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Phagocytic Cells in the Taste Buds of Rat Circumvallate Papillae after Denervation

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Abstract

Phagocytic cells in the taste buds of rat circumvallate papillae after the sectioning of bilateral glossopharyngeal nerves were examined by electron microscopy and immunohistochemistry. Electron micrographs taken 1 day after denervation revealed that flat-shaped cells were present just beneath the taste buds and that their cellular processes extended toward the debris from the degenerating taste buds. At 2–6 days after denervation, long and thin processes of the flat cells surrounded the debris and appeared to have taken them up into the cytoplasm as small vesicles. Evidence for phagocytosis by the flat cells was seen up to 9 days after denervation and again at 24 and 40 days, in correlation to the degeneration and regeneration of the taste buds. Pre-embedding immunohistochemistry using anti-vimentin antibody showed that flat cells strongly reacted with vimentin. Light microscopic immunohistochemistry using anti-macrophage antibodies (ED1, ED2) showed that throughout the post-operative days macrophages were not present underneath or within the taste buds. Most of the ED2-immunoreactive resident macrophages were located in the deep layer of connective tissue, and a few were found in the nerve bundle. ED1-immunoreactive cells were seen in the duct cells of von Ebner's glands and a few were in the trench wall of circumvallate papillae; however, they were also immunoreactive for anti-OX62 antibody, which recognizes dendritic cells. The results indicate that the phagocytic cells of the taste buds are fibroblasts, not macrophages. Moreover, resident macrophages participate in phagocytosis of degenerated nerves together with Schwann cells. **Chem. Senses 21: 467–476, 1996.**

Introduction

It is well known that taste buds die and disappear from the lingual epithelium after denervation (Guth, 1957; Farbman, 1969; Jeppsson, 1969; Fujimoto and Murray, 1970; Naga *etal.*, 1970; Suzuki and Takeda, 1987). In a recent study using the TUNEL method (terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling) for the histochemical detection of apoptotic cells (Gavrieli *et al.*, 1992), we clarified that the death of taste bud cells occurs by apoptosis (Takeda *et al.*, 1996). Electron microscopic observation of mouse circumvallate papillae after the sectioning of the glossopharyngeal nerve has shown that apoptotic bodies, which show nuclear and cytoplasmic condensation, are phagocytosed by neighboring taste bud cells (Takeda *et al.*, 1996). In normal taste buds of monkey foliate papillae, the taste bud cells have been reported to contain dead cells, suggesting that taste

bud cells themselves have a phagocytic function; and the phagocytic cells are the only cell type in the taste buds that is considered to secrete a dense substance into the taste pore (Farbman *et al.*, 1985). However, taste bud cells shrink and disappear from the epithelium after denervation; thus, other cells that phagocytose the dead cells or cellular debris might also be present. Following denervation, homogeneous electron-dense substances of various sizes are observed in the connective tissue beneath the basement membrane of the taste buds. They are also found between the taste buds and the basement membrane and may be cellular debris from degenerated taste buds. We observed that the cells in the connective tissue beneath the epithelium phagocytosed dense substances (Takeda *et al.*, 1996); however, the cell type is not known. Moreover, nerve fibers disappear from the taste buds and connective tissue after sectioning of the glossopharyngeal nerve, and these degenerated nerve fibers are probably removed by phagocytosis.

Macrophages are known to be 'professional' phagocytes in various tissues; however, they are not the only cell type that can phagocytose cellular debris. For example, epithelial cells, tumor cells, fibroblasts and Schwann cells also participate in the removal and clearance of dead cells (cf. Savill *et al.*, 1993). In addition, we found that supporting cells in the olfactory epithelium participated in phagocytosis: in the first week after unilateral bulbectomy of adult rats the phagocytosis by macrophages was active and at longer post-bulbectomy periods, 30–90 days, the participation of supporting cells in phagocytosis was observed (Suzuki *et al.*, 1995). Furthermore, in Wallerian degeneration in the peripheral nervous system, both Schwann cells and macrophages are known to phagocytose degenerated axons and myelin debris (Pellegrino *et al.*, 1986; Stoll *et al.*, 1989; Reichert *et al.*, 1994; Liu *et al.*, 1995).

In the present study we examined the disposal and clearance of dying taste buds and associated nerves in the rat circumvallate papillae after sectioning of the glossopharyngeal nerve.

Materials and methods

Adult Wistar rats were anesthetized i.p. with Nembutal (Abbott Labs., North Chicago, IL), and the bilateral glossopharyngeal nerves sectioned. The surgery was carried out according to a previous paper (Suzuki and Takeda, 1987). The rats were housed in rooms with controlled temperature and humidity and received food and water *ad libitum*. They were allowed to survive for 1, 2, 4, 6, 9, 12, 17, 24 and 40 days after the operation. Normal unperturbed rats were used for the control. For immunohistochemistry, the rats were killed by an overdose injection of Nembutal, and small blocks of tongue tissue containing circumvallate papillae were excised and frozen in Freon 22 (Spray freezer, Oken). Some blocks were immersed in periodate–lysine–paraformaldehyde (PLP) solution for 6 h at 4°C. After having been rinsed in phosphate-buffered saline (PBS) containing 25% sucrose overnight at 4°C the specimens were sectioned on a cryostat at 10 μm. The fresh-frozen sections were fixed in acetone. The sections were blocked with normal swine serum for 20 min, and incubated for 2 h at room temperature in one of the following primary antibodies diluted with PBS (1:100), monoclonal anti-rat macrophages ED1 and ED2, anti-rat veiled (dendritic) cells, OX62 (Serotec, Indianapolis, IN) or anti-rat vimentin (Dako, Kyoto, Japan), 1: 100. After rinsing in PBS, a labelled streptavidin–biotin (LSAB) kit (Dako) was used to stain the sections. The immunoreactive product was colored by incubation with hydrogen peroxide (0.3%) in the presence of 50 mg/ml diaminobenzidine (DAB). As a negative control, PBS was used instead of a monoclonal antibody. For double staining, the sections were stained with ED2 antibody, reacted with the reagents of the LSAB kit, and colored by the DAB reaction. The sections were rinsed in PBST (PBS containing 0.3% Triton X-100) overnight at 4°C and subsequently incubated with anti-glial fibrillary acidic protein (GFAP) antibody (Dako), stained with the LSAB kit, and colored with 4-chloro-1-naphthol.

For electron microscopy, PLP-fixed tongue tissues were cut with a cryostat at a 10 μm thickness and incubated with anti-vimentin antibody for 6 h at room temperature. The sections were stained with the LSAB kit and the immunoreactive products were colored by the DAB. The sections were postfixed in 2% OsO_4, dehydrated through a graded series of ethanol and embedded in Epon 812. Ultrathin sections were cut but not counterstained in order to obtain better contrast of the immunodeposits. The observations were made under a Hitachi H-7000 electron miroscope. Some specimens were prepared for conventional electron microscopy as follows: rats were deeply anesthetized with Nembutal and perfused through the heart with buffered 2% glutaraldehyde and 1.6% paraformaldehyde. Small blocks of the tongue containing circumvallate papillae were excised, postfixed in 2% OsO_4, dehydrated, and embedded in

Epon 812. Semi-thin (2 μm) and ultrathin sections were cut. Semi-thin sections were stained with toluidine blue and observed under a light microscope, and ultrathin sections were stained with uranyl acetate followed by lead citrate, and examined under an electron microscope.

Results

One to two days after the sectioning of the bilateral glossopharyngeal nerves, many dense bodies were found in the cytoplasm of the taste bud cells and dense substances were present in the connective tissue along the basement membrane beneath the taste buds (Figure 1A). In the control rats, there was a small amount of dense substances. Under the electron microscope, flat-shaped cells with long and thin cytoplasmic processes were located beneath the basement membrane of the epithelium, and were in contact with dense substances (Figure 1B). In the cytoplasm of these flat cells, ribosomes were observed; however, other organelles were poorly developed. The phagocytosis of debris by the flat cells was evident at 2–6 days, and thin processes of cytoplasm had elongated and surrounded the debris (Figure 1C). Many small pits were seen at the tip of cellular processes, and the debris was seen in small vesicles in the cytoplasm. Phagosomes and large lysosomes were no longer observed in the cytoplasm (Figure 1D). At 12–17 days, taste buds were not present in the epithelium of the trench wall of circumvallate papillae; thus phagocytosis by flat cells was not observed. By 24 days, regenerated taste buds appeared in the epithelium. The taste buds did not possess taste pores, suggesting that they were immature. A small amount of debris was observed beneath the taste buds (Figure 1E) and the processes of flat cells phagocytosed the debris. At 40 days, the debris was also observed. Positive immunoreactivity for vimentin was found in the flat cells in the control (Figure 1F) and throughout the postoperative days. Under the light microscope, the vimentin-immunoreactive cells, located just beneath the epithelium, were flat in shape, whereas other vimentin-immunoreactive cells in the deep layer of connective tissue were not flat-shaped in the control animal (Figure 2A). The flat cells, which were responsible for phagocytosing the degenerated cellular debris, exhibited branched or ramified processes at 6 days after denervation (Figure 2B).

Two types of anti-macrophage antibodies, anti-ED1 and anti-ED2, were used. ED1 is a cytoplasmic antigen, which is associated with lysosomes in activated, monocyte-derived macrophages, whereas ED2 is a membrane antigen, binds to resident macrophages, and is used to examine phenotypic differences between activated (i.e. ED1-immunoreactive) and resident macrophages (Dijkstra *et al.*, 1985; Damoiseaux *et al.*, 1989). ED2-immunoreactive resident macrophages were widely distributed in the connective tissue of the circumvallate papillae in control animals (Figure 3A). At higher magnification, ED2-immunoreactive macrophages were seen to be large and irregularly shaped, and were located in the deep layer of the lamina propria (Figure 3B). At 6 days after denervation, macrophages were still present in the deep layer of the lamina propria (Figure 3C) and were not observed just beneath the epithelium on any post-operative day. In comparison with the distribution of vimentin-immunoreactive cells (Figure 2), it is clear that ED2-positive cells are not the flat cells beneath the epithelium. The ED1-immunoreactive cells were found in the duct of von Ebner's glands, the trench wall and connective tissue at 6 days after denervation (Figure 3D); however, the immunoreactivity was weak. The distribution of ED1-immunoreactive cells in the connective tissue was different from that of vimentin-positive flat cells, which were located just beneath the trench wall. Since ED1 monoclonal antibody is known to react with not only macrophages but also dendritic cells, the OX62 antibody, which reacts with dendritic cells, was also used. The OX62-immunoreactive cells were observed in the duct of von Ebner's glands, and in both the lamina propria and the trench wall at 6 days after denervation (Figure 3E). In the control, most OX62-immunoreactive cells were spindle shaped, and were observed in the lamina propria (Figure 3F) and the ducts of von Ebner's gland.

In control specimens treated with PBS instead of the antibody, no staining was observed in the circumvallate papillae.

After denervation the nerve fibers disappeared rapidly from the taste buds and connective tissue, and only Schwann cells, which were stained with anti-GFAP antibody, remained in the connective tissue. A few ED2-positive resident macrophages were present in the nerve bundles in the deep layer of lamina propria, but were not found in the smaller nerve fibers near the trench wall (Figure 4). The immunoreactivity for the ED1 antibody was not detected in the nerve bundles or thin nerve fibers. Schwann cells were observed in the connective tissue on all of the post-operative days. In electron micrographs of sections at 2 days after the

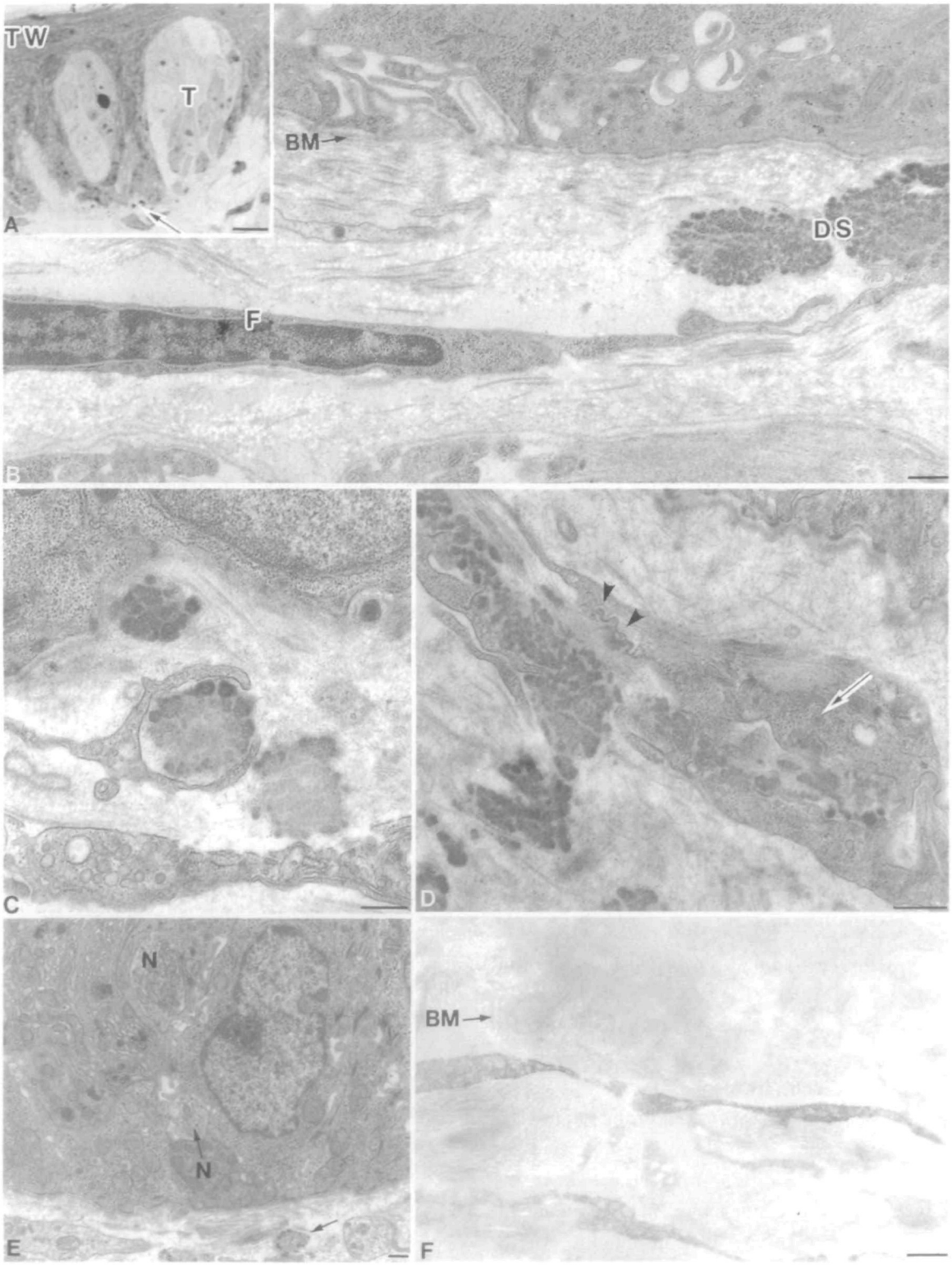

Figure 1 **(A)** Toluidine blue staining of semi-thin section of trench wall of rat circumvallate papillae at 2 days after sectioning of the glossopharyngeal nerve. The arrow indicates dense substance. **(B)** Electron micrograph of the trench wall of circumvallate papillae at 1 day after denervation. A flat cell (F) in the lamina propria has phagocytosed dense substance (DS). **(C)** Electron micrograph of flat cells at 4 days after denervation. **(D)** Electron micrograph of flat cells at 6 days after denervation. The arrow indicates dense substance in the cytoplasm of a flat cell. Arrowheads indicate small pits. Many filaments are seen in the cytoplasm. **(E)** Electron micrograph of taste buds at 24 days after denervation. Regenerated nerve fibers (N) are seen in the taste buds. The arrow indicates dense substance. **(F)** Electron micrograph of vimentin-immunoreactive cells in the lamina propria. Control. T, taste buds; TW, trench wall; BM, basement membrane. Scale bar: A, 10 μm; B–F, 0.5 μm.

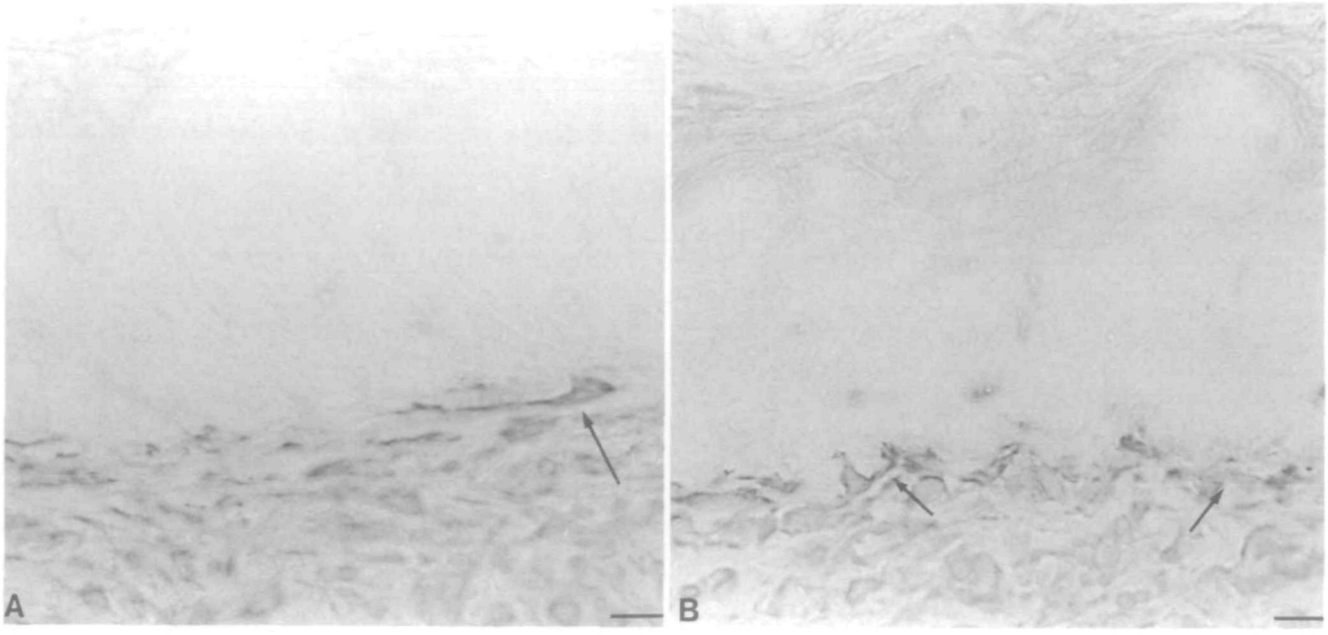

Figure 2 Immunoreactivity for vimentin in the trench wall of circumvallate papillae in the control **(A)** and at 6 days after denervation **(B)**. Vimentin-immunoreactive flat cells are seen just beneath the epithelium (A, arrow). The long and branched cytoplasmic processes of flat cells are seen at 6 days after denervation (B, arrows). Scale bar: 10 mm.

sectioning of the nerve, macrophages in the nerve bundle, which were identified as large and irregularly shaped cells, contained degenerated axons and degraded myelin (Figure 5A). At 4–9 days, macrophages did not contain debris or phagosomes, but had a few lysosomes. Schwann cells, which were distinguished from macrophages by the presence of basement membrane, also contained myelin debris and phagosomes at 1 day after denervation. The phagocytosis by Schwann cells was evident at 2–6 days after denervation (Figure 5B) and was observed up to 9 days.

Discussion

The main finding of the present study is that the flat cells that phagocytose debris from dying taste buds are not macrophages and are suggested to be fibroblasts. They were immunoreactive for vimentin, the intermediate filament protein present in cells of mesenchymal lineage (Osborn *et al.*, 1981), but were negative for the anti-macrophage antibodies ED1 and ED2. Furthermore, ultrastructurally, the cytoplasmic organelles were poorly developed. The cells of mesenchymal origin which are supposed to be localized in the lamina propria of rat circumvallate papillae, i.e. leukocytes, lymphocytes, plasma cells, mast cells and dendritic cells, have characteristic organelles in their cytoplasm; therefore, they were distinguished from macrophages and fibroblasts by electron microscopic examination. The uptake of cellular debris into the cytoplasm of fibroblasts was active at 2–6 days after the sectioning of the glossopharyngeal nerve and seems to be pinocytosis rather than phagocytosis. Small debris-containing vesicles were observed in the cytoplasm, but phagosomes and large lysosomes were not seen. Phagocytosis and pinocytosis are considered types of endocytosis when cells take up large or small elements respectively. The endocytosis of fibroblasts has been observed particularly when small foreign bodies such as acid dyes, ink particles and latex beads have been injected into the dermis and subcutis of mice (Fujita *et al.*, 1988) and into the corneal stroma of rabbits (Fujita *et al.*, 1987). Under some conditions fibroblasts can phagocytose larger things, such as apoptotic neutrophils (Hall *et al.*, 1990).

The uptake of debris by fibroblasts was observed not only in early stages (1–9 days) after denervation but also in late stages, i.e. at 24 and 40 days. Taste bud cells are continuously replaced under normal conditions; they live for ~10 days (Beidler and Smallman, 1965). The death and replacement of taste bud cells might occur in regenerated taste buds as well. It is known that regenerated nerve fibers enter the trench wall of circumvallate papillae at 17 days after denervation and that taste buds begin to regenerate at that time (Suzuki and Takeda, 1987). At 24

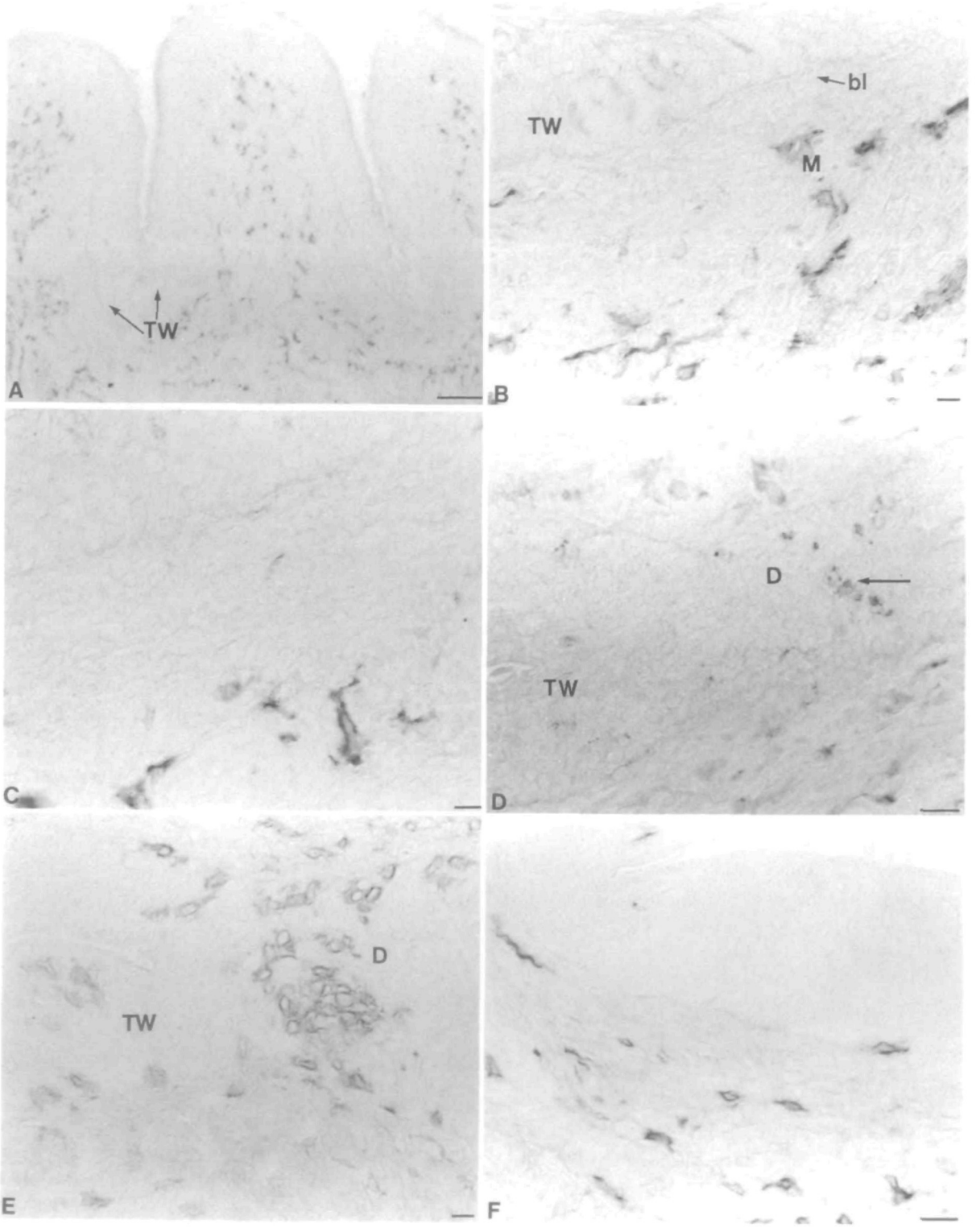

Figure 3 Immunoreactivity for ED1 and ED2, markers of macrophages, and for OX62, a marker of dendritic cells, in the circumvallate papillae. **(A)** Immunoreactivity for ED2. ED2-positive macrophages are widely distributed in the connective tissue of the circumvallate papillae. Control. **(B)** Higher magnification of (A), showing large, irregularly shaped macrophages (M). **(C)** Immunoreactivity for ED2 at 6 days after denervation. **(D)** Immunoreactivity for ED1 at 6 days after denervation. Weak reactions are seen in the duct cells of von Ebner's glands (arrow), the trench wall, and lamina propria. **(E)** Immunoreactivity for OX62 at 6 days after denervation. Most of the OX62-positive cells are seen in the trench wall and in the duct cells of von Ebner's glands. **(F)** Immunoreactivity for OX62 of control animals. The OX62-positive cells are seen in the lamina propria near the trench wall. D, ducts of von Ebner's glands; TW, trench wall; bl, basal lamina. Scale bar: A, 100 mm, B–F, 10 mm.

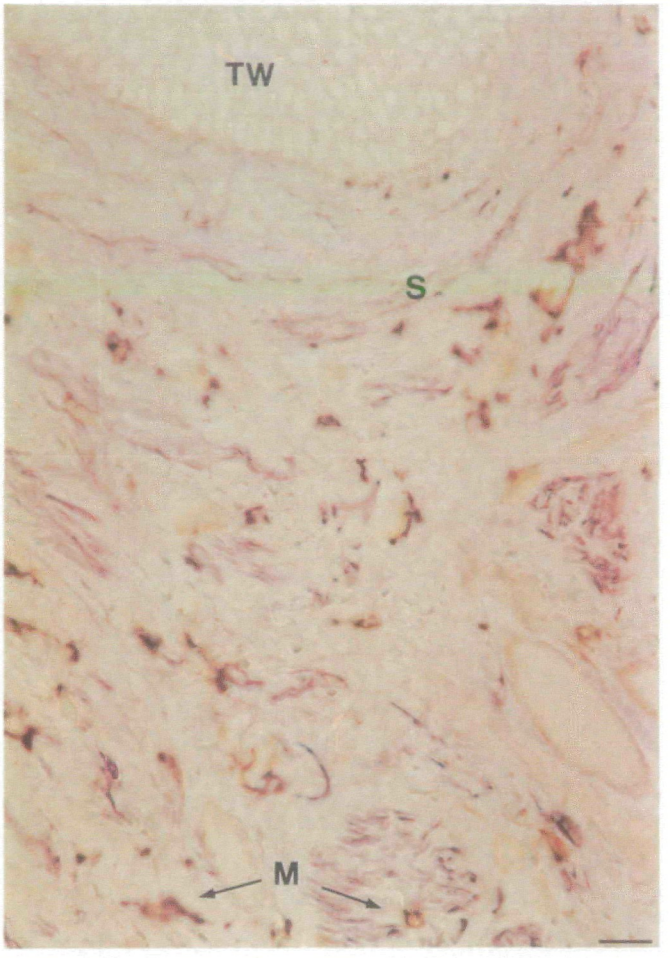

Figure 4 Double immunostaining for ED2 and GFAP in the lamina propria at 12 days after denervation. Macrophages (M) stained by ED2 antibody are colored by DAB (dark brown). Schwann cells (S) which react with anti-GFAP, are stained by 4 chloro-1-naphthol (purple). ED2-positive macrophages are seen in the nerve bundle. TW, trench wall. Scale bar: 20 μm.

days after denervation, taste bud cells are <7 days old. Since the average life span of taste bud cells is 10 days (Beidler and Smallman, 1965), some cells in the taste buds at 24 days die before they reach maturity. The death of the cells which do not reach maturity is also observed in the adult rat olfactory epithelium after bulbectomy; some precursor cells die before they differentiate the olfactory neurons (Carr and Farbman, 1993).

The endocytotic activity of fibroblasts is weaker than that of macrophages (Baur *et al.*, 1979; Svoboda and Deporter, 1980). There were few lysosomes in the cytoplasm of fibroblasts, suggesting that the cellular debris taken up into the cytoplasm might be stored for a long time. In fact, ink particles or latex beads can be recognized in the dermis and subcutis for up to 9 months after they are injected (Fujita *et al.*, 1988). In contrast, macrophages contain many more apoptotic bodies, large phagosomes and lysosomes in the cytoplasm, as shown in rat olfactory epithelium (Suzuki *et al.*, 1995). Another difference between fibroblasts and macrophages is mobility: macrophages are motile and phagocytose dead cells very quickly, whereas fibroblasts do not actively move. Fibroblasts respond to the cellular degeneration or injection of foreign bodies, and their long and slender cellular processes extend to take up dead cells and foreign bodies. In the present study the immunoreactivity to vimentin showed that the cellular processes of fibroblasts branched and ramified when they phagocytosed actively. Although fibroblasts usually play a role in phagocytosis together with macrophages *in vivo* and *in vitro* (Svoboda and Deporter, 1980; Fujita *et al.*, 1987, 1988; Ishizuya-Oka and Shimozawa, 1994), in rat taste buds the fibroblast appears to be a discrete type of phagocyte. Macrophages are known to secrete cytokines and growth factors, which may act on other cells (Lindholm *et al.*, 1992; Griffin *et al.*, 1993; Kasai *et al.*, 1994). The survival of taste buds depends on the presence of the gustatory nerve, which is considered to release a trophic substance (Kinnman and Aldskogius, 1988); thus growth factors or cytokines released from macrophages may not be necessary to regenerate the taste buds. Futhermore, the disposal and clearance of dead taste bud cells by fibroblasts is carried out in the absence of an inflammatory response.

The present study revealed the presence of dendritic cells (OX62 immunoreactive) in the trench wall of circumvallate papillae and duct cells of von Ebner's glands. The dendritic cells are antigen-presenting cells and are distributed in many tissues, mainly lymph nodes, skin and keratinized stratified squamous epithelium. They contain lysosomes in their cytoplasm, which may be related to ED1 immunoreactivity but do not have a phagocytic function (Osada and Shamoto, 1994). The reason for the infiltration of dendritic cells into the trench wall after denervation is not clear. Further experiments are in progress to examine the role of these dendritic cells.

In contrast to the disposal and clearance of dead taste bud cells, degenerated nerve bundles were disposed of and cleared by both Schwann cells and macrophages. Schwann cells phagocytosed degenerated nerves as early as 1 day after the sectioning of the glossopharyngeal nerve. The phagocytosis by Schwann cells was observed up to 9 days after denervation. Schwann cells remained in the connective tissue throughout the post-operative period; the regenerated nerve fibers may extend toward the epithelium along this

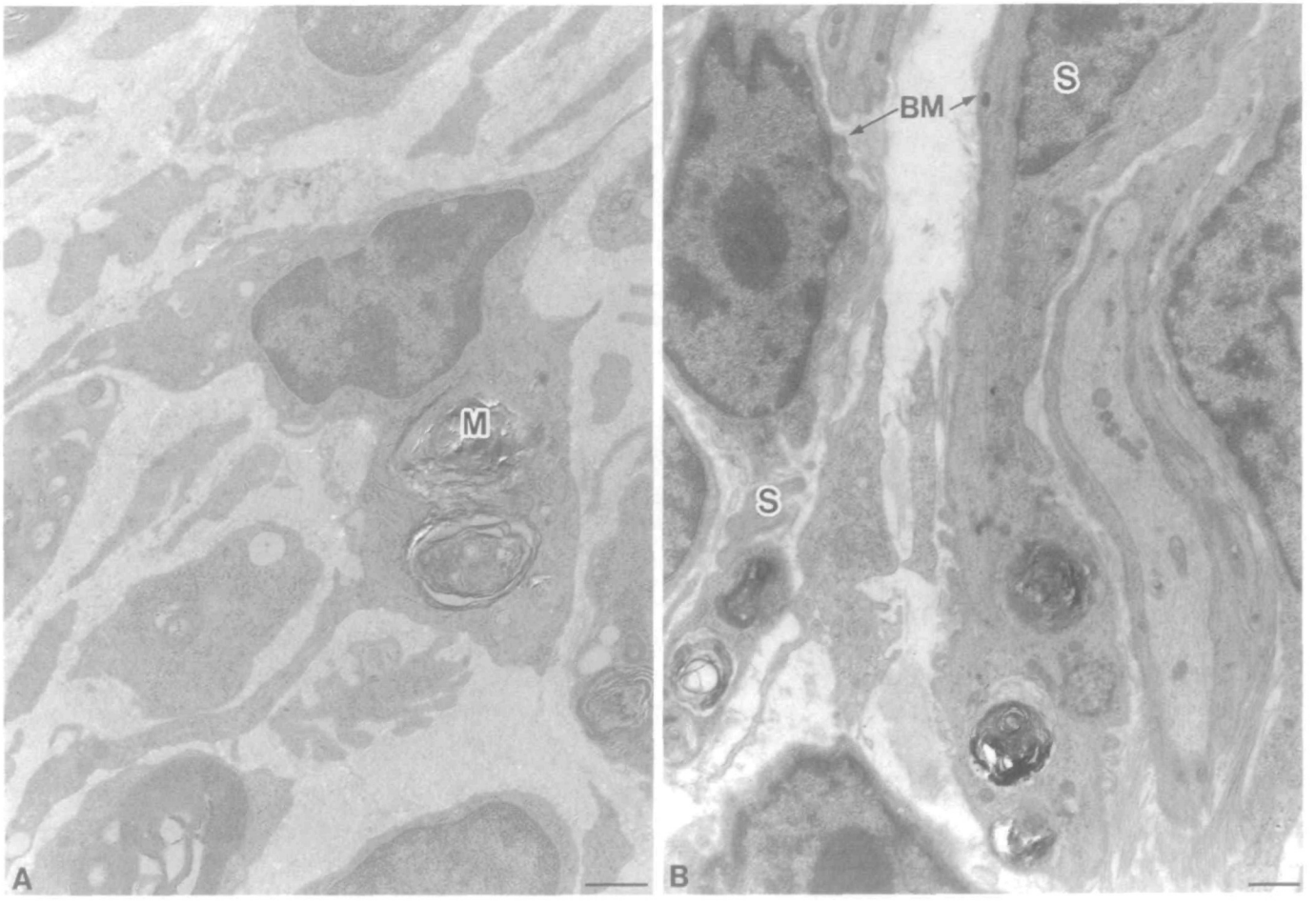

Figure 5 Electron micrographs of a nerve bundle in the lamina propria of circumvallate papillae. **(A)** Two days after denervation. Macrophages (M) contain myelin debris. **(B)** One day after denervation. Schwann cells (S) contain myelin and phagosomes. BM, basement membrane. Scale bar: 1 mm.

Schwann pathway. The phagocytotic activity of resident macrophages (ED2-positive) was observed on the day 2 after denervation. The timing of phagocytosis is similar to that of Wallerian degeneration in the peripheral nervous system, where Schwann cells and macrophages are responsible for phagocytosing degenerated axons after the transection of the rat sciatic nerve (Monaco *et al.*, 1992; Liu*et al.*, 1995). Furthermore, in the peripheral nervous system the invasion of monocyte-derived macrophages (ED1- positive) is seen at the transected site (Monaco *et al.*, 1992; Liu *et al.*, 1995). In the present study we could not detect the immunoreactivity for ED1 in the nerve bundles ofcircumvallate papillae. Interaction between Schwann cellsand macrophages has been reported: macrophages secrete cytokines and growth factors that play a role in inducing Schwann cells to proliferate, migrate to the transection site and produce neurotrophic factors for regeneration of nerve fibers (Pellegrino *et al.*, 1986; Stoll *et al.*, 1989; Griffin *et al.*, 1993; Liu *et al.*, 1995). Thus, the participation of macrophages and Schwann cells in phagocytosis is important for the removal of degenerated axons and probably for the regeneration of glosso-pharyngeal nerve.

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