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A Novel Monoclonal Antibody that Recognizes Apical Membrane of Frog Taste Cells

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Abstract

We established a hybridoma clone 1N1 that produced a monoclonal antibody to stain the apical portion of frog taste cells, by directly immunizing taste discs of the bullfrog (*Rana catesbeiana*) without any dispersion procedure of the taste organ. The antibody stained discrete regions on the surface of the taste discs, but did not stain the epithelium sheet of the tongue devoid of taste discs. The antibody stained ~93% of the taste discs tested (172/184) derived from nine frogs, showing that distribution of the antigen was common to most of the taste discs. The following observations strongly suggested that the antibody recognized a certain antigen on the apical membrane of the taste cells. (i) The antibody selectively stained cross points of intermucus areas on the surface of the taste disc. Neither the mucus cells nor the wing cells that mainly cover the surface were stained with the antibody. (ii) Dispersed taste cells were prepared by calcium ion chelating and subsequently by collagenase treatment to avoid digestion of the antigen. The antibody stained the apical end of the taste cells.

Introduction

Taste substances are initially recognized on the apical membrane of taste cells. To further understand the taste recognition system, it is important to identify the taste cell-specific molecules that must be potentially responsible for taste recognition. Molecules expressed on the apical membrane of the taste cells are especially interesting because they may possibly function as taste receptors.

A monoclonal antibody recognizing taste cells is of great use in the identification and isolation of the cells. In addition, it may be useful in the purification and gene cloning of taste cell-specific molecules possibly responsible for taste recognition, including putative taste receptors, as reported (Abe *et al.*, 1993; Matsuoka *et al.*, 1993).

With conventional methods, it has been difficult to generate such a monoclonal antibody, because it is difficult to obtain the large number of taste cells needed for complete immunization and screening. For proper immunization, it is preferable to prepare a pure taste cell population from tongue epithelium. However, we have no established technique for such a preparation. In the present research, we have attempted direct immunization of dissected taste discs of the bullfrog, and established a hybridoma clone that produced a monoclonal antibody which stained the apical end of the taste cells.

Materials and methods

Reagents.

A phycoerythrin (PE)-labelled second antibody against mouse IgG (U-M35004) was purchased from Dainippon Pharmaceutical Co. Ltd (Osaka, Japan). Normal goat serum was purchased from Cedarlane (Ontario, Canada). Murine IgG was obtained from OEM Concepts, Inc. (Toms River, NJ). Mouse myeloma cell line P3X63-Ag8.653 was maintained in RPMI 1640 medium (Gibco, Rockville, MD) supplemented with 5 mM glutamine and 10% FCS.

Immunization of frog taste discs

Dissected frog's taste discs as immunogen were prepared as follows. A wild bullfrog (*Rana catesbeiana*) weighing ~200–300 g was anaesthetized with an i.p. injection of 4 ml of 20% urethane solution. The tongue was excised and washed thoroughly with tap water and then with Ringer solution (112 mM NaCl, 3.4 mM KCl, 3.6 mM $MgSO_4$, 0.2 mM $CaCl_2$ and 2.5 mM $NaHCO_3$) in order to remove

the mucus from the tongue surface. Taste discs were dissected using fine scissors under a stereoscopic microscope and kept in Ringer solution at 4°C. Approximately 250 taste discs were collected from a single frog, washed with Ringer solution for three times and suspended in 0.5 ml of the solution. A 0.5 ml quantity of adjuvant (MPL and TDM emulsion, RIBI Immunochem Research Inc., Hamilton, MO) was added, and the mixture was injected i.p. into a BALB/c mouse with a thick needle (23 G, 0.65 × 32 mm) to avoid leaving any of the immunogen in the syringe. The taste discs were immunized four times at 2 week intervals. Seven days after the third injection, the immunized serum was collected to check the titre with immunofluorescent staining.

Cell fusion

Three days after the final immunization, splenocytes (3.4×10^8) obtained from the immunized mouse were fused with the murine myeloma cell line P3X63-Ag8.653 (5×10^7) by electrofusion. Hybridoma cells were plated into 96-well microtitre plates (Sumilon MS-80960, Sumitomo Bakelite Co., Japan), with each well containing 100 μl of HAT medium (RPMI 1640 medium containing 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin and 1.6×10^{-5} M thymidine), 20% FCS and 4% BriClone (BioResearch, Ireland). Murine thymocytes obtained from BALB/c mice were added as feeder cells. After 2 weeks, the culture supernatant was screened with immunofluorescent staining as described below. All positive colonies were subcloned two or three times according to standard protocol.

Immunostaining

Immunostaining was performed with the dissected taste discs or surgically prepared tongue epithelium sheet from the frog's tongue. The samples were washed three times with Ringer solution, suspended in 1 ml of Ringer solution containing 2% goat serum and stored for 90 min at room temperature for blocking. After removing the blocking solution, the samples were incubated with diluted serum (1/2500), culture supernatant of a hybridoma clone, or partially purified monoclonal antibody 1N1 or control murine IgG (5 μg/ml) for 12–16 h at 4°C with occasional shaking. After washing the samples three times, antibody binding was visualized using diluted PE-labelled antibody against mouse IgG (1/200) or a streptavidin–biotin–peroxidase kit (Histofine SAB-PO-M, Nichirei, Tokyo, Japan). After the staining, the samples were washed twice, transferred to a small well in a glass slide (Iuchi seieido Co. Ltd, Osaka, Japan) and observed using a fluorescence microscope (Optiphoto 2, Nikon, Tokyo, Japan).

Alternatively, frozen sections of the stained tongue epithelium sheet were cut in a cryostat set at 20 μm, mounted on glass slides coated with gelatin and observed with a fluorescence microscope.

Preparation of dispersed taste cells

Taste discs were washed three times with Ringer solution, suspended in Ca^{2+}-free Ringer solution containing 2 mM EGTA and incubated at 37°C for 20 min. The treated taste discs were washed three times with Ringer solution to eliminate EGTA, an inhibitor of collagenase, and incubated in collagenase solution (S-1, Nitta Gelatin Co. Ltd, 2 mg/ml in Ringer solution containing 1 mM of $CaCl_2$) for 20 min at 37°C. After the collagenase treatment, the dispersed cells were prepared by gently pipetting 30–40 times, washed a further three times with Ringer solution and observed using a fluorescence microscope.

Results

Immunized serum staining of frog taste disc

After the three repeated immunizations, the immunized serum was obtained to check the titre with immunofluorescent staining. The immunized serum stained the taste disc strongly, while the preimmunized serum did not. The stained areas were restricted in the intermucus areas, especially the cross points of the areas supposed to be the apical portions of taste cells and wing cells (Figure 1A), the ciliated cells surrounding the surface of the taste disc (Figure 1B) and unidentified cells located on the lateral face of the taste disc (Figure 1C). In contrast, mucus cells, which mainly cover the surface of the tongue, were scarcely stained. Figure 1D represents the location of the stained cells in the taste disc structure. The diluted serum (1/10000) clearly stained the taste disc, supporting the conclusion that the immunization was completed for obtaining a hybridoma that produced a monoclonal antibody to stain the taste disc.

Monoclonal antibody 1N1 staining of the frog taste disc

After repeated screening with immunofluorescent staining, we obtained a hybridoma clone (1N1) which produced a monoclonal antibody to stain the surface of the taste disc. We failed to obtain a clone that produced antibody to stain the mucus cells, wing cells, ciliated cells or cells on the lateral face of the disc that were stained with the immunized serum. The subtype of the antibody was identified as IgG_1 containing a κ-chain, using an Amersham mouse monoclonal antibody typing kit (RPN29).

The frog taste disc was stained with the monoclonal antibody 1N1. The stained regions appeared as discrete spots on the surface of the taste disc. Cells on the lateral face of the disc were scarcely stained (Figure 2A, D). The taste disc was scarcely stained with control IgG solution (Figure 2B, E). The antibody stained ~93% of the taste discs tested (172/184) derived from nine frogs, showing that distribution of the antigen was common to most of the taste discs. To

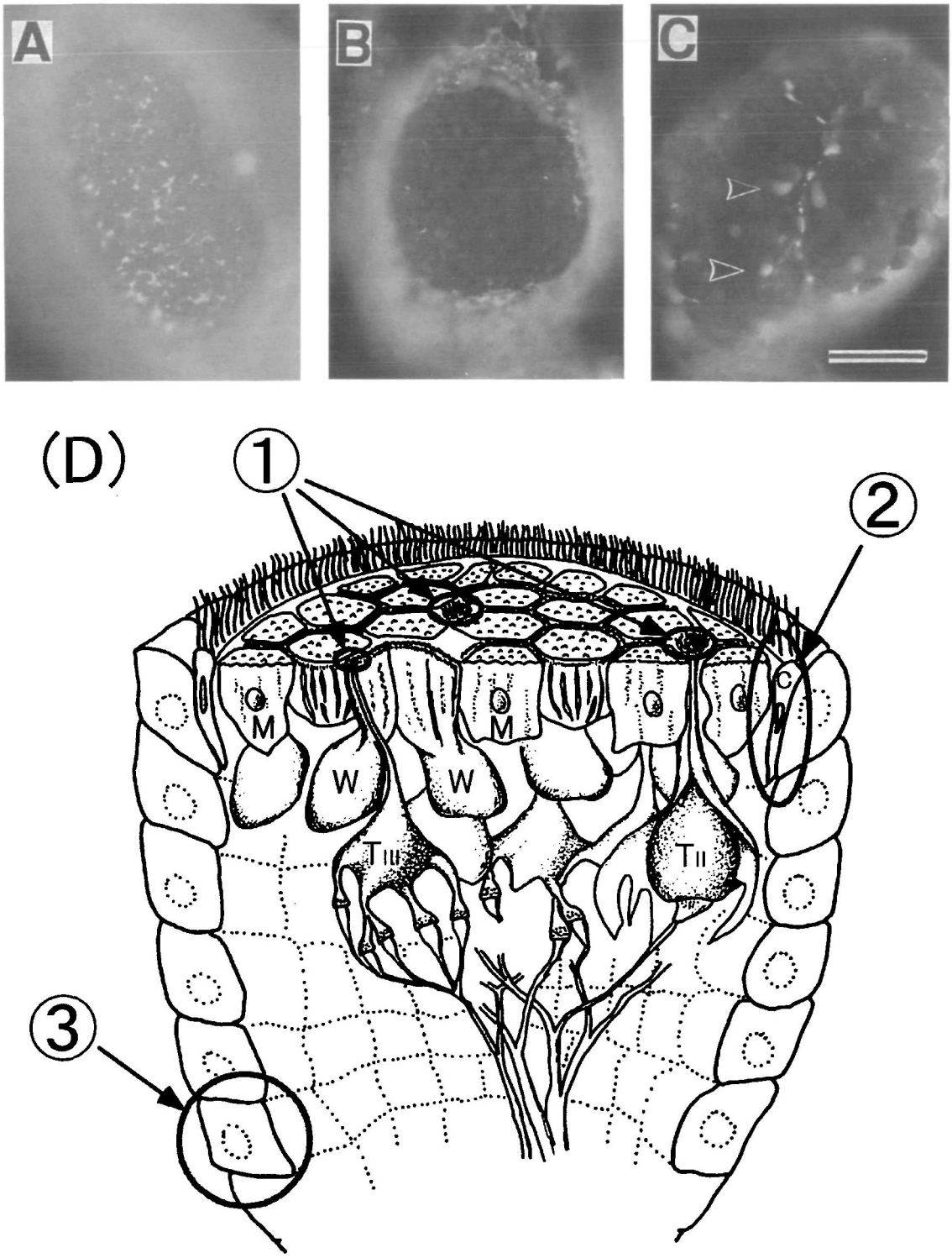

Figure 1 Immunized serum staining the frog taste disc. **(A)** The diluted serum (1/2500) stained intermucus areas on the surface of the disc. The staining was visualized with fluorescein isothiocyanate-labelled anti-mouse IgG antibody. **(B)** The diluted serum stained ciliated cells surrounding the surface of the taste disc. The staining was ambiguous in the image because of rapid movement of the stained cilia. Note that the staining of the intermucus area was not clear because of an inadequate focus position for observation of the area. **(C)** The diluted serum stained unidentified cells on the lateral face of the disc (arrowheads). The micrograph was taken from the bottom of the taste disc. The bar represents 100 μm. **(D)** Schematic drawing representing the frog taste disc [modified from the originals illustrated by Graziadei and DeHan (1971) and Osculati and Sbarbati (1995)]. Numbers 1, 2 and 3 indicate cross points of intermucus areas, a ciliated cell and an unidentified cell on the lateral face of the disc respectively. T_{II}, taste cell (type II); T_{III}, taste cell (type III); M, mucus cell; W, wing cell; C, ciliated cell.

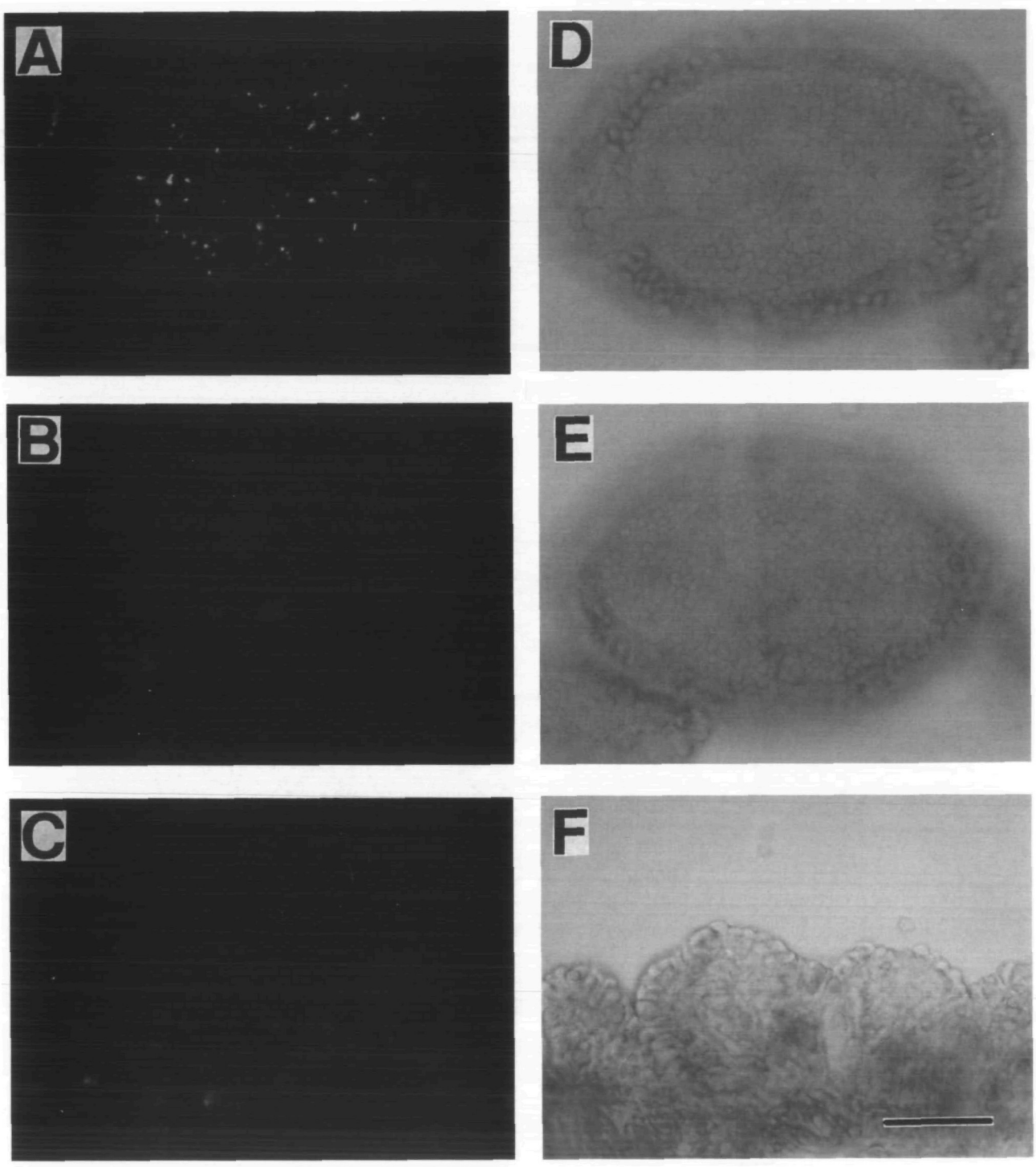

Figure 2 Monoclonal antibody 1N1 staining discrete regions on the surface of the frog taste disc. **(A–C)** Fluorescence micrographs; **(D–F)** light micrographs. (A, D) A frog's taste disc incubated with monoclonal antibody 1N1. The fluorescence-positive regions appeared to be discrete spots on the surface. (B, E) A frog's taste disc incubated with control IgG. (C, F) A tongue epithelium sheet incubated with monoclonal antibody 1N1. The bar represents 100 μm.

confirm that the staining was restricted on the taste disc, the epithelium sheet of frog tongue devoid of taste disc was incubated with the monoclonal antibody. The staining was negative (Figure 2C, F).

Monoclonal antibody 1N1 staining cross points of intermucus areas on the taste disc surface

To examine the distribution of the antigen more precisely, the staining on the surface of the taste disc was visualized

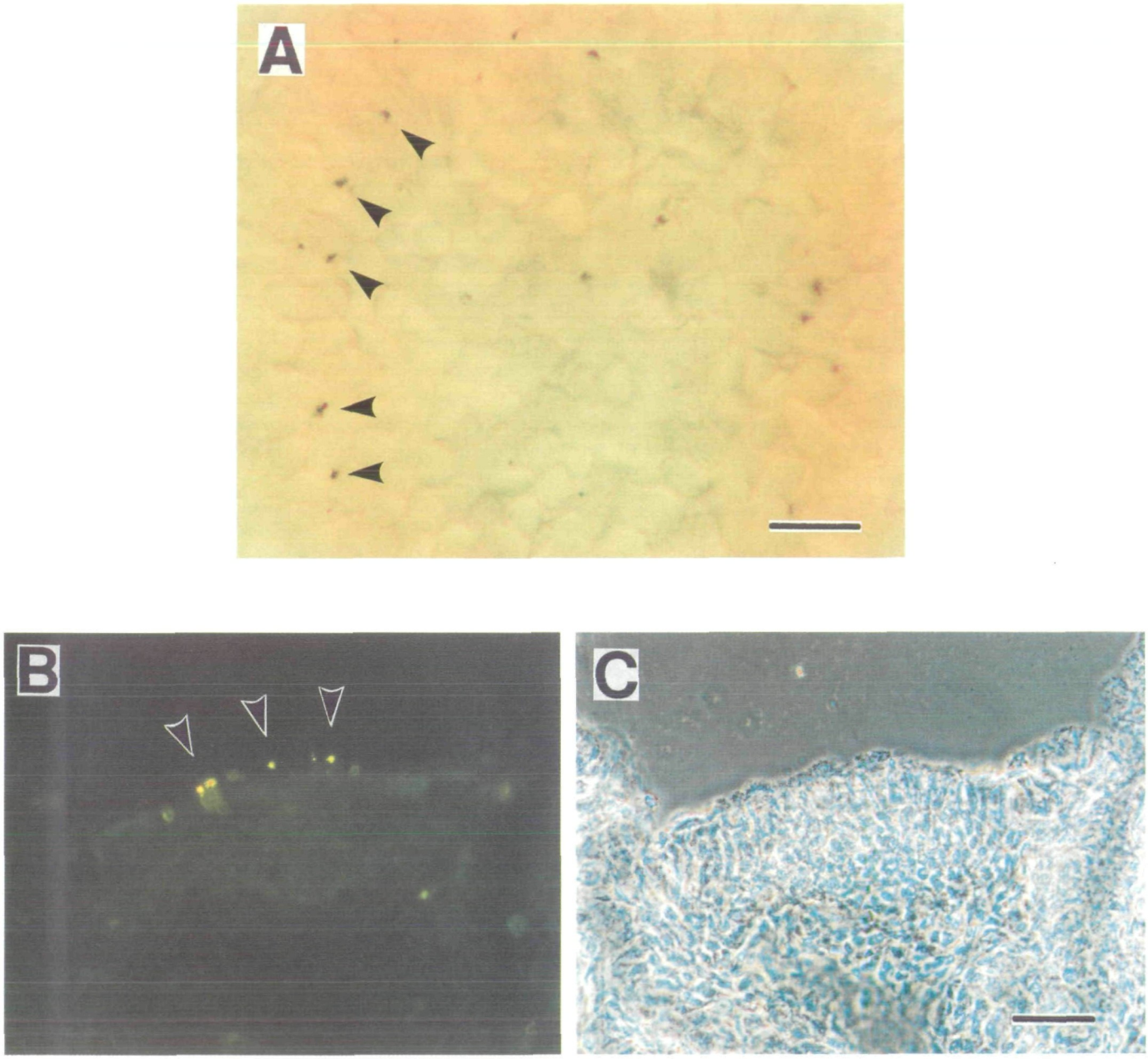

Figure 3 Monoclonal antibody 1N1 staining the cross points of intermucus areas on the surface of the frog's taste disc. **(A)** An enlarged image of the surface of a taste disc stained with monoclonal antibody 1N1. Staining was visualized using diaminobenzidine as the chromogen. The antibody stained cross points of intermucus areas on the surface (arrowheads). The bar represents 20 μm. **(B, C)** A sliced section of a tongue epithelium sheet stained with monoclonal antibody 1N1. The fluorescent-positive regions appeared to be discrete spots on the taste disc surface (arrowheads). The thickness of the section was 20 μm. (B) Fluorescent micrograph; (C) light micrograph. The bar represents 100 μm.

using diaminobenzidine as the chromogen. In an enlarged image, the antibody stained a small part of the cross points of intermucus areas on the surface (Figure 3A), which corresponded to the exposed apical portion of taste cells (Sbarbati *et al.*, 1990; also indicated as 1 in Figure 1D). Neither the mucus cells nor the wing cells that mainly cover the surface were stained with the antibody.

Next we prepared sliced sections of the stained tongue epithelium sheet containing a taste disc for observation from the lateral view. The stained regions appeared as discrete spots on the surface of the taste disc (Figure 3B).

Monoclonal antibody 1N1 staining of the apical portion of dispersed taste cells

We tested the staining of dispersed taste cells to confirm that the antibody stained the apical portion of the taste cells. In a preliminary trial, taste cells dispersed with collagenase treatment lost their elongated shape during the

staining procedure. To prevent morphological change of the taste cells, we prepared dispersed taste cells after the complete immunostaining of the taste discs. Dispersed taste cells were prepared by calcium ion chelation with EGTA and subsequent collagenase treatment to avoid digestion of antigen molecules and antibodies labelling the taste discs.

The antibody stained the top of the dendrite of the elongated cells, i.e. the apical end of the taste cells (Figure 4A–C, E–G). A large part of the stained cells possessed branched processes at the basolateral end (Figure 4E, F). The rest of the stained cells lacked such a branched process (Figure 4C, G). Neither mucus cells with a round shape (Figure 4A–C, E–G) nor wing cells (Figure 4D, H) were stained in the preparation. This strongly suggested that the antibody recognized a certain antigen on the apical membrane of the taste cells. Taste cells dispersed with papain lost the staining, which was probably due to proteolytic digestion of antigen molecules or antibodies labelling the taste discs (data not shown).

Discussion

In the present paper, we demonstrated the generation of a monoclonal antibody to stain the apical portion of frog taste cells with the following observations. First, the antibody stained cross points of intermucus areas on the surface of the taste disc, where the apical membrane of the taste cells is localized (Figure 3). Second, the antibody stained the apical end of the dispersed taste cells (Figure 4). The results strongly suggested that the antibody recognized a certain antigen on the apical membrane of the taste cells. So far as we know, this is the first trial to establish a monoclonal antibody that recognizes a taste cell-specific molecule by direct immunization of the taste organ.

For the immunization, we directly injected frog taste discs as immunogen without any dispersion procedure. We expect that the frog system was more suitable for obtaining a monoclonal antibody than a mammalian system for the following reasons. (i) The frog has larger number of taste cells on a tongue than mammals (von Düring and Andres, 1976; Miller and Smith, 1984). Thus we have continuously used the frog system for our biochemical studies (Nakamura *et al.*, 1994). (ii) The very small exposed surface of the mammalian taste bud is not suitable for immunization because of poor accessibility to the immune system. On the other hand, the apical portion of the frog's taste cells is distributed on a wide and plain surface of the taste disc (~200 μm in mean diameter), and is well exposed to the extracellular environment.

We performed the direct immunization with dissected taste discs but not with dispersed taste cells. It is preferable to prepare a pure taste cell population from tongue epithelium. However, we have no established technique for this preparation. Furthermore, dispersion with any enzyme gives rise to the following disadvantages. (i) The dispersion with proteolytic enzymes may lead to digestion of taste cell-specific molecules. (ii) The dispersed taste cells easily lose their elongated shape, which might affect the expression of taste cell-specific molecules. (iii) The cells hidden inside the disc will be exposed after the dispersion, which may decrease the immunization efficiency of the apical portion of the taste cells.

The antibody stained a small part of the cross points of intermucus areas on the surface (Figure 3A), suggesting that it recognizes a small subset of taste cells that express the antigen. The staining pattern of the dispersed taste cells (Figure 4A–C) supported this suggestion. The stained cells showed the following morphological features. (i) They appeared as elongated cells with a long dendrite. (ii) The top of the dendrite was quite thin. The size of the top was estimated as ~1–2 μm in Figures 3A and 4A–C. (iii) A large part of the stained cells possessed branched processes at the basolateral end. It is likely that the cells are defined as type III cells in the definition of Sbarbati *et al.* (1990) and Osculati and Sbarbati (1995). (iv) The rest of the stained cells lacked such a branched process (Figure 4C, G). It is uncertain whether the cells should be defined as type II cells or type III cells that have lost the branched structure during the dispersion procedure. In the preparation of the dispersed cells, the antibody did not stain all the elongated cells. This may be because some of the cells lost their stained region during the dispersion procedure, or because the antibody recognized only a small subset of taste cells.

The monoclonal antibody was raised against a restricted apical portion of the taste cells, but not against the mucus cells or wing cells that are more abundant on the surface of the taste disc. This is interesting because the apical portion of the taste cells occupies only 6% of the total surface of the taste disc (Sbarbati *et al.*, 1990). Our findings suggest the following possibilities. First, highly antigenic molecules, including potent receptor molecules for taste substances, exist on the apical portion of the taste cells. Second, the apical portion of the taste cells is efficiently incorporated in antigen-presenting cells, as the size of the apical portion is only 2–3 μm or even smaller (Sbarbati *et al.*, 1990; Osculati and Sbarbati, 1995). Especially, vesicles with a diameter of 10 nm on the apical membrane of the frog's taste cells (Röhlich and Pevzner, 1982) must be efficiently incorporated in the antigen-presenting cells. On the other hand, mucus cells are large (~20 μm in diameter) and completely covered with mucus, which functions as a barrier against antigen-presenting cells.

The antigenic moiety recognized by the monoclonal antibody remained unclear. Surface antigen molecules, such as Lewis^b antigen (Akabas *et al.*, 1988), the amiloride-sensitive sodium channel (Simon *et al.*, 1993), the cyclic nucleotide-activated channel (Misaka *et al.*, 1997), Ca^{2+}-dependent ATPase (Barry, 1992), calcium binding proteins (Kershbaum and Hermann, 1992) and cytokeratin (Zeng *et al.*, 1995), have been identified on the apical membrane of

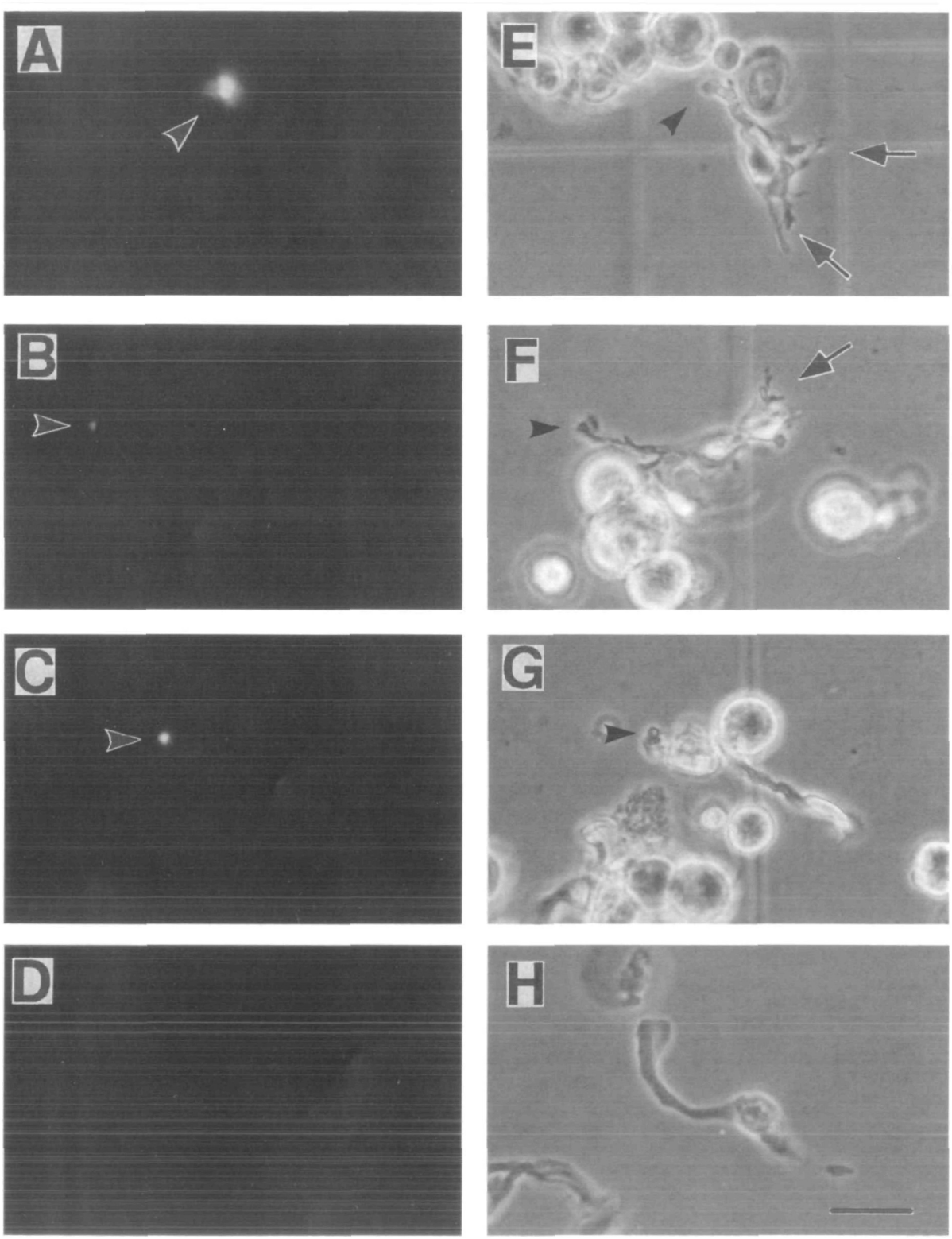

Figure 4 Monoclonal antibody 1N1 staining of the apical portion of dispersed taste cells. **(A–D)** Fluorescence micrographs; **(E–H)** light micrographs. The antibody stained the apical end of the taste cells. Arrowheads show the corresponding position in the images. (A, B, E, F) The stained type III taste cells possessed branched processes at the basolateral end (E, F, arrows). (C, G) The stained taste cells, probably defined as type II cells, lacked such a branched process. (D, H) Wing cells were not stained with the antibody. The bar represents 20 μm.

mammalian taste cells. The monoclonal antibody may recognize a counterpart of the molecules in the frog. Alternatively, the antigen may be cell-associated extracellular material on the surface of the taste disc. The surface of the frog taste disc was strongly stained with lectins such as *Dolichos biflorus* agglutinin or soybean agglutinin (Witt and Reutter 1988), indicating that the surface is rich in carbohydrates. Sbarbati *et al.* (1990) showed that the apical portion of the frog's taste cells was heavily stained with ruthenium red, cationized ferritin and potassium pyroantimonate. They speculated that the microenvironment was rich in phospholipid, anionic sites suitable for Ca^{2+} trapping, and the Ca^{2+}. Such particular extracellular material itself, or a molecule associated with the material, may be the antigenic moiety recognized by the antibody. We attempted to detect the antigen by Western blotting, but no clear band was observed (data not shown). We speculate that the antigen determinant was easily unfolded with detergent, or the antigen was a large molecule so that the blotting was not successful.

The antibody is available for the following research. First, we can identify a subset of taste cells with the antibody. It is possible to examine the involvement of the stained cells in taste recognition with physiological techniques. Next, we may be able to identify a molecule specifically expressed on the apical membrane of taste cells. Our trial of Western blotting was unsuccessful, but the antibody may be available for affinity chromatography or immunoprecipitation, because the antigen will keep its native structure under the experimental procedures. Research is ongoing to clarify the molecular properties of the antigen recognized by the antibody.

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