

Keratin 18 is associated with a subset of older taste cells in the rat

Chunxiao Zhang, Murray Cotter, Anne Lawton, Bruce Oakley, Lianna Wong*, Qun Zeng

Department of Biology, University of Michigan, 3127 Natural Science Building, Ann Arbor, MI 48109, USA

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Abstract. All or nearly all intragemmal (elongated) cells of rat taste buds were immunopositive for keratins 7, 8, and 19. In contrast, keratin 18 was detected in 19 ± 5 cells per taste bud (mean \pm sd), or about 25% of the intragemmal cells. During taste bud development keratins 7, 8, and 19 were evident initially in polygonal cells and later in elongated taste cells. Keratin 8 appeared in vallate taste cells at P0 (postnatal day 0), followed by keratins 7 and 19 at P1, and keratin 18 at P2–P3. Keratin 18 was always limited to elongated cells. The assemblage of elongated taste cells comprising a taste bud began with a single elongated cell, rather than with the synchronous elongation of a cluster of cells. Developmental errors were observed at P2–P3, e.g., some vallate taste cells had a misoriented axis. In order to study the pace of keratin differentiation during cell turnover we injected bromodeoxyuridine (BrdU) into adult rats to monitor taste cell age. Keratin-19-positive intragemmal cells differentiated within 1 day. In contrast, keratin 18 was first detected in cells aged 3 days. Hence, both in taste cell development and replacement, keratin 18 was restricted to the older cells; it was the last taste cell keratin to become expressed during differentiation.

Introduction

Keratin expression changes as epithelial cells progressively differentiate [9, 15, 19, 21, 27, 33]. The diversity of keratins and their transient expression make them the best differentiation markers of oral epithelial tissues [19, 20, 30].

Epithelia are either simple (one layer) or stratified (multiple layers). Simple epithelia contain some combination of keratins 7, 8, 18 and 19 [18, 19]. These four keratins have been associated with taste buds [1, 13, 15,

22–25, 31, 34, 35, 37]. Thus, both the morphology and the immunocytochemistry of the oral epithelium suggest that taste buds are islets of simple epithelia embedded in stratified squamous epithelia [25, 30, 35].

Lane [15] was the first to report keratin 18 in taste buds (rat fungiform; monoclonal antibodies [MAbs] *LE61* and *LE65*). Sawaf et al. [31] confirmed these observations for human fungiform taste buds (MAb *Ks18.174*). Takeda et al. [34] (MAb *PKK3*) and Toh et al. [35] (MAb *LE61*) observed keratin-18-like immunoreactivity in mouse vallate taste buds. In contrast to keratins 7, 8, and 19, we report that keratin-18-like immunoreactivity is absent from 75% of intragemmal cells—the elongated cells within a taste bud [13, 25]. We quantified the subset of taste cells that had keratin 18 and measured the timing of keratin 18 occurrence during development and cell replacement.

Methods

Tissue collection. Sprague-Dawley albino rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were kept on a 12-h light: 12-h dark cycle and housed with *ad libitum* food and water. For tissue collection, neonatal rats (postnatal ages 0, 1, 2, 3, 4, 7, and 10 d) were euthanized with carbon dioxide gas. Adult rats were deeply anesthetized with an i.p. injection of sodium pentobarbital (70 mg/kg b.w.). The anesthetized animals were perfused intracardially with either 0.9% NaCl solution containing 0.02% sodium heparin and 0.5% procaine hydrochloride or TRIS-buffered mammalian Ringer's solution [26], followed by acid alcohol fixative (70% ethanol, 10% acetic acid).

Tongues were excised and immersed in acid alcohol fixative for at least 1 h. Tongue tissue was cryoprotected in a mixture of 20% sucrose and the embedding compound OCT. Ten-micrometer-thick cryosections were cut at -25°C , mounted on gelatin-coated slides, and stored at -20°C . The methods of animal care and euthanasia were approved by the University of Michigan Committee on the Use and Care of Animals.

Immunohistochemistry. The primary monoclonal antibodies (MAbs) were: MAb *RCK105* to keratin 7 (Monosan, [28]); MAb *4.62* to keratin 19 (Sigma, [10]); MAb *LE41* to keratin 8 (Amersham, [15]); MAb *LE65* to keratin 18 (Amersham, [15]), MAb

* Present address: Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA 94720, USA

Correspondence to: B. Oakley

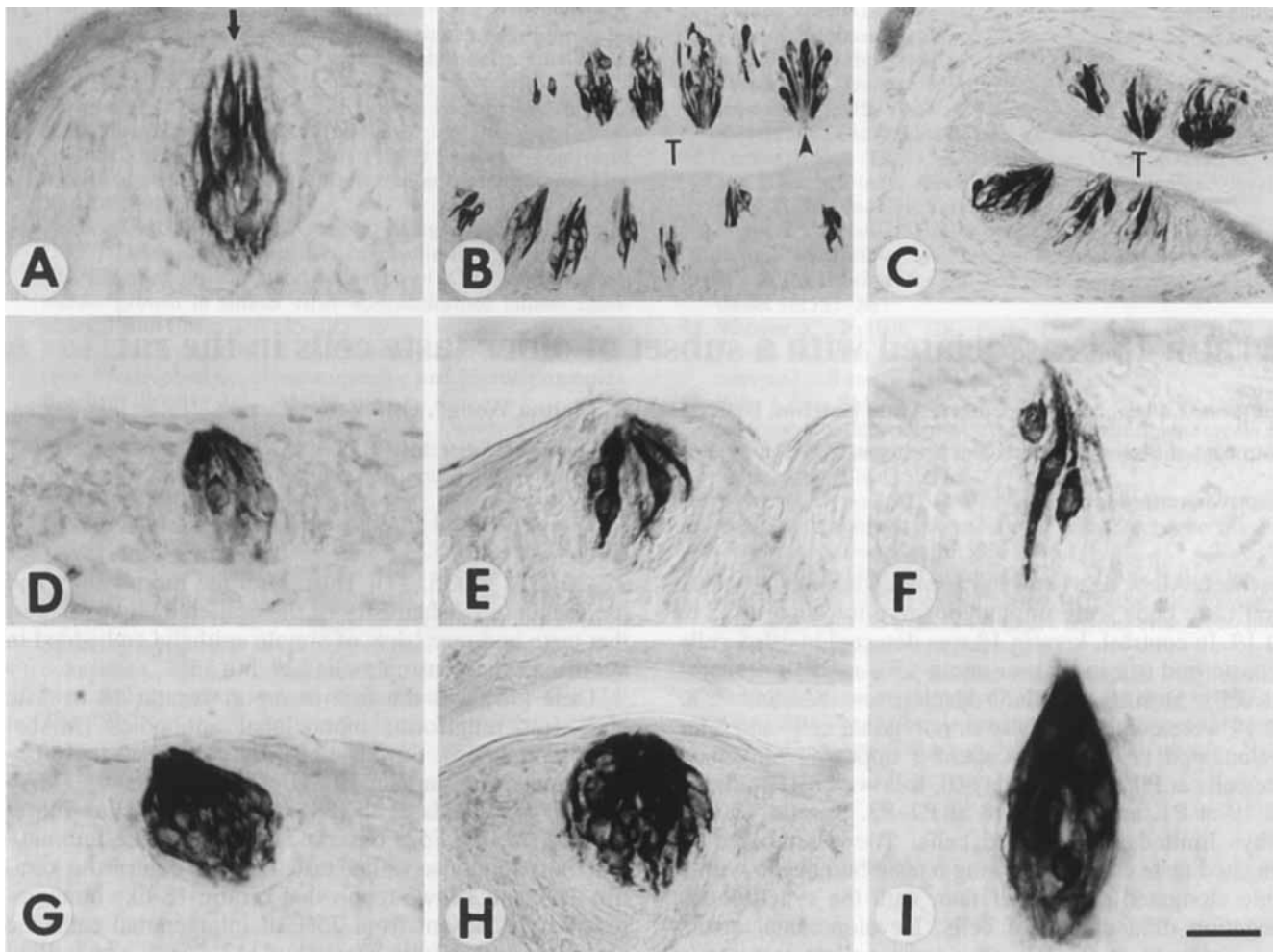


Fig. 1A-I. Keratin-18-like immunoreactivity is present in a subset of intragemmal cells in taste buds of: fungiform papillae (A), valvate papilla (B), foliate papillae (C), epiglottis (D), nasopalatine papilla (E), and palate (F). The *arrow* in A indicates the apical tips of keratin 18-positive intragemmal cells. In order to stimulate

the apex of taste cells (*arrowhead* in B), taste solutions must enter the trenches (T) of the foliate and valvate papillae. G Keratin 19 in an epiglottal taste bud. H Keratin 7 in a nasopalatine taste bud, I Keratin 8 in a taste bud of the palate. *Scale bar* in I is 50 μm for B, C and 20 μm for other panels

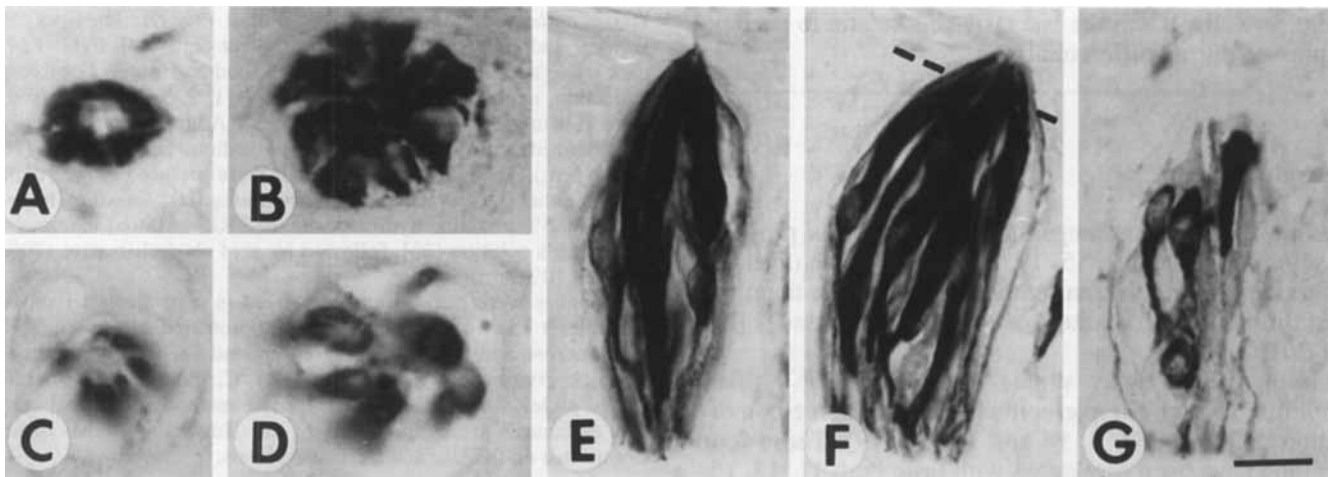


Fig. 2A-G. Keratin 19 immunoreactivity in a vallate taste bud cross-section that passed through (A) and immediately beneath (B) the taste pit. Keratin 18 immunoreactivity in a vallate taste bud cross-section through (C) and beneath (D) the taste pit. E-G

Three serial, longitudinal-sections of a vallate taste bud containing 23 keratin-18-positive cells. The *dashed line* in F indicates the approximate location of the base of the taste pit. *Scale bar* in G is 8 μm for A-D and 10 μm for E-G

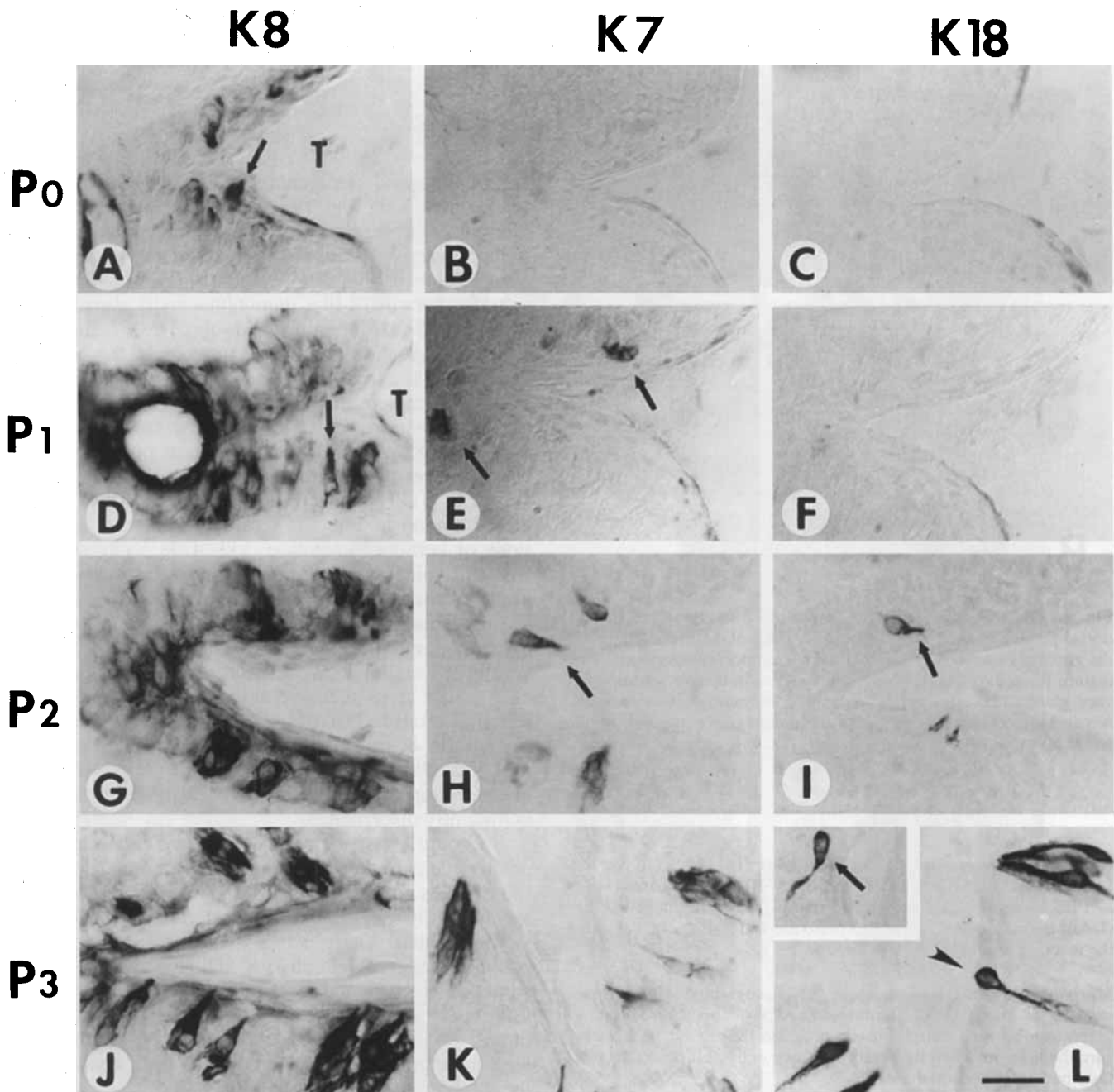


Fig. 3A–L. Immunoreactivity for keratins 8, 7, and 18 in neonatal vallate papillae at postnatal days 0, 1, 2, and 3 (A–L). **A** At P0 there were polygonal taste cells immunoreactive only for keratin 8 (arrow). At P0 there was no immunoreactivity for keratin 7 (B), or keratin 18 (C). By P1 (D), taste cells (arrow) and the luminal surface of circular expansions of the developing trench were keratin-8-positive. At P1 (E) there were keratin-7-positive polygonal cells

but no keratin-18-positive cells (F). **G–I** At P2 there were taste cells reactive for keratin 7, 8, and 18. At P2 and P3 several taste cells were oriented roughly parallel to the trench (arrows in H, I and inset in L). Taste cell nuclei occasionally migrated to the surface of the trench (arrowhead in L). The scale bar in L is 50 μ m for A–L

PKK3 to keratin 18 (Labsystems, [18]), and MAb *Bu1/75* to BrdU (Sera-Lab, from Accurate Chemical Sci.).

Immunohistochemistry was carried out using an avidin-biotin peroxidase (ABC) method (Vector Labs). For the ABC-peroxidase method, mounted tissue sections were hydrated in four 5-min washes of 0.1 M phosphate-buffered saline (PBS), pH 7.4, containing 0.4% Triton-X 100 (TX-100, Sigma). The slides were then

incubated for 30 min with 3% normal goat serum (Cappel, Organon Teknika) in PBS/TX-100, followed by a 1-h incubation with the primary antibody at room temperature. The slides were washed with PBS four times for 5 min each, followed by a 45-min application of the secondary antibody, biotin-conjugated goat anti-mouse IgG preadsorbed with rat serum proteins (B-8774, Sigma). Three additional washes in PBS preceded both the 30-min appli-

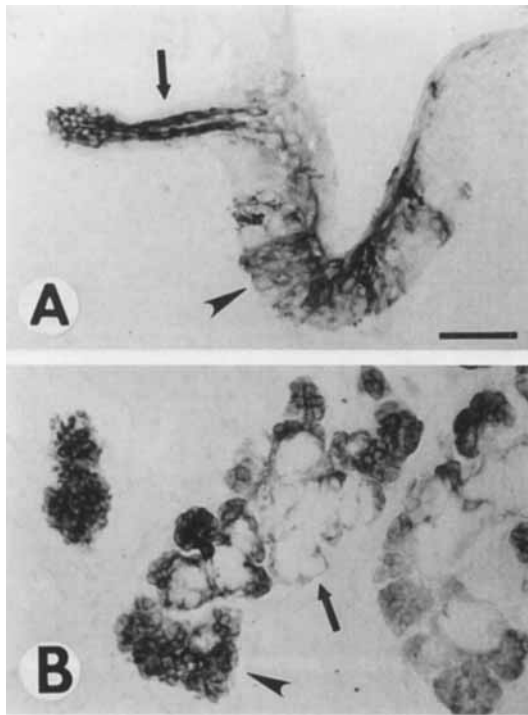


Fig. 4A, B. **A** A salivary duct (*arrow*) at P3 is connected to the dorsal portion of the vallate trench. Regions of diffuse staining of the gustatory trench (*arrowhead*) were a temporary developmental feature limited to keratin 8. **B** Only for keratin 8 were serous salivary glands beneath the vallate papilla immunopositive (*arrowhead*). Mucus glands were always immunonegative (*arrow*). Scale bar is 50 μ m for **A** and **B**

cation of Vectastain avidin-biotin complex (PK-4000 kit, Vector Laboratories, Burlingame, CA) and the 5–10 min incubation with a PBS solution containing 0.5 mg/ml 3,3'-diaminobenzidine (DAB, Sigma), 0.01% hydrogen peroxide and 0.04% NiCl_2 to tint the reaction product blue.

Morphometric analysis. We analyzed photographs of transverse sections of the apex of immunostained taste buds. We used a graphics tablet and morphometric program (Sigma Scan) to measure the area occupied by keratin-positive cells as a percentage of taste bud cross-sectional area.

BrdU injection and detection of BrdU and Keratin 18 or 19. At 9 a.m. on day 0 adult rats were injected intraperitoneally with 5-bromo-2'-deoxy-uridine (BrdU, Sigma) at 25 mg/rat ($n=9$ rats) or 5 mg/rat ($n=7$ rats). Rats were euthanized 1 h, and 1, 2, 3, 4, and 5 days after BrdU injection. Mounted, 10 μ m thick, tongue sections were denatured in 2N HCl; hydration in PBS/TX-100 preceded (four 4-min washes) and followed (three 10-min washes) the DNA denaturation. A 60-min incubation with anti-BrdU antibody (1:20, Accurate Chemical and Scientific Corp.) was followed by the secondary antibody, biotin-sp-conjugated goat anti-rat IgG (1:1000, Jackson ImmunoResearch Lab.). After three 4-min washes in PBS, the slides were subjected to a 30-min application of the Vectastain avidin-biotin complex. Prior to treatment with DAB/nickel chloride, the sections were exposed for 30 min to 1% cobalt chloride. Next the sections were subjected to the protocol described above for keratin staining, except that the nickel chloride was omitted. The BrdU-labeled nuclei were blue while the keratin 18 or keratin 19-like immunoreactivity was yellow/brown.

The seven rats injected with 5 mg BrdU provided the quantitative data. Fifty taste buds were each examined 1 h and 1 day after BrdU injection. One hundred taste buds were examined on each of days 2–5.

Results

All, or almost all, intragemmal cells were immunoreactive for keratins 7, 8, and 19. In contrast, using MAb LE65 (keratin 18) fewer than half of the intragemmal cells were immunoreactive in adult taste buds of rat tongue, palate, nasopalatine papilla, and epiglottis (Fig. 1). Similar results for keratin-18-like immunoreactivity were obtained with MAb PKK3 for vallate and foliate taste cells.

In cross-sections of vallate taste buds, the MABs for keratins 7, 8, and 19 generated immunopositive rings or disks. A complete ring of stained cells was present when the cross-section passed through the taste pit. A solid disk, except for the outer margins, was present when the cross-section passed immediately beneath the taste pit (Fig. 2A, B). There were no well-formed rings or disks after immunostaining for keratin 18. Instead, cross-sections of keratin-18-positive cells were scattered among frequent voids and took no preferred position within the circular cross-section of the taste bud (Fig. 2C–D).

We evaluated cross-sections immediately below the taste pit in our first method of quantifying the extent of keratin 18 staining. We measured the percentage of a taste bud's cross-sectional area that was stained for a specific keratin. The mean percentages were $98\% \pm 1\%$ for keratin 8, $n=12$ buds and $40\% \pm 8\%$ for keratin 18, $n=9$ buds ($P < 0.01$, t test). Taste cells were not counted in cross-section, because the cell boundaries could not be confidently discerned.

To count cells, we counted the nuclei of keratin-18-positive cells in serial, longitudinal sections (Fig. 2E–G). (Antibodies for keratins 7, 8 and 19 stained so ubiquitously that it was impossible to identify unstained intragemmal cells.) There was a mean of 19 ± 5 keratin-18-positive intragemmal cells per vallate taste bud (mean \pm sd nuclei, uncorrected for split nuclei, $n=46$ buds). Small taste buds had as few as 10 keratin-18-positive cells; the largest buds had up to 27. Since rat vallate taste buds have a mean of 75 intragemmal cells [11], about 25% of the intragemmal cells are keratin 18-positive. Taste buds elsewhere in the oral cavity had comparable numbers of keratin-18-positive taste cells. However, there was wide variation among fungiform taste buds—some had as few as five keratin-18-positive cells.

We examined the developmental occurrence of keratins 7, 8, 18, and 19 in the vallate papilla at birth (postnatal day 0=P0), and at P1, P2, P3, P4, and P6. From P0–P6 immunoreactivity for keratin 19 (not shown) and keratin 7 developed in tandem. At P0 staining for keratins 7, 18, and 19 was negative, but for keratin 8 there were a few immunoreactive salivary duct cells and polygonal taste cells (no marked elongation). By P1 the immunoreactivity of vallate trench cells included keratins 7 and 19. At P2 isolated keratin-18-positive cells were occasionally observed; they were always elongated. It was not until P2–P3 that taste cells regularly ex-

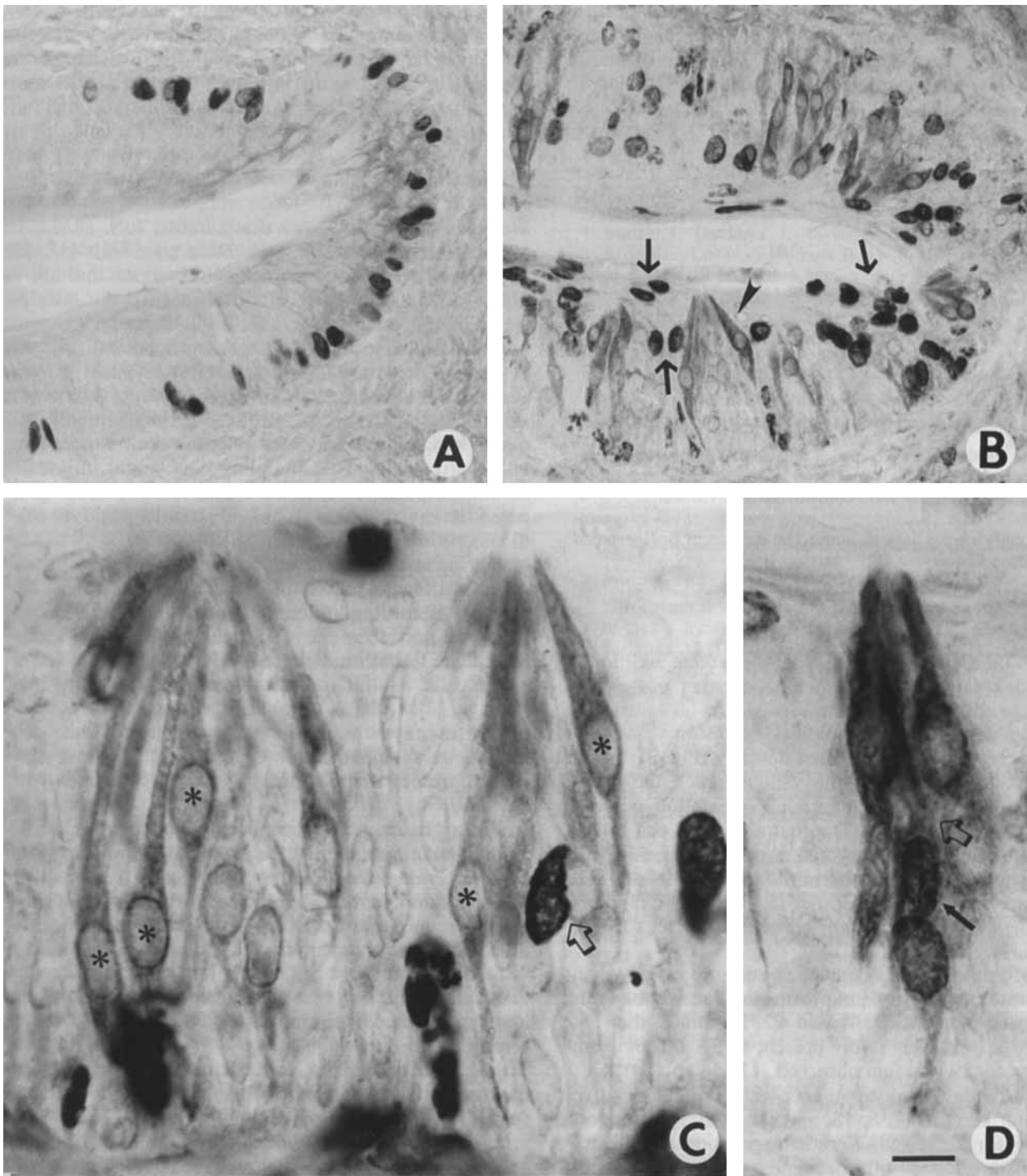


Fig. 5A–D. Animals were euthanized at time t , after bromodeoxyuridine (BrdU) injection. **A** At $t=1$ h, BrdU-labeled cells were restricted to the basal layer. **B** By $t=2$ days, labeled cells had migrated toward the trench surface (arrows). One of several keratin-18-positive cells is evident (arrowhead). **C** At $t=2$ days, a BrdU-positive, keratin-18-negative cell is present in a taste bud (open ar-

row). Five keratin-18-positive cells with BrdU-negative nuclei (asterisks) are prominent. **D** A taste bud at $t=4$ days contains a keratin-18-positive cell (open arrow) with a BrdU-positive nucleus (solid arrow). Scale bar in **D** is 25 μm for **A**, **B** and 8 μm for **C**, **D**

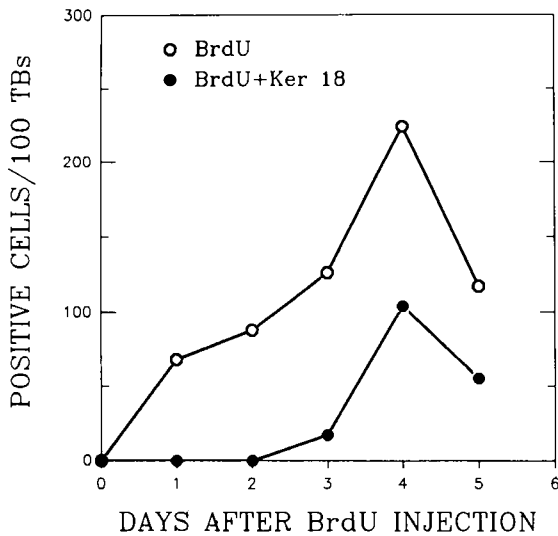


Fig. 6. The number of BrdU-labeled and double-labeled intragemmal cells per 100 taste buds increased as a function of days after BrdU injection. Animals were euthanized after 1 h and 1, 2, 3, 4, and 5 days. Keratin 18 immunoreactivity was present in intragemmal taste cells aged 3 days or more. The decline in BrdU-labeled cells at 5 days may reflect BrdU toxicity

tended to the trench surface. By P3 there were additional dispersed keratin-18-positive cells; clusters of several elongated cells were also seen (Fig. 3J–L).

During development the apical extension of vallate taste cells was generally directed dorsally (Fig. 3J). With continued downgrowth of the vallate trench the intragemmal cells shifted into their adult orientation—perpendicular to the trench wall. The first elongated cell to appear was typically a solitary cell. Apparent errors in positioning were observed: the initial elongated taste cell might have its long axis parallel to the trench wall surface or less commonly might elevate its nucleus to the trench surface (Fig. 3H, I, L).

Fungiform taste buds developed no more rapidly than vallate taste buds. Fifty fungiform papillae were examined at each of ages P0, P1 and P2. Keratin 8, the first keratin to appear, was rarely present at P1 in fungiform cells. Keratin 19 was first observed at P2.

Mature cells of salivary ducts were immunopositive for all four keratins (7, 8, 18, and 19) with the strongest reactivity for keratin 8. Keratin expression in salivary duct cells developed with the same timing and sequencing as for taste cells. In neonates some salivary ducts entered the upper portion of the vallate trench (Fig. 4A). In young animals the salivary glands were mixed clusters of immunonegative mucous secreting cells and immunopositive (only for keratin 8) serous cells (Fig. 4B). Neither type of salivary gland cell acquired immunoreactivity for keratins 7, 18, or 19.

In adult rats, cell division monitored by BrdU was conspicuously associated with basal cells. Within 1 day after BrdU injection, some fusiform intragemmal cells were double-labeled for BrdU and keratin 19. By 2 days numerous BrdU-positive cells had reached the surface of

the epithelium between taste buds. By 3 days there were a few intragemmal cells double labeled for BrdU and keratin 18. It was not until 4 days after BrdU injection that cells double-labeled for BrdU and keratin 18 were regularly observed (Figs. 5, 6).

Discussion

We have previously shown that keratins 7, 8, 18 and 19 are present in rat, gerbil, and rabbit taste buds [13, 25, 37]. Here, we have confirmed observations that all, or nearly all, intragemmal cells that reached the taste pit were immunopositive for keratins 7, 8, and 19. Apical sections excluded those younger intragemmal cells not yet extending to the taste pit. Keratin-18-positive intragemmal cells were older; they were at least 3 days old. Hence, sections at the level of the taste pit might over-represent keratin-18-positive cells in relation to keratin-18-negative cells. This sampling bias would inflate the keratin-18-positive cross-sectional area (40%) compared to nuclear counts of keratin-18-positive cells (25%).

During taste bud development keratin 18 was not detected in taste cells until 2–3 days after keratins 7, 8 and 19. Accordingly, keratin 18 immunoreactivity was restricted to elongated taste cells. Similarly, during adult taste cell turnover, keratin 18 was rarely detected in cells less than 4 days old. With a lifespan of 10 days [7], vallate taste cells could spend 60% of their lifespan with keratin 18 (4–10 days). Since only 25% of intragemmal cells were keratin 18-positive, they must represent a subset of intragemmal cells aged 4–10 days.

The percentage of keratin-18-positive cells varied among vallate taste buds. We estimate that after normalization for taste bud volume, there remained up to a two-fold variation in the abundance of keratin-18-positive cells. Some of this variation may be statistical fluctuation in the cell population age structure across vallate taste buds. Fungiform taste cells turn over more rapidly than vallate taste cells [2, 7]. This could contribute to the lower number of keratin-18-positive cells observed in some fungiform taste buds.

MAbs *LE65* and *PKK3* detected keratin-18-like immunoreactivity in fewer than half of the intragemmal cells. The younger intragemmal cells may have been unstained because they contain little keratin 18 or because the keratin 18 is conformationally or biochemically altered [14, 32]. Where keratin 18 is present it is likely to modulate the abundance of other keratin polypeptides (7, 8, and 19). The proportions of keratins 7, 8, 18, 19 are known to differ with cell type in other tissues [6, 12, 14]. As a rule, pairs of basic (keratins 1–8) and acidic (keratins 10–19) keratin polypeptides form heterodimers [16, 36]. If intragemmal cells obey this rule, regulation of keratin 7, 8, or 19 would adjust to accommodate the up-regulation of keratin 18 in older cells. Conceivably, keratin-18 might form heterotrimers, like 8/18/19, 7/8/18, and 7/8/19, known in other simple epithelial cells [3, 6].

Keratins 7, 8, and 19 are expressed when the developing taste cell is still polygonal. Only later does the nucleus migrate and the taste cells extend apical and basal processes. Keratins may be permissive for cell elongation and migration, or they may contribute strength and rigidity that assist in the extension of cell processes and in tissue penetration.

Previous studies have demonstrated that developing vallate taste buds require about 10 days to form a taste pore [22]. This pace has been confirmed for fungiform taste buds [17]. By continuing to add cells, vallate taste buds ultimately doubled the number of fusiform cells that were present when the pore formed [11]. Keratin staining has revealed that the intragemmal cell assemblage begins as one or two elongated cells. The assemblage of fusiform cells, known as a taste bud, appears to develop and enlarge by the prolonged sequential addition of individual cells rather than by the synchronous constriction and elongation of a cluster of polygonal cells.

Taste bud development does not mirror taste cell turnover or regeneration [23]. In the present research the keratin 7, 8, or 19-positive polygonal cells observed in development were not detected in adults. Such polygonal cells may be unique to development. Alternatively they may be obscured by the ubiquitous keratin-positive intragemmal cells in adults, or have a fleeting existence given the faster pace of differentiation during adult taste cell replacement and regeneration. Within one day an adult basal cell can give rise to a replacement cell that enters the taste bud, elongates, and expresses keratin 19. Regeneration of an entire taste bud after reinnervation may require only 2–3 days in an adult vs 10 days for development [4, 5, 22].

The spatial errors observed during the development of taste buds, such as having the cell axis parallel to the trench wall, appear to have been eliminated in adult taste cells.

Previous studies of taste bud development showed that P0 rats have no vallate taste buds with a pore to the epithelial surface [11, 22]. We employed antibodies against keratins 8 and 19 to determine that individual taste cells only extended to the trench surface by P2–P3, not at P0 or P1. Examination of the palate at P2 indicated a similar rate of taste bud development (unpublished observations). These findings suggest that the rat's oral taste system is too immature to respond to milk (lactose) at birth [23]. However, the fungiform epithelium is leaky at P7, shortly before taste pores appear. At that time, slow chemical modulation of nerve endings may be possible [17]. Electrophysiological recording at birth would directly evaluate taste axon sensitivity and response latency to lactose.

Salivary duct cells are the only other lingual cells with keratins 7, 8, 18, and 19. The development of keratin immunoreactivity follows the same progression in taste cells and salivary duct cells: positive staining at P0 for keratin 8, at P1 for keratins 7 and 19, and at P2 for keratin 18. The ducts of von Ebner's salivary glands merge with the vallate and foliate trench epithelium. Taste buds have been reported in salivary ducts and may regenerate from salivary duct tissue [29]. These studies

of tissue structure, keratin expression patterns, and taste bud regeneration point to the communality of cells comprising the von Ebner's salivary ducts and gustatory trenches [8]. It is possible that some taste cells and salivary duct cells arise from the same founding population.

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