Human blood group antigen H is not the specific marker for type I cells in the taste buds*

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Summary. We examined the localization of human blood antigen H (AbH) and its correlation with other cell type markers in the taste buds of circumvallate papillae of the adult rat. Immunoreactivity for AbH was localized in the membrane of two cell populations in the taste buds: in spindle-shaped cells extending from base to the apical portion of the taste buds as well as in round-shaped cells at the basal portion of the taste buds. Quantitative analysis revealed that approximately 47.8%, 24.4%, and 14.6% of cells within the taste buds displayed AbH-, α -gustducin- or protein gene product 9.5 (PGP 9.5)-immunoreactivity, respectively. Approximately 16.3% and 6.6% of AbH-immunoreactive taste bud cells displayed α -gustducin- or PGP 9.5-immunoreactivity, respectively. Although previous studies proposed that AbH immunoreactivity was specific for type I cells (dark cells or supporting cells), the present results indicate that AbH immunoreactivity is also present in some type II cells (α -gustducin immunoreactive cells) and type III cells (PGP 9.5-immunoreactive cells).

Introduction

In mammals, the taste buds are localized mostly within two populations in the oral epithelium, i.e. the lingual and palatal epithelium. In addition, a few taste buds are present in the laryngeal surface of the epiglottis. In the lingual epithelium of the rat, taste buds are localized in three lingual papillae: fungiform, foliate and circumvallate. Taste buds contain proliferative basal cells (progenitor cells) as well as 50–80 elongated epithelial cells (taste cells). Taste cells have traditionally been classified into three types of cells: dark, light and basal. Dark cells or type I cells have an electron-dense cytoplasm, and are thought to be supporting cells. Light cells are characterized by the presence of an electron-lucent cytoplasm, and are further divided into two types in regard to the presence of synaptic vesicles. Light cells without apparent synaptic vesicles are called type II cells, while those with synaptic contact with intragemmal nerve fibers and containing synaptic vesicles are termed type III cells. A number of histochemical studies have focused on distinguishing the cell types on the basis of their expression of chemical substances. It has been shown that many bioactive substances are contained in light cells. For example, type II cells display immunoreactivity for α -gustducin (Boughter *et al.*, 1997; Cho *et al.*, 1998; Yang *et al.*, 2000b), and type III cells reveal protein gene product 9.5 (PGP 9.5) (Iwanaga *et al.*, 1992; Kanazawa and Yoshie, 1996; Yee *et al.*, 2001), a neural cell adhesion molecule (N-CAM) (Takeda *et al.*, 1992; Nelson and Finger, 1993), neuron-specific enolase (NSE) (Yee *et al.*, 2001), SNAP-25 (Yang *et al.*, 2000a), and serotonin (5-HT) (Takeda *et al.*, 1981; Kim and Roper, 1995; Yee *et al.*, 2001). In contrast, little is known about the histochemical properties of type I cells. To our knowledge, carbonic anhydrase isoform II (Daikoku *et al.*, 1999) and glutamate-aspartate transporter (GLAST; Lawton *et al.*, 2000) have been localized in type I

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cells. Smith *et al.* (1994) reported that human blood group antigen H (AbH) is localized on the surface of all types of taste cells. Pumplin *et al.* (1999), however, demonstrated that AbH is likely present on the surface of dark cells where processes lie between the light cells. Consequently, most researchers designated AbH as a reliable marker for dark cells. This study aimed to demonstrate the expression pattern of AbH in the circumvallate papillae of adult rats to investigate this discrepancy. Moreover, the relationship between AbH, α -gustducin, and PGP 9.5 in the taste cells was examined by the double immunofluorescence method.

Materials and Methods

A total of 7 male Sprague-Dawley rats, weighing 150–200 g, were used in this study. All animals were deeply anesthetized with chloral hydrate (600 mg/kg, i.p.) and transcardially perfused with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in a 0.1 M phosphate buffer (PB) followed by 0.02 M phosphate-buffered saline (PBS). Their tongues were carefully removed, and circumvallate papillae were dissected out and further fixed in 4% paraformaldehyde in 0.1 M PB for additional 2–3 days. Following a rinse in 20% sucrose/PBS overnight at 4°C, some of the tongues (n=5) were dehydrated through an ascending series of ethanol, embedded in paraffin, cut at a thickness of 3 μ m, and processed for the indirect immunofluorescence method or avidin-biotin-horseradish peroxidase (ABC) method. Parallel to this, the other tongues (n=2) were cut at a thickness of 50 μ m with a cryostat, collected in cold PBS, treated as free-floating sections, and processed for the ABC method. All animal experiments were reviewed and approved by Intramural Animal Use and Care Committee at the Osaka University Graduate School of Dentistry prior to the experiments (Project # 03-002).

Paraffin sections were deparaffinized with xylene, rehydrated through a descending series of ethanol, rinsed in PBS, and processed for the ABC method. Sections were treated with PBS containing 0.09% H₂O₂ for 30 min to block the endogenous peroxidase activity, and then with PBS containing 3% normal horse serum (NHS; Vector Laboratories, Burlingame, CA, USA) and 1% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) to reduce the non-specific binding. Sections were then incubated with monoclonal anti AbH (1:1000; clone 92-FR-A2, Dako, Copenhagen, Denmark) for 16–18 hours at 4°C. Sections were subsequently incubated with biotinylated anti-mouse IgM (1:100; Vector), and further with an ABC complex (Vector) for 90 min each at room temperature. The antigen-antibody binding sites were visualized by incubation with

0.04% diaminobenzidine (DAB) and 0.008% H₂O₂ in a 0.05 M Tris-HCl buffer (pH 7.5) with nickel ammonium sulfate (NAS) enhancement. The immunostained sections were counterstained with methyl green, dehydrated through an ascending series of ethanol, cleared with xylene, and cover-slipped with Permount (Fisher Scientific Inc., New Jersey, USA).

Floating sections were treated for ABC immunohistochemistry as mentioned above, except that NAS enhancement was omitted. After visualization of the HRP products, sections were further fixed in 1% OsO₄ reduced by 1.5% potassium ferrocyanide, dehydrated through an ascending series of ethanol, transferred to propylene oxide, and finally embedded in Epoxy resin. Semithin sections were prepared and stained with toluidine blue.

For double immunofluorescence staining, deparaffinized sections were first incubated with a monoclonal antibody against AbH (1:200) for 90 min at room temperature, and then with FITC-conjugated anti-mouse IgM (1:100; Vector) for 60 min. Sections were subsequently incubated with polyclonal anti- α -gustducin (1:200; Santa Cruz Biotechnologies Inc., Santa Cruz, CA, USA) or with polyclonal anti-PGP 9.5 (1:1000; Ultraclone, England) and then with Cy3-conjugated anti-rabbit IgG (1:1000, Molecular Probes, OR, USA) for 90 min each at room temperature. Following cover-slipping with Vectashield (Vector), they were examined with a fluorescein microscope (Axioskop 2 plus; Carl Zeiss, Germany). Images were captured by CCD camera (AxioCam, Carl Zeiss), and transferred to Adobe Photoshop (Ver. 5.0). After observation, cover-slips were carefully removed by extensive rinses in PBS, and the sections were subjected to hematoxylin-eosin (HE) staining for general histological observation.

Quantitative analyses were performed from 20 randomly selected fields of taste buds of cross-cut sections, mostly taken from the middle portion of the taste buds. The numbers of cells showing immunoreactivity for AbH, α -gustducin, and PGP 9.5 were counted. Only cells having nuclear profiles were analyzed. As the immunoreaction for AbH did not surround the surface of the cells completely (see Results), cells exhibiting immunoreactivity over more than three fourths of the cellular outline were considered "immunopositive cells". The percentages of immunoreactive cells to the total number of cells within the taste buds were calculated and presented as mean $\pm$ SD.

The characterization and origin of primary antibodies against AbH and PGP 9.5 have been reported in the literature (Smith *et al.*, 1994 for AbH; Gulbenkian *et al.*, 1987 for PGP 9.5).

Results and Discussion

In the present study, the immunoreactivity for AbH was constantly localized in taste bud regions. Intensive immunoreactions apparently outline the whole taste bud, and these were detected on the majority of taste bud cells. Occasional immunoreactions were also demonstrated on cells in the spinous layer of the lingual epithelium of the circumvallate papillae (Fig. 1A). Within the taste buds, semithin sections revealed that AbH-immunoreactivity was restricted to the membranes of two precise populations of cells: spindle-shaped cells extending from basal to the apical portion, and rounded cells at the basal portion (Fig. 1B). It was evident that the immunoreactions for AbH surrounded the cell surface of individual cells; however, this was incomplete for the most of them as revealed in cross-sections (Fig. 1C).

Cross-sections also clearly revealed that AbH-immunoreactivity was localized on the surface of cells (Fig. 2A, 3A). Immunoreactivity for α -gustducin was homogeneously distributed in the cytoplasm of taste bud cells (Fig. 2B). Some of the AbH-immunoreactive cells showed α -gustducin immunoreactivity, and some α -gustducin-immunoreactive cells had an AbH-immunoreactive membrane (Fig. 2C). A total of 320 cells (16.0 ± 4.8 cells/taste bud) were analyzed. Approximately 48.8% (156/320) of the cells (7.8 ± 3.1 cells/taste bud) and 24.4% (78/320) of the cells (3.9 ± 1.5 cells/taste bud) showed immunoreactivity for AbH and α -gustducin, respectively. Approximately 16.3% (44/320) of the cells exhibited immunoreactivity for both AbH and α -gustducin. Two thirds of the α -gustducin immunoreactive cells showed AbH-immunoreactivity, and one third of AbH-immunoreactive cells exhibited immunoreaction for α -gustducin (Fig. 2E).

Protein gene product 9.5-immunoreactivity was displayed by some taste bud cells and dot-like structures (Fig. 3B). The cellular membrane of some PGP 9.5-immunoreactive cells displayed AbH-immunoreactivity. Dot-like PGP 9.5 immunoreactive structures were closely associated with the cell membrane (Fig. 3B, C). For quantitative analysis, a total of 302 taste bud cells having nuclear profiles were analyzed (15.1 ± 4.3 cells/taste bud) to examine the correlation between AbH and PGP 9.5. Approximately 47.4% (143/302; 7.2 ± 2.2 cells/taste bud) and 14.6% (44/302; 2.2 ± 1.2 cells/taste bud) of the taste cells displayed AbH- and PGP 9.5-immunoreactivity, respectively. About 6.6% (20/302; 1.0 ± 1.0 cells/taste bud) exhibited both AbH- and PGP 9.5-immunoreactivity. About 45.5% (20/44) of the PGP 9.5-immunoreactive cells showed AbH-immunoreactivity, while 14.0% (20/143) of the AbH-immunoreactive cells exhibited PGP 9.5-immunoreactivity (Fig. 3E).

Fig. 1. Microphotographs of immunoreactivity for AbH. **A:** AbH-immunoreactivity is present at the membrane of the taste cells and in the spinous layer of the trench wall (arrow). Scale bar: 50 μ m. **B and C:** Higher magnification of AbH-immunoreactivity in the taste buds. Immunoreactions are restricted to the cell membrane of the majority of taste bud cells in longitudinal and cross sections, respectively. Some cells are completely surrounded and others are incompletely surrounded by AbH-immunoreactivity. Scale bars: 10 μ m

The present immunohistochemical study demonstrated the localization of AbH, which thought to be a specific marker of type I cells (dark cells or supporting cells), and its correlation with other types of cells in the circumvallate papillae of adult rats. The localization of AbH immunoreactivity in the lingual papilla and epithelium of the trench wall of the circumvallate papillae agrees with the results by Smith *et al.* (1994) and Pumplun *et al.* (1999). In the taste buds, AbH-immunoreactivity was detected in the cell membrane of some taste bud cells. The population of type I cells

Fig. 2. Paired photographs of AbH- (A) and α -gustducin-immunoreactivity (B). C: Merged image of A and B. D: The same section stained with HE. Asterisks indicate the same cell profiles showing both AbH- and α -gustducin-immunoreactivity. Scale bar in A: 20 μ m. E: A bar graph showing the frequency of taste bud cells with AbH only (green), AbH and α -gustducin (yellow), and α -gustducin only (red).

has been thought to comprise approximately 60% of the entire taste bud cell population. The present quantitative analysis revealed that approximately 47.8% of the taste buds showed AbH-immunoreactivity, and that 16.3% and 6.6% of the AbH-immunoreactive cells have α -gustducin and PGP 9.5-immunoreactivity, respectively. A rough calculation indicates that approximately 25% of the taste bud cells have AbH-immunoreactivity only. These cells might belong to type I cells, since type I cells do not express α -gustducin or PGP 9.5 immunoreactivity. However, the percentage calculated in the present study might be underestimated since we defined AbH-immunoreactive cells as

Fig. 3. Paired photographs of AbH- (A) and PGP 9.5-immunoreactivity (B). C: Merged image of A and B. D: The same section stained with HE. Asterisks indicate the same cell profiles showing both AbH- and PGP 9.5-immunoreactivity. Note that many dot-like PGP 9.5-immunoreactive structures (arrows in B and C) are closely related to the cell membrane. Scale bar in A: 20 μ m. E: A bar graph showing the frequency of taste bud cells with AbH only (green), AbH and PGP 9.5 (yellow) and PGP 9.5 only (red).

"cells having immunoreactivity over more than 3/4 of the entire cell surface". It is speculated that not all type I cells have AbH immunoreactivity.

Yang *et al.* (2000b) demonstrated α -gustducin in the some light cells lacking the synaptic contact with afferent nerve fibers. This type of cell is categorized as a type II cell. It is generally believed that approximately 20–25% of the taste bud cells are type II cells. In the present quantitative analysis, approximately 24.4% of the taste cells exhibited α -gustducin, suggesting that most of the type II cells do show α -gustducin immunoreactivity (see below). Although some type II cells have been reported to lack the

immunoreactivity for α -gustducin (Yang *et al.*, 2000b), in our study, we conclude that the percentage of type II cells without α -gustducin immunoreactivity likely seems very low.

PGP 9.5-immunoreactivity is present in the nerve fibers and cytoplasm of some spindle-shaped cells (Iwanaga *et al.*, 1992; Kanazawa and Yoshie, 1996; Yee *et al.*, 2001). In the present study using cross-sections, round-shaped and dot-like PGP 9.5-immunoreactive structures were recognized. The round-shaped structures were enclosed within the cell outline, suggesting the immunoreactions were localized within the cytoplasm. Dot-like PGP 9.5-immunoreactive structures in close association with the cell membrane have been distinguished as nerve fibers. Previous ultrastructural studies showed that PGP 9.5-immunoreactive cells having synaptic contact with afferent nerve fibers are type III cells (Kanazawa and Yoshie, 1996; Yee *et al.*, 2001). In addition, Yee *et al.* (2001) reported that this protein is also present in a small number of type II cells, and provided evidence that PGP 9.5-immunoreactive type II cells are immunonegative for α -gustducin. These lines of evidence suggest that there are probably at least two types of type II cells: α -gustducin (+)/PGP 9.5 (-) cells and α -gustducin (-)/PGP 9.5 (+) cells. In the present study, we could not determine whether PGP 9.5-immunoreactive cells belong to type II cells or type III cells. However, approximately 15% of the cells displayed immunoreactivity for PGP 9.5, indicating that the majority of type III cells exhibit PGP 9.5.

In conclusion, the present study clearly demonstrated that the AbH immunoreactivity generally considered a specific marker for type I cells (dark cells or supporting cells) is also localized in two other types of cells in the circumvallate papillae of adult rats.

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