# Cytokeratin polypeptide expression during the histogenesis of guinea pig submandibular salivary gland

GABRIEL MARSHAK[1,2], ORITH LEITNER[2] and BENJAMIN GEIGER[2]

[1]*Department of Otolaryngology, Kaplan Hospital, Rehovot, Israel, affiliated with the Hebrew University and Hadassah Medical School, Jerusalem*
[2]*Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel*

## Summary

The present study was directed towards the characterization of cell-specific histogenetic markers for the various epithelial elements of the adult and the developing guinea pig submandibular salivary gland. We have employed immunofluorescent labelling using three cytokeratin monoclonal antibodies, for which the polypeptide specificities towards guinea pig cytokeratins were determined. All the epithelial elements of the adult gland were positively labelled with two monoclonal antibodies, namely KG 8.13 ('broad spectrum' anti-cytokeratin) and antibody Ks B.18 (reactive with a simple cytokeratin-specific polypeptide of $49 \times 10^3 M_r$). Antibody KS 8.58 (reactive with a guinea pig cytokeratin polypeptide of $50 \times 10^3 M_r$) labelled the basal cells of the large ducts, as well as the myoepithelium. During development of the gland, the submandibular anlage and its primary and secondary branches with their terminal buds, were uniformly labelled with the three antibodies; however, the cytokeratin polypeptides reactive with antibody KS 8.58, which were apparently expressed in all cells of the developing ducts, gradually disappear from most of the ductal cells, starting at about 6 weeks of gestation, and remain only in the basal or reserve cells of the large ducts and the myoepithelium. These observations support the notion that the basal cells retain at least some of the properties of the embryonic glandular epithelium and could be considered as pluripotent reserve cells which may function as progenitors for other epithelial elements in the salivary glands epithelia.

Key words: cytokeratin, salivary gland, histogenesis, guinea pig, monoclonal antibody, polypeptide.

## Introduction

During the last 20 years it has been commonly postulated that all the epithelial cells of salivary glands arise from pluripotent reserve cell progenitors which are present along the excretory duct (Tamarin & Greenberg, 1965; Shackleford & Schneyer, 1971; Eversole, 1971; Young & Van Lennep, 1978). Furthermore, it was proposed that neoplastic transformation of these cells gives rise to diverse salivary gland neoplasms (Eversole, 1971; Batsakis, 1980; Regezi & Batsakis, 1977), with either monomorphic or pleomorphic nature. To study these developmental processes, attempts have been made to identify specific molecular markers for the various glandular epithelia.

In previous studies, we have shown that immunocytochemical labelling for specific intermediate filaments (IF) may be used to distinguish the different epithelial elements of human salivary glands (Geiger, Geiger, Leitner & Marshak, 1987).

Recent studies have indicated that IF may be used as reliable histogenetic markers for normal and malignant tissues (Anderton, 1981; Lazarides, 1980; Franke et al. 1982; Osborn, 1983). Thus, immunolabelling with antibodies directed towards the five classes of IF enabled the identification of epithelial mesenchymal, myoid, glial and neuronal cells, as well as their neoplastic derivatives, regardless of the particular morphological appearance of these cells and tissues (Osborn, 1983; Osborn, Altmansberger, Debus & Weber, 1984; Vogel & Gown, 1984; Ramaekers et al. 1982; Franke, Schmid, Osborn & Weber, 1978). Furthermore, extensive biochemical and immunochemical analyses of a large variety of epithelial tissues indicated that each of the various

epithelia contains a unique combination of 2–10 polypeptides out of the 19 distinct cytokeratins present in humans (Moll et al. 1982; Sun et al. 1986; Quinlan et al. 1985; Fuchs, 1983). These findings have suggested that polypeptide-specific anti-cytokeratins may serve as useful histogenetic markers for different epithelia of normal, metaplastic and neoplastic origins (Moll, Krepler & Franke, 1983; Gigi et al. 1982; Debus, Weber & Osborn, 1982; Ramaekers et al. 1983; Gigi-Leitner et al. 1986).

As we have previously shown (Geiger et al. 1987; Marshak et al. 1987), positive labelling with a broad spectrum cytokeratin antibody (KG 8·13) and antibody to the human cytokeratin polypeptide, no. 18, was observed in essentially all the epithelial elements of the human submandibular salivary gland. Antibody KS 8.58, specific for human cytokeratins nos 13 and 16, selectively labelled basal cells along the large ducts of the human submandibular gland. Polypeptide no. 19, on the other hand, was detected in ductal cells only, the acini and the associated myoepithelial cells being unlabelled (Geiger et al. 1987).

In the present study, we have employed immunofluorescence microscopy using a battery of cytokeratin-specific monoclonal antibodies for characterization of the various epithelial filaments of the adult guinea pig submandibular salivary gland and of the prenatally developing gland, with particular reference to the possible existence, in mature gland, of pluripotent stem cells that retain the properties of the embryonic tissue and may serve as progenitors for the various glandular epithelia.

## Materials and methods

### Tissues

Male Dunkin-Hartley guinea pigs, about 1 year old, were obtained from the Experimental Animal Center of the Weizmann Institute where they were maintained under controlled environmental and nutritional conditions. Salivary glands were removed under chloroform anaesthesia through a midline incision according to standard procedures.

Pregnant guinea pigs at various gestational stages were anaesthesized as above and the fetuses individually delivered by laparotomy. For 4-week-old fetuses, the complete heads were frozen and used for the immunohistological study. While, in the case of the 6-week-old and full-term fetuses, the floor of the oral cavity including the tongue was excised and the mandible carefully removed. Tissues were snap frozen in liquid nitrogen – cooled isopentane and stored at −80°C until use. Parallel samples were fixed in 4 % buffered formaldehyde, dehydrated and embedded in paraffin according to routine histological procedures.

### Antibodies

#### (a) KG 8.13

A broad-spectrum cytokeratin monoclonal antibody which reacts with the cytokeratin filaments present in essentially all human epithelial cells so far tested (Gigi et al. 1982). Its reactivity with guinea pig and bovine cytokeratins will be shown below.

#### (b) KS 8.58

A monoclonal antibody that reacts with human cytokeratin polypeptides nos 13 and 16 which are present in stratified nonkeratinizing human epithelia (Geiger et al. 1987; Sun et al. 1986).

#### (c) Ks B.18

A monoclonal antibody raised against the cytokeratins of cultured bovine mammary gland epithelial cells (BMGE). This antibody was shown to react in bovine and human tissues exclusively with cytokeratin polypeptide no. 18.

#### (d) Other antibodies

Affinity-purified goat antibodies to mouse Fab were prepared and conjugated to lissamine rhodamine sulphonyl chloride as described (Geiger & Singer, 1979; Brandtzaeg, 1973).

### Gel electrophoresis and immunoblotting analysis

Analysis of the cytokeratin composition of various tissues from guinea pig was carried out using one-dimensional gel electrophoresis (Laemmli, 1970). Relevant regions of thick (30 $\mu$m) frozen sections were dissected under the microscope and extracted with high-salt buffer and detergent (Moll, Franke, Volc-Platzer & Krepler, 1982) and the cytoskeletal residues were analysed electrophoretically in 8 % polyacrylamide gels. Immunoblotting transfer was carried out according to the method of Towbin et al. (Towbin, Staehelin & Gordon, 1979) using $^{125}$I-labelled goat anti-mouse Fab.

### Immunofluorescent labelling

Frozen sections of 6–7 $\mu$m were cut at −20°C in a Frigocut 2800 cryostat (Jung Reichert, FRG). The sections were recovered on clean glass slides, air dried, fixed in acetone at −20°C and immunolabelled as described (Geiger & Singer, 1979; Brandtzaeg, 1973). Antibody-stained sections were briefly dehydrated in absolute ethanol, mounted in Entellan (Merck, FRG) and examined in a Zeiss Axiophot photomicroscope equipped for epifluorescence observations, using ×40/1·0 or ×100/1·3 oil-immersion plan neofluar objectives.

## Results

### Specificities of the monoclonal cytokeratin antibodies

Two of the antibodies used in this study (KG 8.13, KS 8.58) were previously characterized by us and by others on human tissues. Their polypeptide specificities for guinea pig cytokeratins are demonstrated
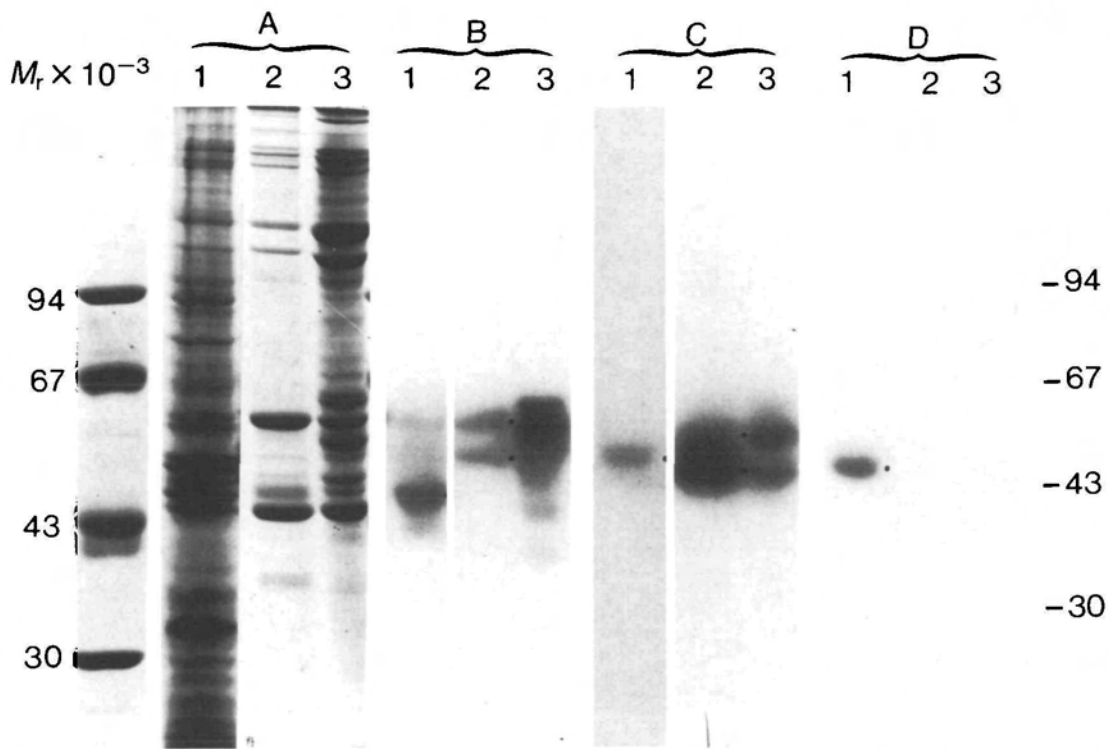
**Fig. 1.** Immunoblotting characterization of the polypeptides specificities of antibodies KG 8.13 (B), KS 8.58 (C) and Ks B.18 (D) on guinea pig tissues including: epidermis (1), floor of the oral cavity (2) salivary gland (3). The lanes on A show the Coomassie blue patterns of the corresponding tissue; the molecular weight markers on the left correspond to the protein gels and their precise location on the nitrocellulose immunoblots marked on the right.

in Fig. 1. Immunoblotting analysis indicated that KG 8.13 recognizes several polypeptides corresponding to relative molecular masses ($M_r$) of 61, 56·5, 53, 50 and 43 × $10^3$.

Antibody KS 8.58, which recognizes polypeptides nos 13 and 16 in human tissues, reacts mainly with two or three guinea pig polypeptides which are abundant in the skin and in the oral mucosa ($M_r$ 53 and 46×$10^3$), as well as with a single polypeptide in the submandibular salivary gland ($M_r$ 50×$10^3$).

Antibody Ks B.18, which recognizes only the polypeptide no. 18 in the human, reacts with one cytokeratin polypeptide in the guinea pig at $M_r$ 49×$10^3$.

*Immunofluorescent labelling of salivary glands of adult guinea pig with cytokeratin-specific antibodies*

Haematoxylin–eosin (HE) labelling of paraffin-embedded submandibular salivary gland sections of the adult guinea pig revealed the different epithelia of the gland. As shown in Fig. 2, the eosinophilic acini are clustered into distinct lobules surrounded by strands of connective tissue. The acini in the submandibular salivary gland were either mucous (Fig. 2B) or serous (Fig. 2A). At the periphery of individual acini and intercalated ducts, flat myoepithelial cells were also detected as shown in Fig. 2A,B (see arrow in Fig. 2B). The conducting system consists of the

intercalated, striated and excretory ducts. The intercalated ducts were mostly enclosed within the secretory lobules in continuity with the acini (Fig. 2A,B). The striated ducts were usually lined with simple cuboidal or columnar epithelium and were predominantly present at the periphery of the lobules (Fig. 2B) as well as in the interlobular space (Fig. 2A,B). The excretory ducts were characterized by a relatively large lumen and often by the presence of pseudostratified or stratified–cuboidal epithelium (Fig. 2B,C). Near the basement membrane of the excretory ducts, small basal cells were often detected (arrows in Fig. 2C).

Immunofluorescent labelling of the submandibular salivary gland of the adult guinea pig with cytokeratin-specific antibodies showed that the majority of epithelial elements, including the acinar and all ductal cells, were positively stained with the broadly cross-reactive cytokeratin antibody KG 8.13 (Fig. 2D–F). The myoepithelium showed only weak labelling with this antibody. Labelling with antibody Ks B.18, which is widely reactive with various simple epithelia, was detected in all the epithelial elements of the mature salivary gland of the guinea pig (Fig. 3A,B). Unique staining patterns were obtained with antibody KS 8.58 which labelled the myoepithelial cells of the
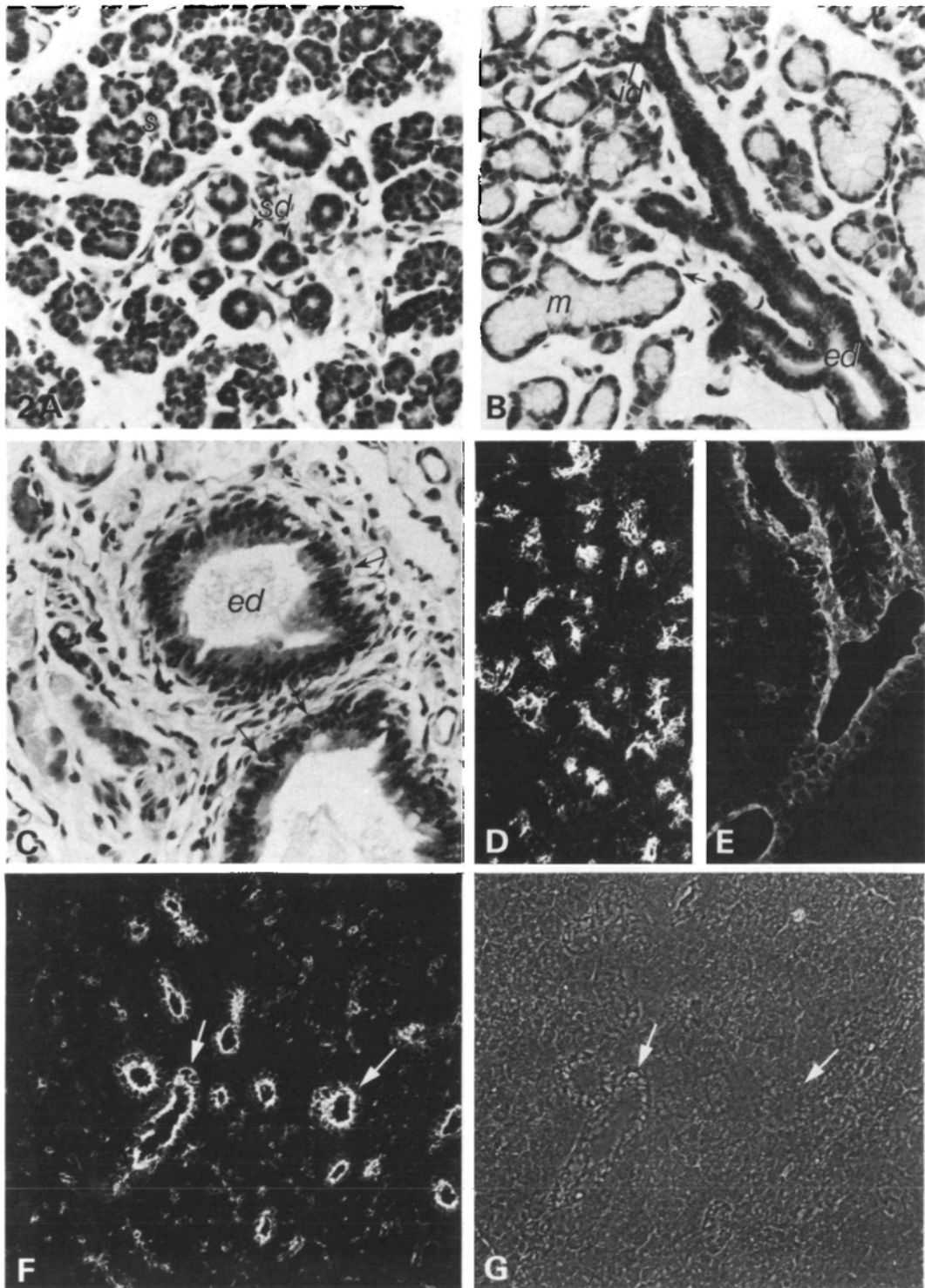
**Fig. 2.** Haematoxylin–eosin stained sections of paraffin-embedded submandibular salivary gland of adult guinea pig (A–C). Immunofluorescent labelling with broad spectrum antibody KG 8.13 (D–F). The phase-contrast micrograph (G) corresponds to the fluorescent micrograph (F). Notice the serous (*s*) and mucous (*m*) acini, as well as the intercalated ducts (*id*), striated ducts (*sd*) and excretory ducts (*ed*) of various sizes, myoepithelial cells (arrow in B) and basal cells along the large ducts that are indicated by arrows in C. Antibody KG 8.13 stains the majority of epithelial elements in the tissue. For orientation see matching arrows pointing to large ducts in F and G. The immunofluorescent labelling shows clusters of acini (D), interlobular excretory ducts (E) and general view (F,G). A–E, ×400; F,G, ×200.
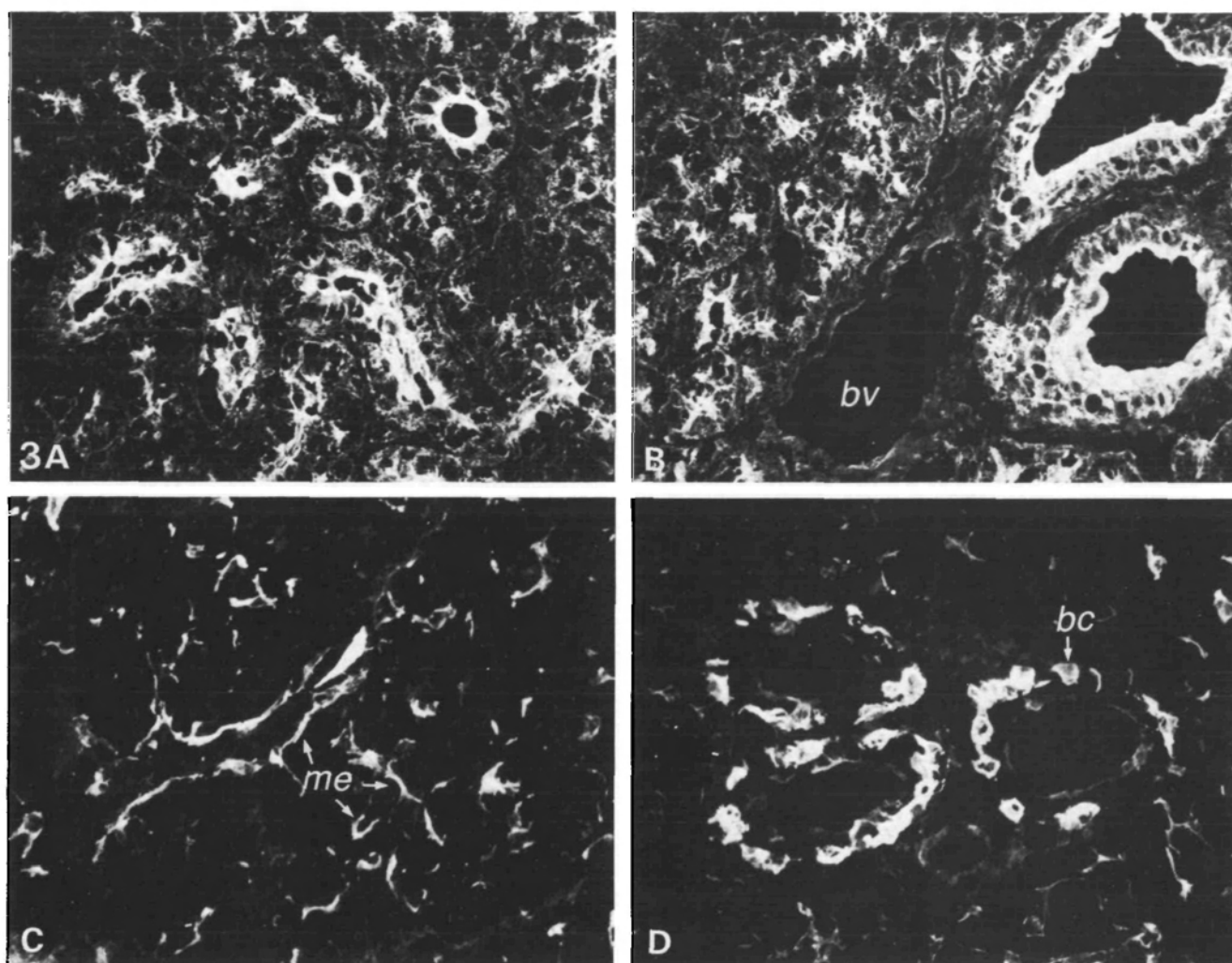
**Fig. 3.** Immunofluorescent labelling of guinea pig submandibular salivary gland with monoclonal antibodies Ks B.18 (A,B) and KS 8.58 (C,D). The micrographs depict acinar clusters and small ducts (A,C) as well as large ducts (B,D). Notice that all epithelial elements are labelled with antibody Ks B.18 while antibody KS 8.58 labels the myoepithelium (*me*) and basal cells (*bc*) only. Blood vessel in B is marked *bv*. ×500.

acini and apparently those present around the intercalated ducts (Fig. 3C). In addition, this antibody labelled the basal cells along the large ducts (Fig. 3D).

### Cytokeratin polypeptide distribution in the developing guinea pig submandibular salivary gland

The morphogenesis of the submandibular salivary gland of the guinea pig begins with a downgrowth of a cell mass from the groove forming the lateral boundary of the tongue. Subsequent to the elongation of this anlage, a process of primary and secondary branching follows where each of the branches ends with a terminal bulb and a lumen is formed in the 'stalk' of the branch (Young & Van Lennep, 1978; Cutler & Chaudhry, 1973, 1974, 1975). These terminal bulbs differentiate into 'terminal tubules' (Jacoby & Leeson, 1959; Leeson & Jacoby, 1959),

which will eventually develop into the acini and possibly into the intercalated ducts.

In the present study, we have divided the period of submandibular gland morphogenesis into three major periods.

(1) The period of primary and secondary branching (about 4 weeks of gestation).

(2) The period of initial apparent differentiation of the 'terminal tubules' into acini and intercalated ducts (about 6 weeks of gestation).

(3) The apparently complete differentiation of the various cellular specializations of the gland that occur towards the end of gestation.

The first phase of morphogenesis is seen in the HE-stained frozen sections of the 4-week-old fetuses (Fig. 4A,B). The primary and secondary branching with their terminal buds are seen with lumenization of the major branches. The large branches are often lined with stratified or pseudostratified epithelia
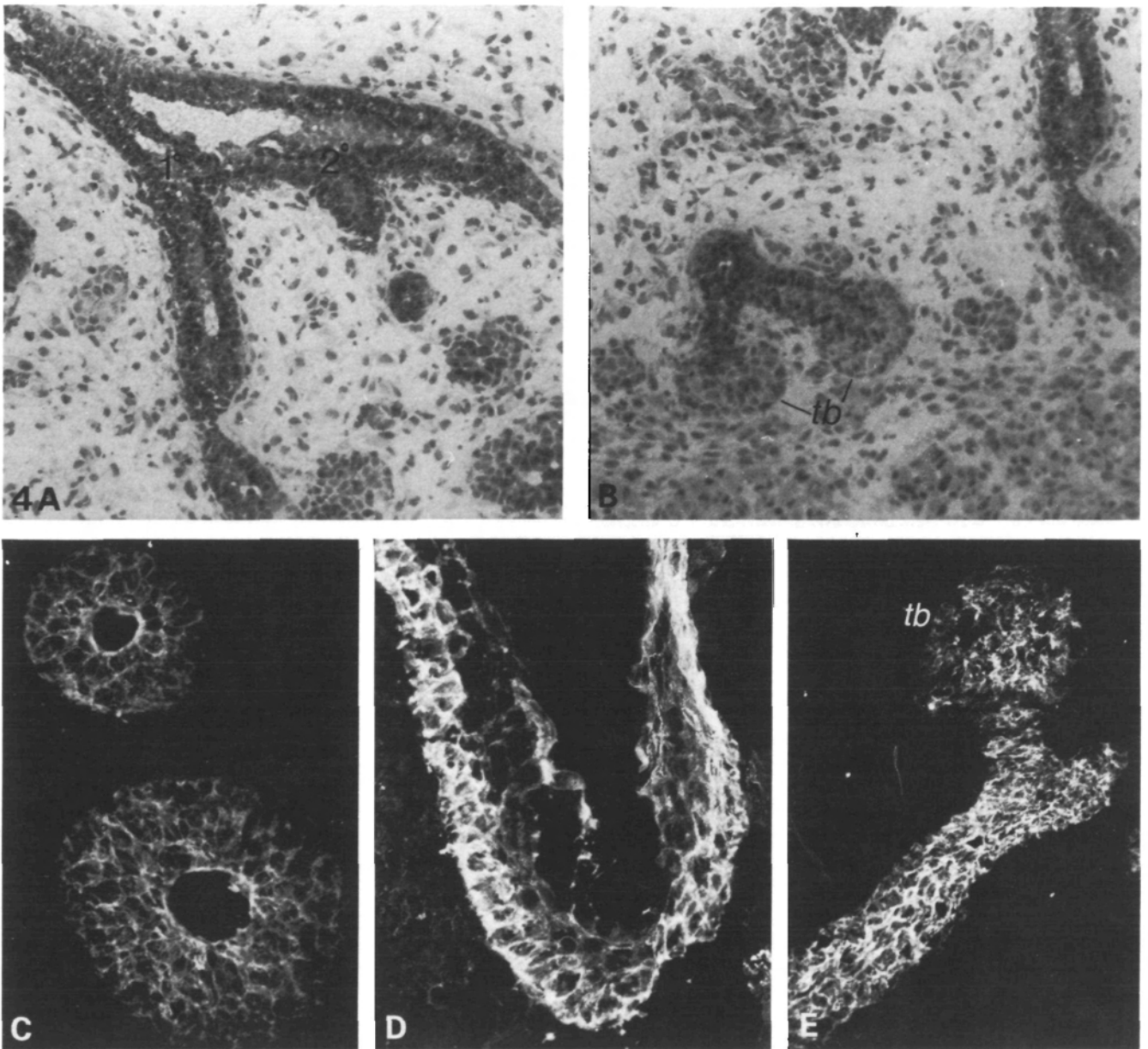
**Fig. 4.** Haematoxylin–eosin (A,B) and immunocytochemical labelling with antibody KG 8.13 (C–E) of the developing submandibular gland in 4-week-old guinea pig fetuses. The HE-stained frozen sections show the primary (1°) and secondary (2°) branching of the salivary gland anlage with their terminal bulbs (*tb*). All the epithelial elements are positively labelled with this broad spectrum anti-cytokeratin antibody. A,B, ×250; C–E, ×500.

whereas the terminal branches are layered by simple cuboidal epithelium. The terminal bulbs contain two cell types: peripherally there is a layer of columnar cells and the central core contains loosely arranged polymorphic cells.

Immunofluorescent labelling of the anlage of the submandibular salivary gland is seen in Fig. 5E where both the contribution of the floor of the oral cavity and of the tongue are positively labelled with antibody Ks B.18. The anlage is also positively labelled with antibodies KG 8.13 and KS 8.58 (Figs 4D, 5B). All the epithelial cells of the primary branches contain cytokeratin polypeptides reactive to antibodies KG 8.13 (Fig. 4C), Ks B.18 (Fig. 5D) and KS 8.58 (Fig. 5A). At this stage, the primary and secondary branches with their terminal bulbs are labelled with the three antibodies (Figs 4E, 5C,F).

Stages in the apparent lobulation and terminal bud differentiation into the glandular end-piece are seen in the HE – stained frozen sections of the 6-week-old fetus (Fig. 6A,B). The large ducts, as well as the small ones, the terminal tubules and the sparsely clustered intercalated ducts and the acini, were all positively labelled with antibodies KG 8.13

(Fig. 6C,D) and Ks B.18 (Fig. 6E,F). It is noteworthy that the myoepithelium also contains the same cytokeratin polypeptides (arrows in Fig. 6C,E).

Immunofluorescent labelling of the submandibular salivary gland of the 6-week-old fetus with antibody KS 8.58 reveals a heterogenous pattern of labelling, as shown in Fig. 7, whereas the stratified epithelium of the large ducts was uniformly labelled (Fig. 7A), the epithelium of the smaller ducts were only partially labelled with this antibody (Fig. 7B–D). There was a gradual, but most conspicuous, disappearance of the positively labelled cells from the walls of the ducts. In many areas, only the basal cells were labelled with

this antibody (Fig. 7C,D). Consequently, the epithelial cells of the endpiece (corresponding to the acini and intercalated ducts) were not labelled with KS 8.58, but the myoepithelial cells which encaged these unlabelled columnar cells were brightly labelled with this antibody (Fig. 7B–D).

The developing submandibular salivary gland of the guinea pig apparently achieves nearly mature morphological differentiation at the end of the gestation period. HE-stained submandibular salivary gland of full-term fetuses is shown in Fig. 8A,B. The lobulation is more definitive, the lobules containing the clustered acini and intercalated ducts with the surrounding myoepithelial cells. The striated ducts
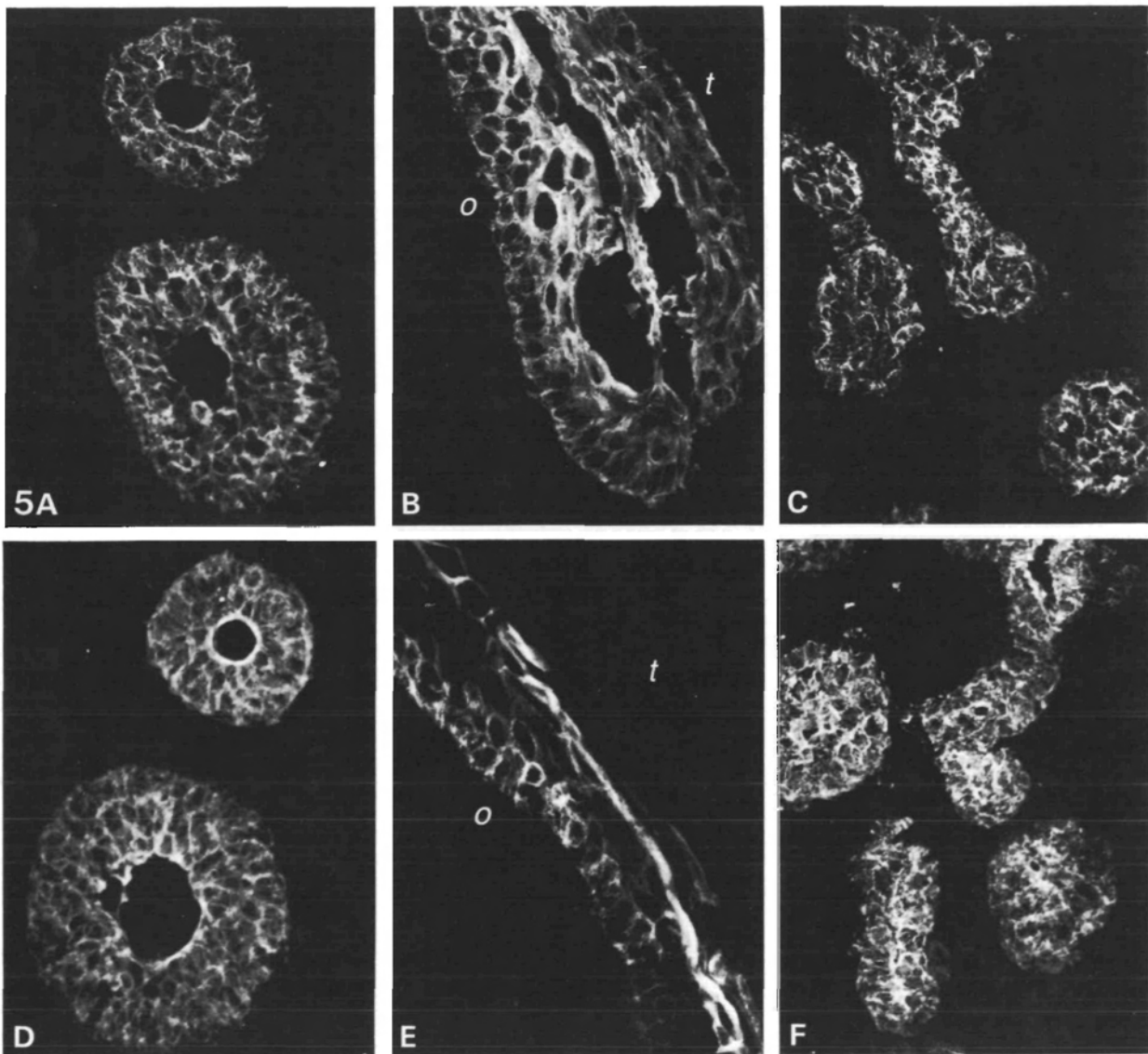


**Fig. 5.** Immunofluorescent labelling of the salivary glands of 4-week-old guinea pig fetuses with antibodies KS 8.58 (A–C) and Ks B.18 (D–F). The micrographs show cross sections at the primary branching area (A,D), the anlage near the oral cavity orifice (B,E) and the terminal bulbs (C,F). The contributions of the oral cavity (*o*) and tongue (*t*) to the anlage are marked in B and E. ×500.
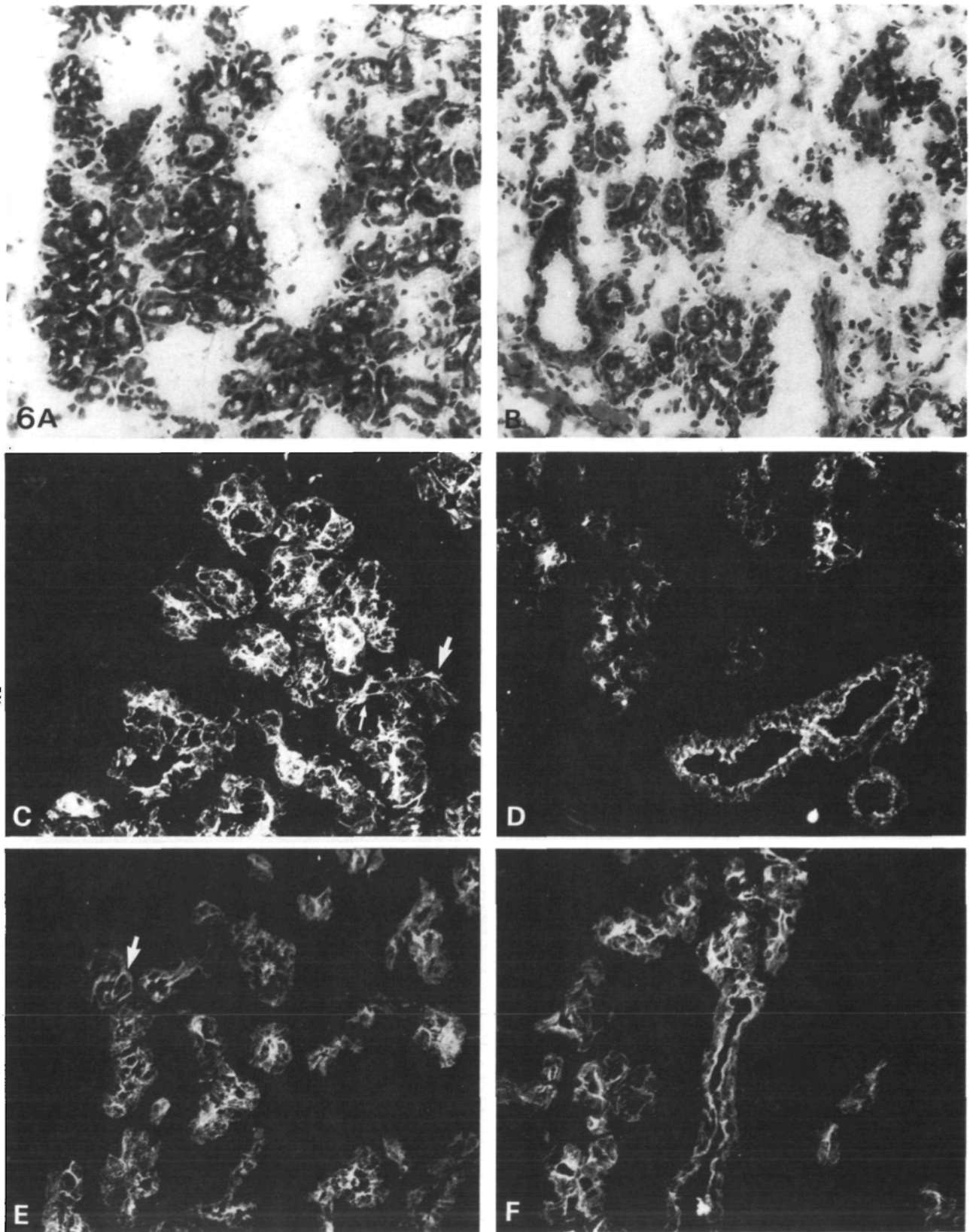
**Fig. 6.** Haematoxylin–eosin (A,B) and immunofluorescently labelled (C–F) frozen sections of salivary glands of 6-week-old guinea pig fetuses. The antibodies used were KG 8.13 (C,D) and Ks B.18 (E,F). The areas selected show clusters of acini (A,C,E) and ducts (B,D,F). At this stage, all epithelial elements, including the nascent myoepithelium (arrows in C and E) are positively labelled with the two antibodies. ×250.
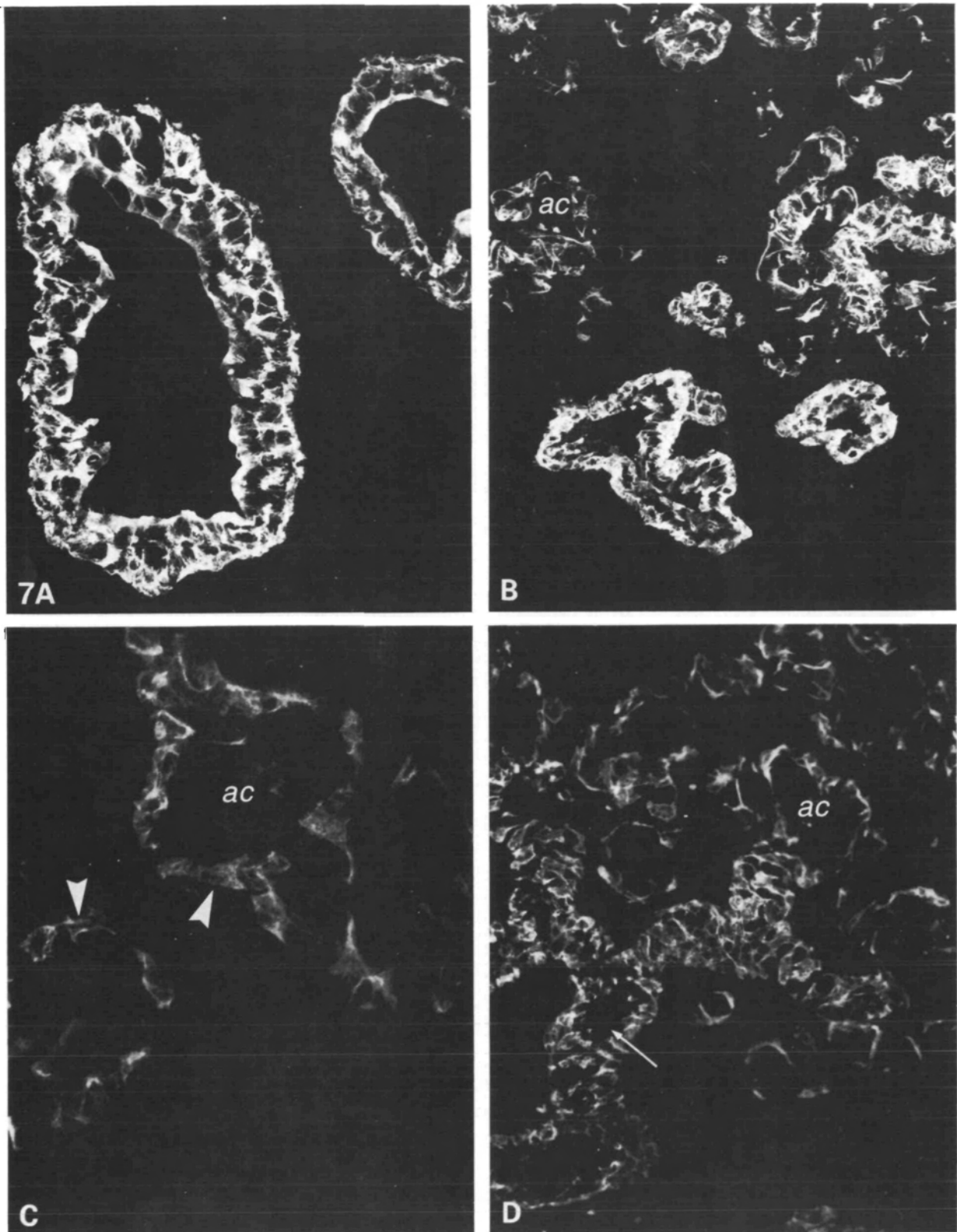
**Fig. 7.** Immunofluorescent labelling of the submandibular gland of 6-week-old fetuses with anti-cytokeratin antibody KS 8.58. At this stage, we detect various transitional stages in the labelling patterns varying from uniform labelling in large ducts (A) through progressive disappearance of labelling from the forming acini (*ac*) and ducts (see arrow in D). The positive labelling of periacinar myoepithelial cells (arrowheads) is shown in high-power photomicrograph (C). A,B,D, ×500; C, ×1250.

and the large excretory ducts are seen predominantly in the interlobular space.

Immunofluorescent labelling patterns obtained with the various anti-cytokeratins in the full-term fetuses were similar to those found in mature glands; all epithelial cells apparently expressed cytokeratin polypeptides reactive with antibody KG 8.13 (Fig. 8C) and antibody Ks B.18 (Fig. 8D) including all ducts, acini and the myoepithelium. At this stage, the cytokeratin polypeptides reactive with antibody KS 8.58 were already confined to the myoepithelial cells and the basal cells of the large ducts (Fig. 8E).

## Discussion

The present study was largely directed towards the characterization of the cellular diversity of the mature and the developing guinea pig submandibular salivary glands using cytokeratins as cell-specific histogenetic markers. Many studies that addressed these aspects in the past have focused on the ultrastructure of the gland and its various elements, as well as on specific molecular markers which show differential distribution throughout the gland. Among these markers were various enzymes, mucins etc. which could be
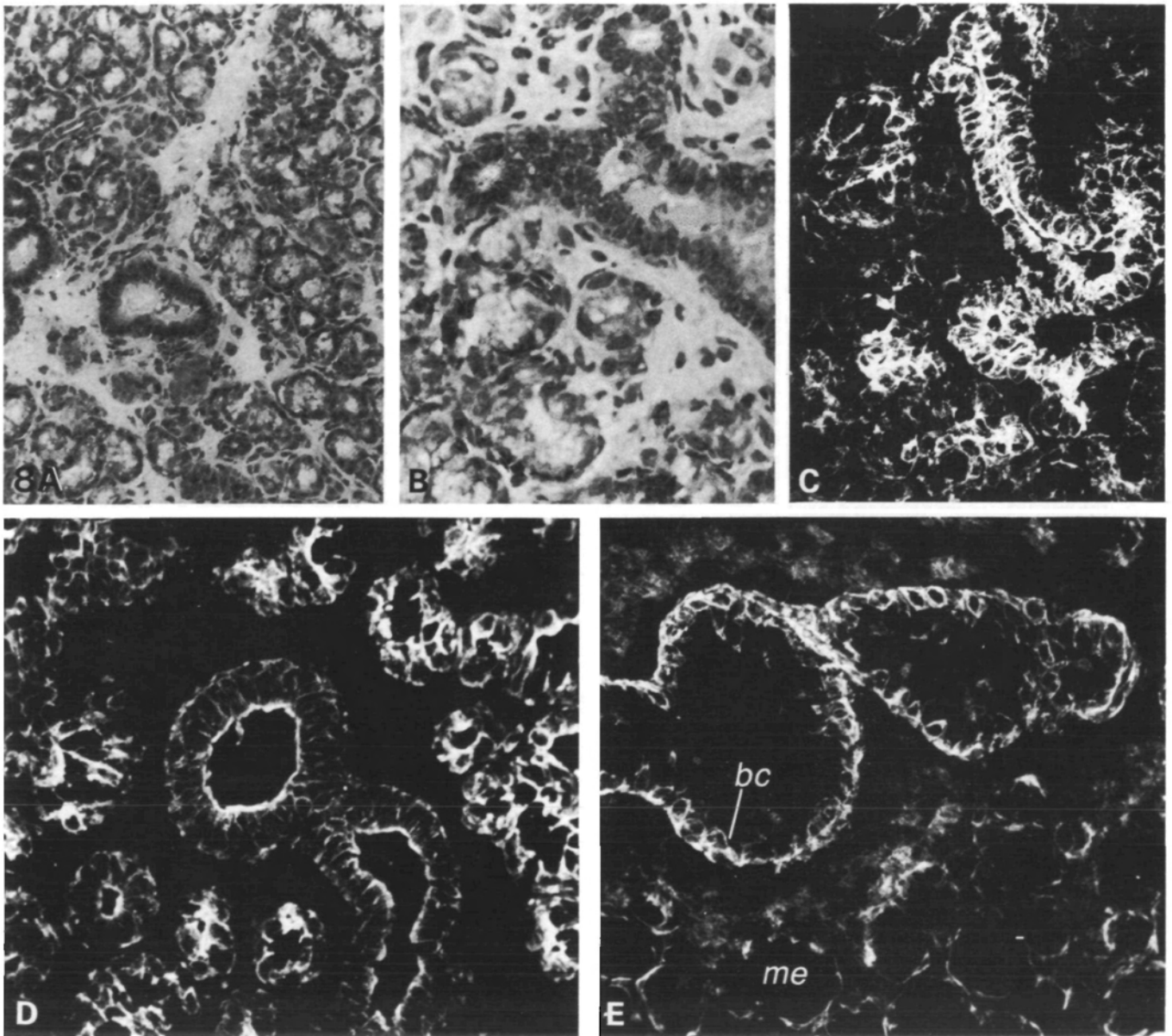


Fig. 8. Haematoxylin–eosin staining (A,B) and immunofluorescent labelling (C–E) of submandibular salivary glands of full-term guinea pig fetuses. The HE staining reveals extensive clustering of the acini into distinct lobules with nearly complete differentiation of the ductal systems (B). Antibodies KG 8.13 (C) and Ks B.18 (D) label all the epithelial elements of the gland. While antibody KS 8.58 (E) conspicuously stains only myoepithelial (*me*) cells and arrays of basal (*bc*) ductal cells. A, ×250; B–E, ×500.

visualized either histochemically or immunohistochemically (Pinkstaff, 1980).

Studies over the last few years have indicated that the subunits of intermediate-sized filaments can serve as most useful markers in studies on epithelial morphogenesis in a variety of systems including salivary glands (Moll *et al.* 1983; Gigi-Leitner *et al.* 1986; Gigi *et al.* 1982; Debus *et al.* 1982; Ramaekers *et al.* 1983). We have recently reported on the localization of specific cytokeratin polypeptides in the different epithelial elements of the human submandibular salivary gland (Geiger *et al.* 1987). Several differences were detected between the various glandular epithelia; for example, we have shown that essentially all the epithelial elements of the human submandibular salivary gland contain cytokeratin polypeptide no. 18, which is abundant in simple epithelia. In contrast, polypeptide no. 19 was present throughout the ductal system, including the intercalated ducts, and was apparently absent from the acini and the myoepithelium. Another set of cells within the ductal system that displayed unique cytokeratin labelling was the basal or 'reserve' cells, which are scattered along the basement membrane of the large ducts. These cells in the human salivary glands were the only cells within the gland decorated with antibody KS 8.58, which has previously been shown to react specifically with human cytokeratin polypeptides nos 13 and 16 (Geiger *et al.* 1987). Since these basal cells are often considered to be the progenitors of different elements throughout the glandular epithelia, we were compelled to use the various anti-cytokeratins (especially antibody KS 8.58) for the study of salivary gland histogenesis. We have chosen guinea pig fetuses as our experimental model, since the morphogenesis of their salivary glands is largely characterized (Young & Van Lennep, 1978) and at least some of their cytokeratin polypeptides are recognized by our monoclonal antibodies. It should, however, be pointed out that, since the guinea pig cytokeratin catalogue is not yet available, as it is for the human (Moll *et al.* 1982), bovine (Franke *et al.* 1981; Cooper & Sun, 1986; Schiller, Franke & Geiger, 1982) and mouse (Franke *et al.* 1981; Schiller *et al.* 1982) polypeptides, we refer here to the molecular weight of the different polypeptides recognized by the various antibodies, without relating them to the better-characterized counterparts in the other species.

Immunofluorescent labelling of the adult guinea pig submandibular salivary gland showed that two monoclonal antibodies, namely KG 8.13 ['broad spectrum' anti-cytokeratin (Gigi *et al.* 1982)] and Ks B.18 (reactive with a single cytokeratin polypeptide $M_r 49 \times 10^3$) labelled essentially all the epithelial elements of the gland. These include the stratified and pseudostratified epithelia of the large ducts and

the epithelium of the striated (interlobular and intralobular) and intercalated ducts, as well as that of the acini and surrounding myoepithelial cells. Antibody KS 8.58 labelled the basal cells as well as the myoepithelium in the adult guinea pig gland. In this respect, labelling with antibody KS 8.58 was similar to that obtained in the human gland with antibody KA-1 (anti-polypeptide 4, 5 and 6) suggesting possible common cytokeratin polypeptides to the basal cells and the myoepithelial cell (Nagle *et al.* 1986).

Immunofluorescent labelling of the 4-week-old gland showed that the submandibular gland anlage, including its primary and secondary branches with their terminal bulbs, were uniformly labelled with antibodies KG 8.13, Ks B.18, as well as with KS 8.58. Two distinct events occur towards the 6th week of gestation as regards the differentiation of the gland. (a) Cells of the terminal bulb gradually differentiate into acinar cells and lose those cytokeratin polypeptides that react with antibody KS 8.58. However, some of the cells in this region retained their reactivity with antibody KS 8.58 and apparently developed into myoepithelial cells at the periphery of the acini (Figs 4E, 5C,F). (b) The cytokeratin polypeptides reactive with antibody KS 8.58, which were apparently expressed in all cells of the developing ducts, gradually disappear from most ductal cells and remain only in the 'basal' or 'reserve' cells of the large ducts (Figs 7B–D, 8E).

These observations support, therefore, the view that the basal cells found along the ductal system retain at least some of the properties of the embryonic glandular epithelium. One of the properties of the latter is its capacity to take different routes of terminal differentiation leading to the establishment of essentially all the epithelial cells of the gland (Eversole, 1971; Young & Van Lennep, 1978). Interestingly, it is commonly believed that salivary gland tumours which display a wide variety of morphological features arise from the ductal basal cells (Batsakis, 1980; Regezi & Batsakis, 1977). The relationships between the latter and a variety of human salivary tumours and their respective cytokeratin expression profiles are currently under study.

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