

Cytokeratin polypeptides expression in different epithelial elements of human salivary glands

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Summary. Immunofluorescent labeling of human salivary glands was carried out with a battery of monoclonal antibodies reactive with specific cytokeratin polypeptides. All the epithelial elements of the glands were positively labelled by a broad-spectrum cytokeratin antibody (KG 8.13) and by antibody Ks 18.18, which reacts with cytokeratin No. 18 exclusively. Labelling of frozen sections with antibody KM 4.62, which is reactive with the 40 Kd (No. 19) cytokeratin, was confined to the ductal system and apparently absent from the acini. Antibody KA-1, reactive with polypeptides 4, 5 and 6 stained both the myoepithelial cells and the basal cells of the large ducts. Antibody KS 8.58, however, reacted with the basal cells exclusively. It is thus proposed that the combined use of the various monoclonal antibodies may provide a most useful probe in studies on epithelial cell diversity in normal salivary glands as well as in pathological disorders of that gland.

Key words: Salivary glands – Intermediate filaments – Cytokeratin – Immunofluorescence microscopy

Introduction

Studies of various structural and physiological aspects of salivary glands have raised much interest over the last years (for general reviews see Young and van Lennep 1978; Pinkstaff 1980). Histological examination of salivary glands at the light- and electron-microscopic levels have pointed to their structural variability, complexity and cellular diversity; the secretory unit of the glands contains

mucus or serous-secreting acinar cells, surrounded by myoepithelium. The conducting system consists of 3 types of ducts, including intercalated, striated, and excretory/ducts (i.e. Tamarin and Sreebny 1965; Young and van Lennep 1978). The first two consist of single-layered simple epithelium and are largely confined to the secretory lobules, while the latter may exhibit pseudostratified or stratified-cuboidal epithelium, and is present in the interlobular areas. Along the basement membrane of the large ducts distinct arrays of basal cells with electron-lucent cytoplasm may be detected (Tamarin and Sreebny 1965; Shackelford and Schneyer 1971).

The presence of multiple types of epithelia have raised interesting questions related to the histogenesis and cellular turnover in salivary glands; is there a single progenitor cell giving rise to all the various epithelia of the gland? Does the ductal epithelium develop into acinar epithelium during the formation of the gland or vice versa? These aspects became especially relevant for studies on the histogenetic origin of salivary gland tumours such as pleomorphic adenoma, acinic cell tumour etc. (Dardick et al. 1985; Thackray and Lucas 1974; Eversole 1971; Regezi and Batsakis 1977; Welsh and Meyer 1968).

The major approach employed here for the study of epithelial diversity in salivary glands was immunohistochemical labeling with antibodies specific for defined intermediate filament subunits; extensive studies over the last several years have established the cell-type restricted expression of intermediate filaments in cells and tissues (Reviews: Franke et al. 1978; Lazarides 1980; Osborn et al. 1982; Anderton 1981; Osborn and Weber 1982). It has also been shown that the most diversified family of intermediate filaments is the cytokeratin group. Thus, each type of epithelium contains a characteristic set of 2–10 cytokeratin polypeptides

out of a repertoire of 19 different polypeptides which are expressed in human tissues ranging in apparent molecular weights from 40 Kd to 69 Kd. (Schiller et al. 1982; Sun et al. 1979; Franke et al. 1981; Moll et al. 1982; Quinlan et al. 1986).

The differential expression of different intermediate filament subunits in cells and tissues has made antibodies to these cytoskeletal elements most useful tools for studies on histogenesis and differentiation. Moreover, antibodies specific for each of the 5 intermediate filament families as well as antibodies reactive with cytokeratin subsets or with individual polypeptides have been used successfully in determining the origin of various human tumours (Osborn et al. 1982; Miettinen et al. 1982; Bannasch et al. 1980; Battifora et al. 1980; Gabbiani et al. 1981; Ramaekers et al. 1981; Vogel and Gown 1984). Recently, the preparation of antibodies of the latter category (mostly monoclonal antibodies) has provided useful probes for studies on the histogenesis of normal and pathological epithelial tissues (Gigi et al. 1982; Huszar et al. 1986; Gigi-Leitner et al. 1986; Debus et al. 1982; Raemakers et al. 1983; Sun et al. 1986) including normal and diseased salivary glands (Caselitz et al. 1981a and b; Caselitz and Löning 1981; Krepler et al. 1982; Gusterson et al. 1982).

In the present study we employ a battery of anti intermediate filament antibodies including 5 different monoclonal antibodies specific for cytokeratin polypeptide subsets, for the study of salivary gland epithelia. The use of the various antibodies has revealed unique patterns of cytokeratin polypeptide expression in acinar cells, ductal cells, myoepithelia and basal cells of the large ducts. We discuss the significance of these results for the histogenesis of the gland and the possible use of the various antibodies for the characterization of salivary gland metaplasias and tumours.

Materials and Methods

Ten normal human submaxillary salivary glands were obtained as a part of the surgical treatment of drooling (Wilkie 1970), in children with congenital cerebral palsy. In these children the excessive salivation is caused by neuromuscular dysfunction of the swallowing mechanism. The histological report of the excised submandibular salivary glands was "normal salivary gland tissue". Five samples of parotid salivary glands were excised from superficial parotidectomy specimens that were removed for pleomorphic adenomas. The areas examined in this study consisted of the cuff of normal parotid tissue surrounding the tumour. In addition 9 cases of pleomorphic adenoma of the parotid gland and three cases of Warthins' tumor were studied, as well as three cases of chronic suppurative sialadenitis secondary to sialolithiasis with squamous metaplasia. The tissues were snap frozen in liquid nitrogen-cooled isopentane im-

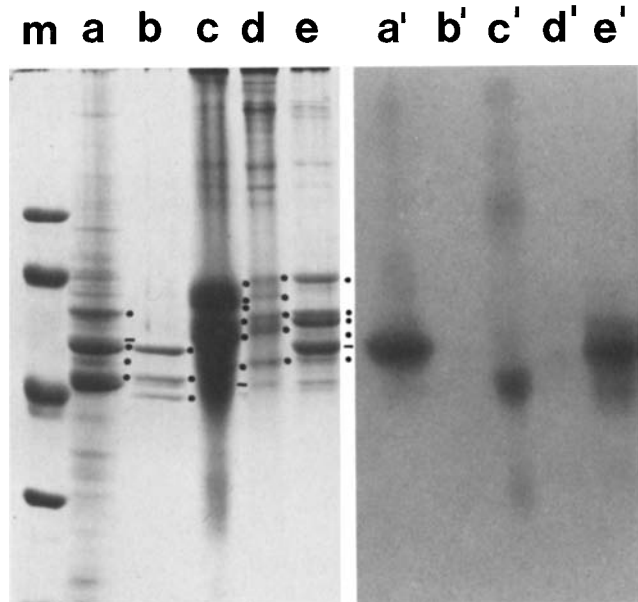


Fig. 1a-m. Characterization of monoclonal antibody KS 8.58 by immunoblotting analysis: **m, a-e**: Coomassie blue staining of one dimensional SDS polyacrylamide gel electrophoresis of molecular weight markers (**m**) and cytoskeletal preparations from various human cells and tissues. **a'-e'**: Immunoblotting analysis of a parallel gel with antibody KS 8.58. Bars on the right of each lane indicate cytokeratin polypeptide showing positive reaction with KS 8.58. Dots indicate cytokeratin polypeptides which do not react. The molecular weight markers include phosphorylase b (96 Kd) BSA (67 Kd) ovalbumin (43 Kd) Soybean Trypsin inhibitor (20 Kd). The arrowheads on the right side indicate the position of positively reacting polypeptides Nos. 13 and 16. The samples, applied to the gel include: slot **a** and **a'**: cultured human epidermoid carcinoma line. A-431 (containing cytokeratin polypeptides Nos. 5, 7/13, 8, 15, 17/18); slot **b** and **b'**: cultured mammary gland carcinoma line MCF 7 (containing cytokeratin polypeptides Nos. 8, 18, 19); slot **c** and **c'**: human foot-sole epidermis (containing cytokeratin polypeptides Nos. 1, 5/6, 9, 10/11, 14, 16); slot **d**: human epidermis (containing cytokeratin polypeptides Nos. 1, 2, 5, 10/11, 14/15); slot **e**: human exocervix (containing polypeptides Nos. 1, 4, 5, 6, 13, 14)

mediately after excision and stored at -80°C until use. Parallel samples were fixed in 4% buffered formaldehyde, dehydrated and embedded in paraffin according to standard histological procedures. Tissues taking for electron microscopic examination were fixed in buffered 2% glutaraldehyde, followed by 1% OsO_4 . Tissue blocks were dehydrated, embedded in Epon (Polybed 812, Polysciences, USA.) sectioned and examined in a Philips electron microscope model 410 at 80 KV.

For immunofluorescent labeling of salivary gland tissues we have used several antibody reagents as follows:

- (a) Monoclonal anti vimentin (Vim 13.2) which was prepared in our laboratory. This antibody reacted only with vimentin-containing intermediate filaments and with the vimentin band in immunoblots (not shown).
- (b) Broad spectrum monoclonal anti cytokeratin (KG 8.13) (Gigi et al. 1982) reactive with polypeptides Nos. 1, 5, 6, 7, 8, 10, 11 and 18, (following the nomenclature of Moll et al. 1981).
- (c) Monoclonal anti cytokeratin polypeptide No. 19 (KM 4.62) Gigi-Leitner and Geiger 1986).
- (d) Monoclonal anti cytokeratin No. 18 (Ks 18.18) (Czerono-

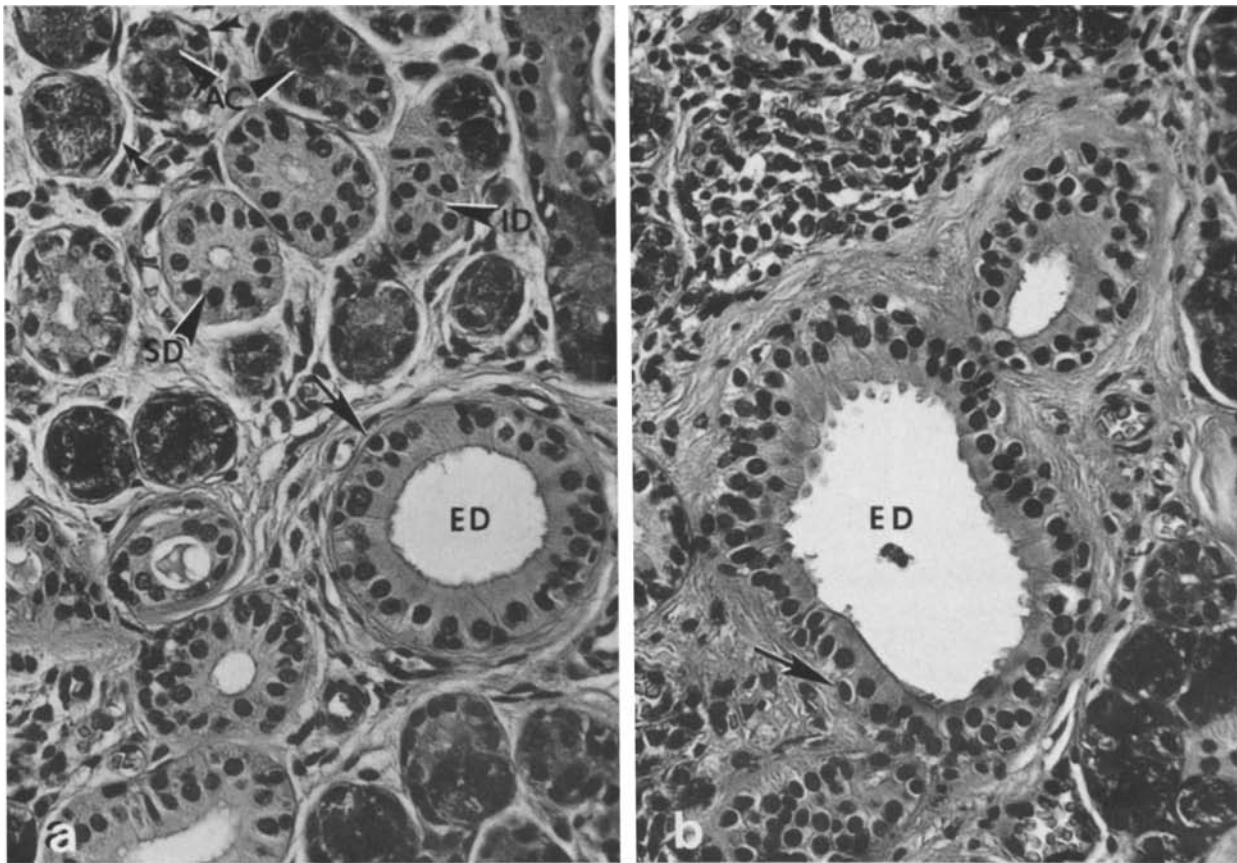


Fig. 2a, b. Haematoxylin-eosin-stained human submaxillary salivary glands. Notice the acini (AC) and the intercalated ducts (ID) which are connected to them. In addition striated ducts (SD) and excretory ducts (ED) may be identified. Notice the presence of basal cells along the basement membrane of the excretory ducts (arrows in **a** and **b**) and the presence of myoepithelial cells at the periphery of acini and small ducts (double arrowhead in **a**). Magnifications: $\times 620$

bilsky et al. 1985) kindly provided by W. Franke from the German Cancer Research Center in Heidelberg, FRG. (e) Monoclonal anti cytokeratins Nos. 4, 5 and 6 (KA-1) (Nagle et al. 1986) kindly provided by R. Nagle from the University of Arizona, Tucson, USA. (f) Monoclonal anti cytokeratins Nos. 13 and 16 (KS 8.58) which is used here for the first time and whose specificity will be shown below. (g) A secondary antibody namely affinity purified, goat anti mouse Fab' linked to lissamine-rhodamine sulfonyl chloride as previously described (Geiger and Singer 1979; Brandtzaeg 1973). (h) Rhodamine-conjugated phalloidin (Wulf et al. 1979, kindly supplied by H. Faulstich, from the Max Planck Inst., Heidelberg, FRG) was used for the localization of actin.

For immunohistochemistry frozen sections (4–5 μm thick) of unfixed tissues were cut in a Frigocut 2700 or 2800 cryostat (Reichert-Jung, FRG) and applied to clean glass slides. After drying for at least 2 h the sections were fixed in cold (-20°C) acetone and dried. Incubation with each antibody was carried out at room temperature for 45–60 min, with a 15 min rinse in phosphate buffered saline. Following the last wash, the stained sections were dehydrated, mounted in Entellan (Merck, Darmstadt, FRG) and examined with a Zeiss Photomicroscope III equipped with epifluorescent illuminator and filter sets for rhodamine and fluorescein fluorescence.

One dimensional gel electrophoresis was carried out on 8% polyacrylamide slab gels in the presence of SDS and mer-

captoethanol. Gels were either stained with Coomassie blue or subjected to electroblotting on nitrocellulose paper. Immunolabeling of the nitrocellulose blots was carried out largely according to Towbin et al. 1979.

Results

Most of the anti cytokeratin monoclonal antibodies used in this study have been previously characterized by us or by others as indicated in "Materials and methods". The antibody KS 8.58 is used here for the first time and its polypeptide specificity is demonstrated in Fig. 1. As shown, this antibody reacts with cytokeratin polypeptides Nos. 13 and 16 which are found in stratified, mostly non keratinizing epithelia (see Moll et al. 1982; Quinlan et al. 1986). Labeling patterns of a variety of human tissues with this antibody were largely similar to those obtained with antibody KS 8.12 (Huszar et al. 1986). However, KS 8.58 exhibited a more intense labeling of cells within salivary

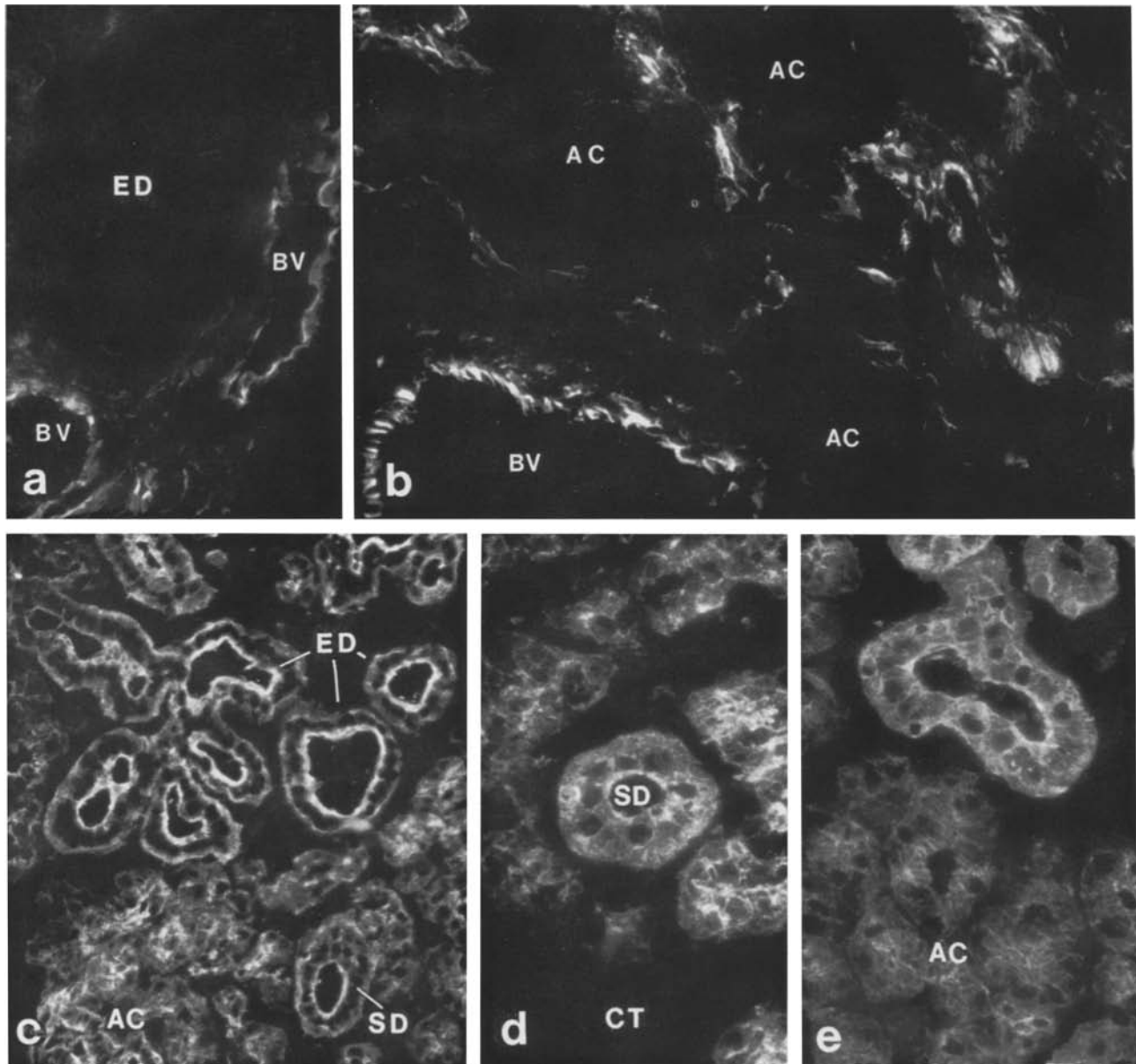


Fig. 3a-e. Immunofluorescent labeling of frozen sections of submaxillary salivary with anti vimentin (**a** and **b**) and with a broad spectrum anti cytokeratin, KG 8.13 (**c-e**). Vimentin was detected in fibroblasts of the inter- and intralobular connective tissues as well as in the walls of blood vessels (**BV**). All the epithelial elements of the tissue including excretory ducts (**ED**), and acini (**AC**) were vimentin negative. Anti cytokeratin labeled all epithelial elements in the acini and in all the striated (**SD**) and excretory ducts. Cellular elements within the connective tissue (**CT**) were not labeled. Magnifications $\times 390$

glands (see below), and was, therefore, selected for the present study.

The cellular elements of human salivary glands. Haematoxylin-eosin (HE) labelling of paraffin-embedded (or frozen) submaxillary or parotid salivary glands revealed the various constituent cell types. As shown in Fig. 2, the eosinophilic acini are mostly clustered into lobules separated by strands of connective tissue. At the periphery of

individual acini and intercalated ducts flat myoepithelial cells may be detected (double arrowhead in Fig. 2a). The conducting system consists of intercalated, striated, and excretory ducts. The former are in direct continuation with the acini and appear to be confined to the secretory lobules. Striated ducts are characteristically larger and predominantly present at the periphery of the lobules as well as in the interlobular areas. The excretory ducts are characterized by a relatively large lumen

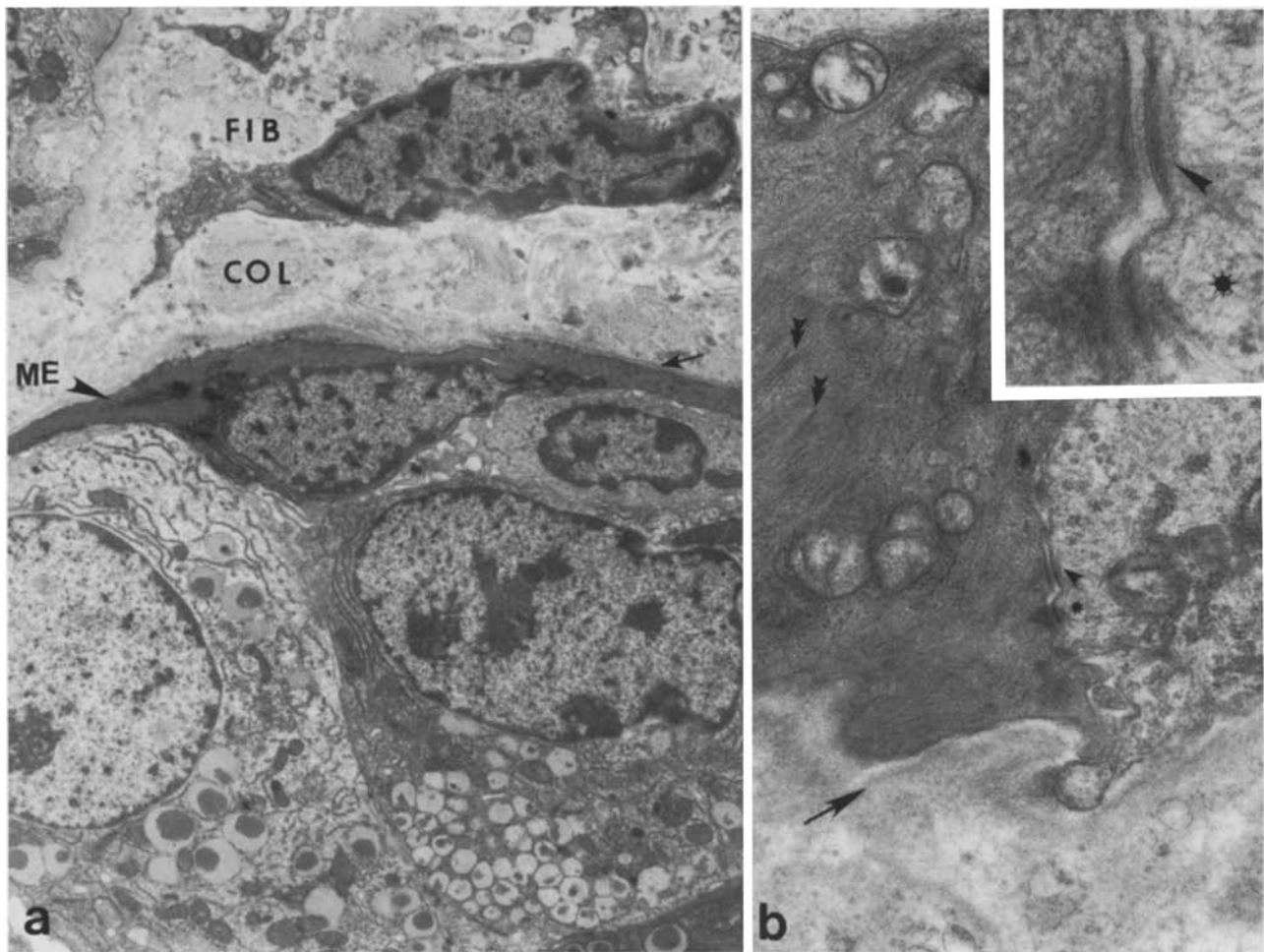


Fig. 4a, b. Transmission electron micrograph of human submaxillary salivary gland showing myoepithelial cells (*ME*) at a low (**a**) and high (**b**) magnifications. Notice that these cells are associated with the basement membrane (*arrows*) extending long processes surrounding the columnar cells. At a high magnification numerous fibers can be detected within these cells, often displaying dense body-like structures [double arrowhead in (**b**)]. Desmosomes are occasionally detected between myoepithelial cells and the columnar epithelium (see matched arrowheads and asterisks in (**b**) and in the insert). *Fib*-fibroblasts of the periacinar connective tissue; *Col*-Collagen. Magnifications: **a**: $\times 7740$; **b**: $\times 2660$ (*insert*: $\times 9720$)

and often by the presence of pseudostratified or stratified-cuboidal epithelium. Near the basement membrane of the excretory ducts small basal cells are often detected (arrows in Fig. 2a and b).

Immunofluorescent labeling with anti vimentin or with the broadly cross-reacting cytokeratin antibody (KG 8.13) have disclosed the mesenchymal and epithelial elements of the gland, respectively. As shown in Fig. 3 the immunolabeling with anti vimentin was confined to stromal fibroblasts and blood vessels and was apparently absent from the acini and ducts (Fig. 3a and b). All the epithelial elements, on the other hand, including the myoepithelium, acinar and ductal cells, reacted positively with the broadly cross reactive cytokeratin antibody (Fig. 3c-e).

Cytokeratin expression in myoepithelial cells. Myoepithelial cells are specifically associated with the secretory unit of the salivary gland and with the intercalated ducts (Young and van Lennep 1978; Cutler and Chaudhry 1973; Tamarin and Sreebny 1965). Though myoepithelial cells may be detected in HE-stained specimens (see Fig. 2) their fine details may be best visualized by electron microscopy (Fig. 4). They are located along the epithelial aspect of the basement membrane extending long processes around the secretory epithelial cells (Fig. 4a). At a higher magnification abundance of cytoplasmic filaments may be detected, occasionally showing dense-body-like structures (double arrowheads in Fig. 4b). Frequently, desmosomes are present between myoepithelial cells and the neigh-

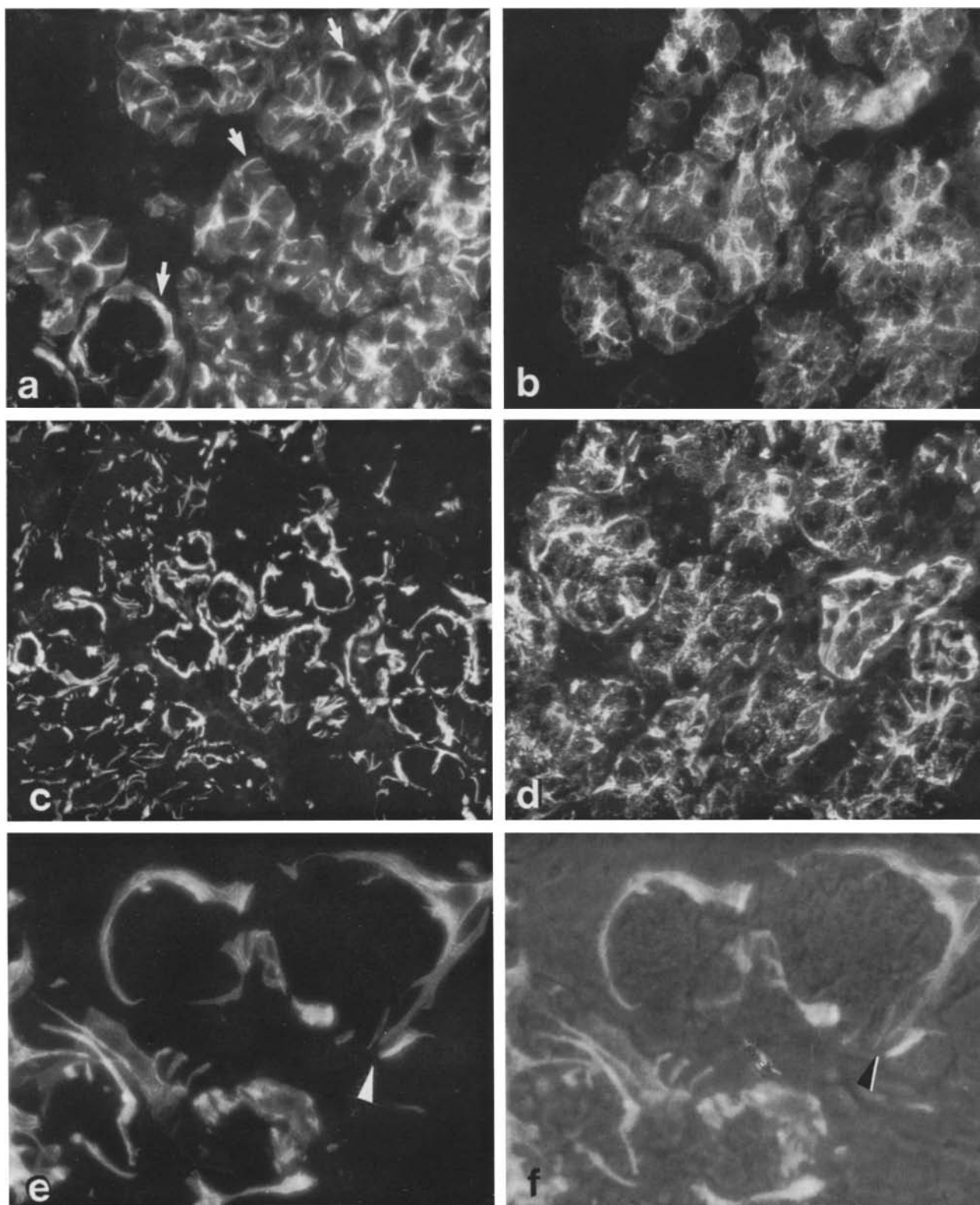


Fig. 5a-f. Immunofluorescent labeling of myoepithelial cells in submaxillary salivary gland. **a** Localization of actin using rhodamine conjugates phalloidine. The arrows point to myoepithelial cells at the periphery of acini; **b-f** Localization of different cytokeratin polypeptides using monoclonal anti cytokeratins as follows: **b** KG 8.13; **c** KA-1; **d** Ks 18.18; **e** Higher power magnification of cells stained with antibody KA-1, disclosing filamentous structures in the cytoplasm. The combined fluorescence – phase contrast photograph (**f**) points to the presence of the KA-1 positive cells predominantly at the periphery of acinar structure (arrowheads in **e** and **f** point to the same region). Magnifications: **a-d**: $\times 390$; **e-f**: $\times 980$

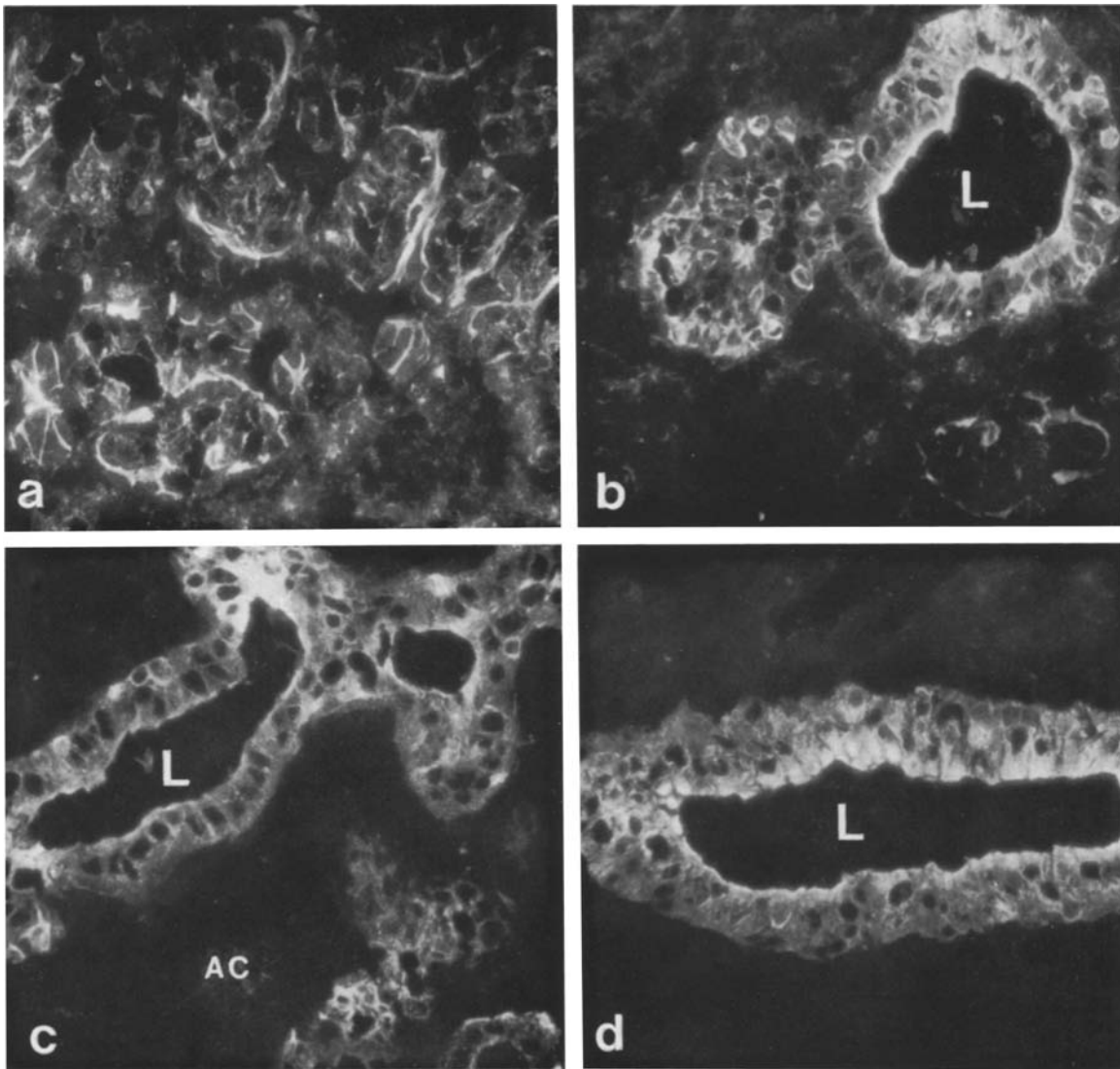


Fig. 6a–d. Immunofluorescent labeling of submaxillary salivary glands with anti cytokeratins Nos: Ks 18.18 (**a** and **b**) and KM 4.62 (**c** and **d**). Notice that antibody Ks 18.18 (specific for polypeptide No. 18) stains both acinar and ductal cells. While KM 4.62 (anti polypeptide No. 19) reacts with ducts only and is negative on the acini (**AC**). **L** lumen of large ducts. Magnifications: $\times 390$

boring columnar epithelium (arrowhead in the insert in Fig. 4b).

As previously documented myoepithelial cells contain large amounts of actin, myosin and several actin associated proteins as well as cytokeratin (Drenckhahn et al. 1977; Franke et al. 1980). This feature was used for the identification of the myoepithelium at the fluorescent microscope level. Incubation of frozen sections with rhodamine-labeled phalloidine for the localization of actin revealed extensive labeling of the myoepithelium as shown in Fig. 5a. Staining with the various cytokeratin antibodies revealed strong reactivity of the myoepithelium with antibodies KG 8.13 (Fig. 5b) and

Ks 18.18 (Fig. 5d) both of which also labeled the secretory acinar cells. Another antibody, namely KA-1 reacted with the myoepithelium specifically and exclusively without staining the neighboring secretory cells (Fig. 5c, e and f). It should be pointed out that monoclonal antibody KA-1 has been previously reported to react with myoepithelial cells in human mammary gland (Nagle and Jarasch 1986). No reactivity with myoepithelium was noted for antibodies KS 8.58 and KM 4.62 (not shown).

Immunofluorescent labeling of salivary gland with antibodies specific for polypeptides Nos. 19 (KM 4.62) and 18 (KS 18.18), both of which are

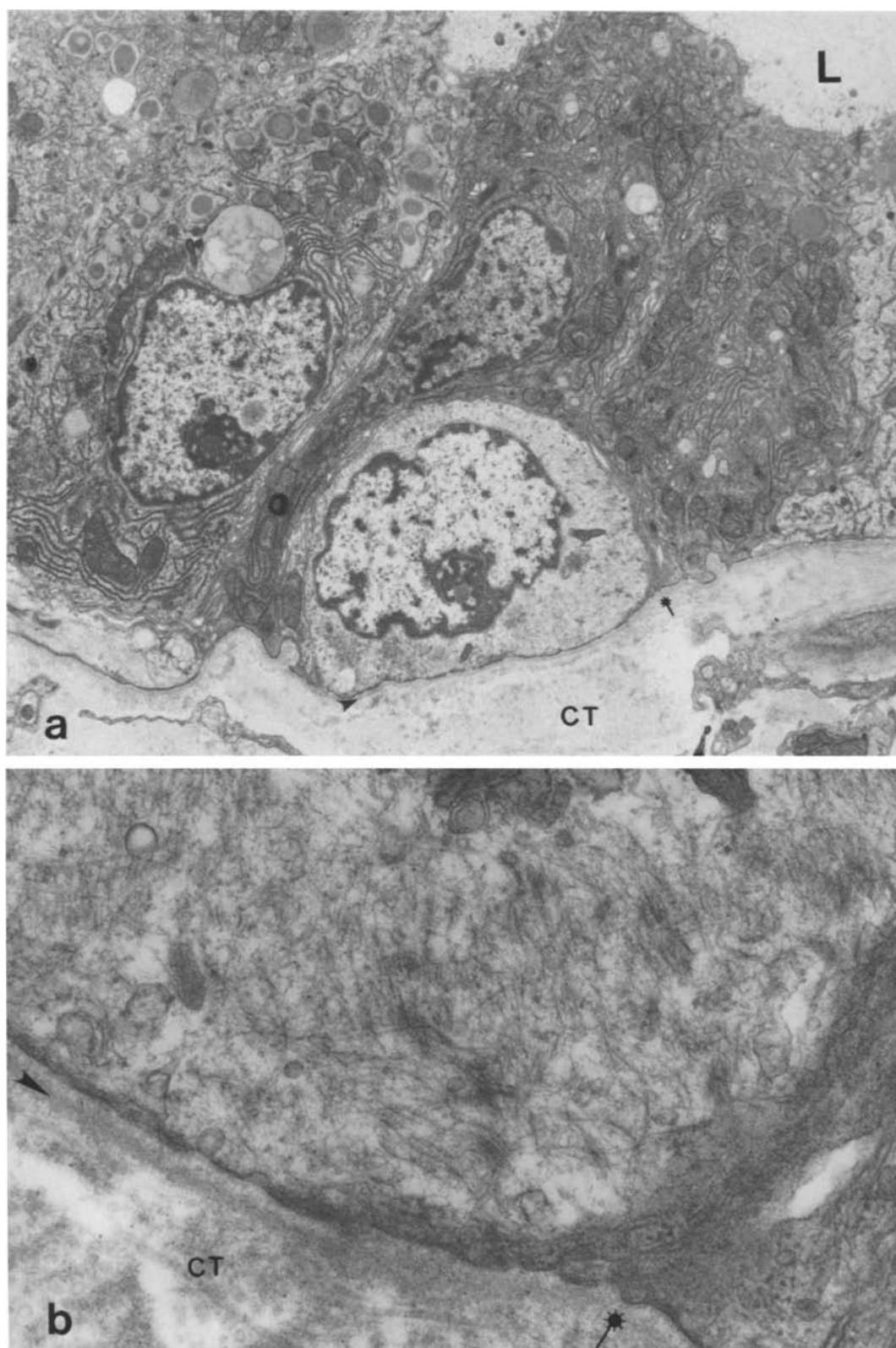


Fig. 7a, b. Electron micrographs showing basal cells of the excretory ducts at a low (**a**) and high (**b**) magnification. The basal cell is associated with the basement membrane (*arrowhead*) and does not reach the lumen of the duct. The cytoplasm is relatively electron lucent with many cytoskeletal filaments. The asterisks in **a** and **b** point to the same location. Magnifications: **a**: $\times 8640$, **b**: $\times 52,200$

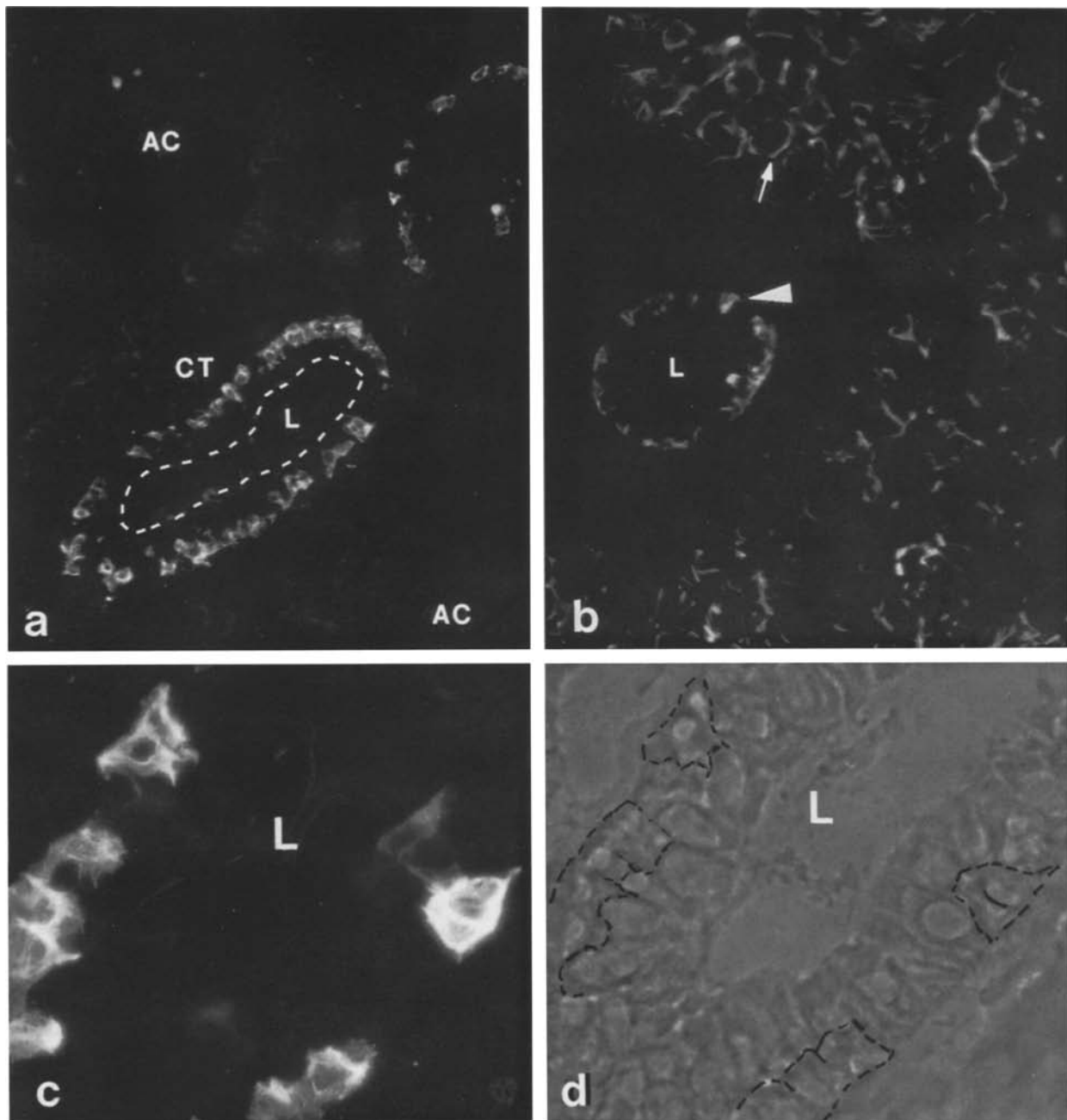


Fig. 8a–d. Immunofluorescent labeling of basal cells with antibodies Nos. KS 8.58 (**a**) and KA-1 (**b** and **c**). The phase contrast micrograph (**d**) is of the same field shown in **c** and the outlines of KS 8.58 – positive cells are marked with black ink on it. Notice that antibody KS 8.58 labels only the basal cells of the large ducts and is apparently negative on all other epithelial cells of the gland. Antibody KA-1 labels both the basal ductal cells and the myoepithelium (see also Fig. 5). AC, acini; CT, connective tissue; L, lumen. Magnifications: **a–b**: $\times 390$; **c–d**: $\times 980$

widely present in various simple epithelia, revealed differential labeling patterns by the two antibodies along the secretory and conducting units. Polypeptide No. 18 was apparently present in all epithelia of the gland, including the acini, ducts and myoepithelium (Fig. 6a and b). Polypeptide No. 19, on the other hand, was apparently confined to the

ductal system and absent from the acini (Fig. 6c and d). It was often difficult to determine unequivocally whether the intercalated ducts or parts of them were positively labeled, and attempts to resolve that problem are now in progress, using immunoperoxidase labeling.

An interesting population of cells found pre-

dominantly along the excretory ducts are the so called "basal" or "reserve" cells. Those cells are scattered along the basement membrane, as may be appreciated from examination of either HE stained sections (see Fig. 2a and b) or transmission electron micrographs (Fig. 7a and b). These cells display a distinctive electron-lucent cytoplasm and are normally not exposed to the lumen of the duct. At high-power magnification dense arrays of tonofilaments were detected in the cytoplasm of these cells. Examination of the staining patterns obtained with antibodies KG 8.13, Ks 18.18 and KM 4.62, (as shown in Figs. 3 and 6 above) indicated that all ductal cells, including the basal cells are positively labeled. Unique staining was obtained with antibody KS 8.58 (reactive with polypeptides Nos. 13 and 16) which selectively labeled only the basal cells (Fig. 8a) and did not react with any other cell type in the gland. Another antibody which labeled the basal cells and was negative on all other ductal cells was KA-1 antibody (Fig. 8b) which, as shown above, also reacted specifically with the myoepithelium. To delineate the specific labeling of the basal cells by the two antibodies we have prepared semi thin (2–3 μ m) frozen sections, labeled them and compared their staining patterns to the corresponding phase contrast images, using a high power ($\times 63/1.4$) objective. As shown in Fig. 8c and d the labelling was confined to the basal cells of the excretory duct.

Discussion

The present study was largely directed towards the characterization of the cellular diversity of salivary gland epithelia and the differentiation of their various cellular elements. Many studies carried out along that line in the past have focused on the fine ultrastructure of the gland as well as on the definition of a series of cell-type specific molecular markers which showed differential distribution. Among the markers used in such studies were various enzymes, mucins etc. which could be visualized both histochemically or immunohistochemically (for review see Pinkstaff 1980).

Another group of molecules which were recently localized in salivary glands includes different cytoskeletal proteins related to the microfilament and intermediate filament systems. Such studies have demonstrated the abundance of actin and myosin in the myoepithelium (Drenckhahn et al. 1977; Franke et al. 1981) as well as the extensive labeling of all epithelial elements of the gland (including the myoepithelium) with anti cytokeratin (Franke et al. 1980; Caselitz et al. 1981). However,

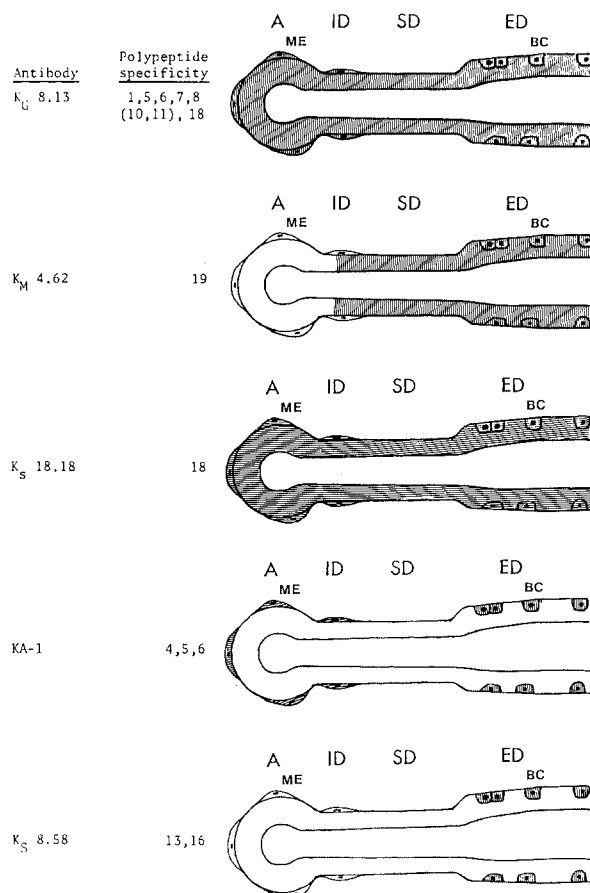


Fig. 9. Schematic summary of the patterns of immunolabeling of salivary glands with the 5 antibodies used here: Represented in this scheme are the acini (A) with the surrounding myoepithelium (ME), intercalated ducts (ID), striated ducts (SD) and excretory ducts (ED) with basal cells (BC). The dashed areas represent regions or cells which are positively labeled by the corresponding antibody

specific characterization of cell-type-restricted expression of individual cytokeratin polypeptides in the different epithelial elements of the gland has not yet been reported.

Such information may, in principle, be derived from either biochemical or immunohistochemical approaches. The former, which has been successfully applied for many epithelia (i.e. Moll et al. 1982), requires a fine microdissection of specific cells out of tissue sections, and the electrophoretic examination of their constituent cytoskeletons. In salivary glands, this approach is not readily applicable due to the close spatial proximity between the distinct cellular entities of the gland. For example, the tight association between the myoepithelium and the secretory acinar cells, or between the basal cells and the columnar or stratified epithelia of the large ducts, render it difficult, if not impossible to separate these cells from each other by mi-

crodissection. An alternative approach which we have used here was based on immunofluorescent labeling of the various glandular elements in situ with a battery of antibodies specific for cytokeratin subsets or for individual cytokeratins.

The polypeptide specificities and labeling profiles of the 5 monoclonal antibodies used in this study are summarized schematically in Fig. 9. The combined use of these antibodies allows us to distinguish between at least 4 different cellular entities according to the cytokeratins they contain. The myoepithelium for example, exhibits specific reactivity with antibody KA-1 but not with KS 8.58. This suggests that myoepithelia contain one or more of the basic polypeptides commonly found in stratified epithelia (Nos. 4–6) but do not express the acidic polypeptide Nos. 13 and 16 which are usually found in stratified non keratinizing epithelia. This feature may turn out to be most useful for the delineation of possible involvement of myoepithelial cells in salivary gland tumours and in particular in pleomorphic adenomas.

Another cell type which may now be specifically identified by its unique reactivity with antibody KS 8.58 (see Fig. 8) is the basal cell of the large (mostly the excretory) ducts. Being the only cell type in the entire gland which reacts with this cytokeratin antibody, we anticipate that hyperplastic or neoplastic derivatives of the basal cells may retain positive labelling with this antibody and may thus be identified. This feature may be especially relevant for salivary gland disorders which contain regions with “stratified” or “epidermoid” cells (Thackray and Lucas 1974; Dardick et al. 1983, 1984, 1985 and Caselitz et al. 1981) whose histogenetic origins have not been unequivocally defined yet. It is noteworthy that the basal cell is commonly considered to be the progenitor cell of the simple epithelia of the ductal system (Eversole 1971; Tamarin and Sreebny 1965; Shackelford and Schneyer 1971 and Young and van Lennep 1978). This implies that when the basal cells differentiate into a ductal columnar cells they stop producing cytokeratin polypeptides Nos. 13 and/or 16. In addition, the differentiating basal cells may coordinately stop expressing the basic “partners” of polypeptide 13 and 16, (Sun et al. 1986) namely cytokeratins nos. 4–6.

Another finding reported here is the selective labelling of different regions along the simple epithelia of the gland with antibody KM 4.62. This antibody, which reacts with polypeptide No. 19 did not stain any acinar cell but was reactive with all the elements of the ductal system (including basal cells). This antibody may thus be used as

a selective marker for metaplastic or neoplastic processes originating in ductal cells (see also Gigi-Leitner et al. 1986).

In conclusion, the combined use of polypeptide-specific cytokeratin antibodies described here provides a new set of cellular markers which may help shed light on the normal development of salivary glands as well as on the histogenesis of salivary gland tumours.

Acknowledgements. We would like to acknowledge with gratitude the excellent help of Mrs. H. Sabanai with the electron microscopy. We would also like to thank R. Nagle and W. Franke for sending us monoclonal antibodies (KA-1 and Ks 18.18) which were used in this study.

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