandibular gland of a newborn mouse. **a** Toluidine blue, **b** immunofluorescent normal serum control, **c** immunofluorescent experimental section reacted with rabbit anti-mucin serum, **d** diaminobenzidine reaction. Secretory terminal-tubule cell (*t*); proacinar cell (*p*). × 1600

cells (Fig. 3d) were present in the toluidine blue-stained section (Fig. 3a). Positive confirmation that these were proacinar cells was obtained by a strong reaction with diaminobenzidine (Fig. 3d), indicative of peroxidase activity in their secretory granules. The peroxidase control showed no reaction. The granules of both secretory cell types in the newborn animal were clearly fluorescent after reaction with rabbit anti-mouse mucin serum (Fig. 3c). The normal serum controls showed very little non-specific fluorescence (Fig. 3b). This indicates that the mature acinar cell-specific mucin was present in both secretory terminal-tubule and proacinar cells of the newborn mouse.

In sections stained with toluidine blue from submandibular glands of adult female mice (Fig. 4a), intercalating-

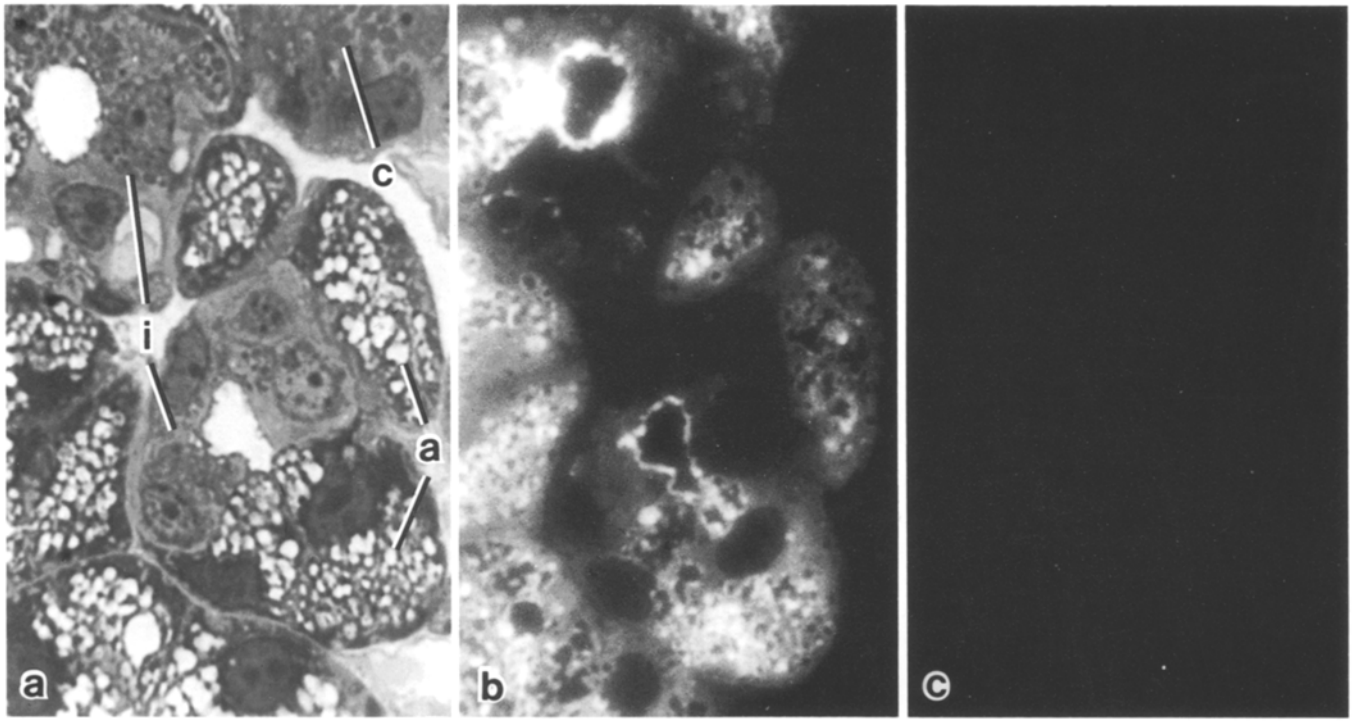

Fig. 4a–c. Adjacent sections (1 μm) from adult submandibular gland. **a** Toluidine blue, **b** immunofluorescent experimental section reacted with rabbit anti-mucin serum, **c** immunofluorescent normal serum control. Granular intercalating-duct cell (*i*); acinar cell (*a*); granular convoluted-tubule cell (*c*). × 1600

duct cells with granules similar in appearance to those of secretory terminal-tubule cells were occasionally noted in the region of the intercalating duct nearest the acinus. Other than the size and staining characteristics of the granules however, these so-called granular intercalating-duct cells show little resemblance to the secretory terminal-tubule cells of the newborn. Granules of acinar cells were much larger, with light-to-medium amounts of violet-staining flocculent material. These showed intense fluorescence when reacted with antiserum against mucin. The granular intercalating-duct cells also showed fluorescent staining of granules, but at a much lower intensity than the acinar cells (Fig. 4b). The granular convoluted-tubule cells exhibited no fluorescence. The normal serum control (Fig. 4c) also showed no fluorescence. These observations indicate that mucin was present in both acinar cells and granular intercalating-duct cells.

Discussion

Results from immunofluorescence and RIA demonstrate that acinar cell mucin is present in the submandibular gland from birth to adulthood. Furthermore, the existence of two secretory cell types at the newborn stage, neither of which is identical to the mature acinar cell, is confirmed. Thus it may be concluded that mucin is produced prior to the final differentiation of acinar cells.

The quantity of mucin produced in newborn animals may more closely approach the value for adults than is indicated in Table 1. While the range in concentration for protein varies little in the adult submandibular gland, values for the concentration of mucin relative to total protein have been shown by Denny and Denny (1984) to vary as much as 12-fold. That study revealed a broad range in concentration of mucin in adult submandibular glands, mostly due to differences among individual animals. The concentration of mucin relative to total protein in adult submandibular glands was observed to vary from 0.4% (w/w × 100) to 4.9%. The value of 1.2% obtained in this study for the newborn group (Table 1), while on the low side of the study, falls within the range for adult concentrations. Those obtained for the 10- and 20-day groups, 6.1% and 6.5% respectively, are higher than any of the previously observed adult values. We interpret these to reflect the time prior to extensive proliferation of granular convoluted-tubule cells, when acinar cells (and thus their product, mucin) are the highest proportion of the cell population (Chang 1974). Menaker and Miller (1973) noted a substantial increase in the ratio of concentration of protein relative to DNA from day 21 through 26 of age in the rat submandibular gland. We see an increase of similar magnitude which begins at an earlier age in mice. The increase in this ratio, accompanied by an increase in mucin concentration, appears to be correlated with a change in the size of the acinar cells, and may reflect their developmental maturation.

As reported previously (Denny and Denny 1982) and confirmed in this study, mature acinar cells show a strong positive reaction with the antiserum against this mucin as visualized by immunofluorescence. Sections from newborn animals showed that mucin was also localized within secretory granules of both secretory terminal-tubule cells and proacinar cells. Based upon the histological similarity and the mucin presence in the secretory granules of proacinar cells, we concur with others (Yamashina and Barka 1973) that proacinar cells are likely to be the immediate precursor of acinar cells at this stage of development of the submandibular gland. However, based also on the presence of mucin in its secretory granule, we feel that the secretory termi-

nal-tubule cell may represent an earlier stage in a continuum of acinar cell development (Cutler and Chaudhry 1974), and not a separate cell line giving rise to the nongranular intercalating-duct cells (Yamashina and Barka 1972; Gresik and MacRae 1975).

Results from drug-stimulation studies have been used to argue against the view that secretory terminal-tubule cells are a part of the acinar cell development lineage. Treatment with isoproterenol shows that β-adrenergic stimulation causes exclusive degranulation of peroxidase-positive cells with no effect on granules of secretory terminal-tubule cells. From this observation, it was concluded that no developmental relationship exists between these two cell types since they are under different physiological control (Yamashina and Barka 1972). Consistent with this are studies by Ball and Redman (1984) which show that within individual acini from submandibular glands of 4-day-old rats, the two secretory cell types were separately regulated. These investigators reported that in vitro, exclusive degranulation of proacinar cells was obtained by β-adrenergic stimulation and degranulation of only secretory terminal-tubule cells by cholinergic stimuli. Cholinergic responsiveness is acquired earlier during rat submandibular gland development than is β-adrenergic responsiveness (Cutler et al. 1981; Bottaro and Cutler 1984). Thus, differential responsiveness does not exclude the possibility that both cell types may represent various stages in the same maturation process rather than multiple pathways of differentiation. Ball and Redman (1984) also detected secretion of a protein that was apparently common to both types of secretory cells from the 4-day-old rat submandibular gland. The presence of mucin in granules of secretory terminal-tubule cells and proacinar cells is consistent with this observation.

Several investigators have noted similarity between the secretory terminal-tubule cells of newborn mice and the granular intercalating-duct cells of adults (Caramia 1966) as well as in adult male and female rats (Yamashina and Barka 1972; Gresik and MacRae 1975). Due to the enhanced sensitivity of immunodetection in this study, mucin was detected in both acinar cells and granular intercalating-duct cells. A recent study indicates that turnover in the acinar cell population is continually replenished by the intercalating-duct cell population (Zajicek et al. 1985). The location of the granular intercalating-duct cells relative to acinar cells, and the presence of low levels of mucin in their granules suggest that the granular intercalating-duct cells may play a role as intermediates in the replacement of acinar cells. This role is somewhat analogous to that which we postulate for secretory terminal-tubule cells in the development of acinar cells.

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