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ORIGINAL ARTICLE

Cholera toxin B subunit-binding and ganglioside GM1 immuno-expression are not necessarily correlated in human salivary glands

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Abstract

Objective. To determine and compare the presence and *in situ* localization of the glycosphingolipid ganglioside GM1 in human salivary glands using the biomarkers for GM1: cholera toxin and antibodies against GM1. ***Materials and methods.*** Immunohistochemical analyses were performed on sections of adult human submandibular, parotid and palatinal glands using cholera toxin sub-unit B and two polyclonal antibodies against ganglioside GM1 as biomarkers. ***Results.*** Immunofluorescence microscopy showed that the toxin and antibodies were co-localized in some acini but not in others. The cholera toxin mainly reacted with the cell membranes of the mucous acini in the submandibular gland, while incubation with the antibody against GM1 gave rise to a staining of the cytoplasm. The cytoplasm in some secretory acinar cells in the parotid gland was stained by the cholera toxin, whereas only small spots on the plasma membranes reacted with anti-GM1. The plasma membranes in the parotid excretory ducts appeared to react to anti-GM1, but not to cholera toxin. ***Conclusions.*** Cholera toxin induces the expression of ion channels and carriers in the small intestine and increases the production of secretory mucins. Although their mutual immunohistochemical localization may differ, both cholera toxin and ganglioside GM1 are present in the mucin-producing acini from salivary glands. This could point to a relationship between ganglioside expression and production of salivary mucins.

Key Words: *ganglioside GM1, cholera toxin, salivary glands*

Introduction

Cholera toxin is a protein produced by *Vibrio cholera*. It consists of two sub-units: sub-unit B is responsible for receptor binding; sub-unit A is the toxic-active sub-unit [1]. The Gram-negative bacterium causes watery diarrhea by producing the toxin that binds to GM1 gangliosides in the plasma membrane of intestinal epithelial cells [2]. GM1 is a glycolipid that belongs to the glycosphingolipid family. It is a monosialo-ganglioside with the structure: Galβ1-3GalNAcβ1-4[Neu5Acα2-3]Galβ1-4Glcβ1-Ceremide. Being located in the outer leaflet of the plasma membrane, its carbohydrate moieties function as receptors for bioactive compounds such as bacteria, bacterial toxins and vira.

In the intestine, the toxin–GM1 complex travels from the plasma membrane to the endoplasmic reticulum; part of the A-sub-unit is transported to the cytosol where it mediates net secretion of electrolytes and water from the serosal to the mucosal side of the epithelium [3].

GM1 is found in small intestine epithelial cells, on thymocytes, neurons and oligodendroglia, in liver cells, nerve cells, etc. [4–6]. GM1 influences a large variety of processes, such as: loss of membrane Na^+, K^+-ATPase in the injured brain, improved motor function in Huntington's disease and the onset of apoptosis [7–9].

The ganglioside is the membrane receptor for cholera toxin. Therefore, *in situ* histochemical detection of GM1 may be attained by using cholera toxin as a marker in sections of the intestine [10]. However, the correlation between cholera toxin and GM1 in other mucin-producing cells is not well established. Although injections of cholera toxin into salivary glands trace the innervating facial nerves [11], there seems to be a lack of studies that demonstrate whether cholera toxin can be used as a marker for GM1 in the

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various human salivary glands and, thus, be used as an indicator for salivary mucin secretion. Carbohydrates, either in the form of glycolipids or glycoproteins, are secreted from many epithelial cells. Nowroozi et al. [12] identified GM1 in the striated ducts and the acinar cells in the human parotid gland. They suggest that this glycolipid plays an important role in the secretory function of the parotid gland. Furthermore, Lencer et al. [13] showed that exposure of the intestinal mucosa to cholera toxin results in mucus secretion from intestinal goblet cells and that this increase in mucin secretion is believed to be regulated via classic transduction pathways [14,15].

Salivary glands express different mucins in both the membrane-associated and the secreted forms. Initial intestinal adherence of *Vibrio cholera* increases mucin secretion in the intestine [16]. One might, therefore, propose the existence of a similar interaction between the adherence of *Vibrio cholera* and mucin production. The rationale for this study was, therefore, to determine whether the biomarkers for GM1: cholera toxin and antibodies against GM1, can be used for *in situ* localization of the glycosphingolipid ganglioside GM1 in mucin-producing human salivary glands.

Materials and methods

Tissue samples and ethics

Human submandibular glands, parotid glands and soft palates were dissected less than 36 h post-mortem. Some tissue samples were fixed for 24 h in either Clark's fixative (1 part acetic acid:3 parts ethanol) or in 10% neutral-buffered formalin. Other tissue samples were fixed in 1.5 % glutaraldehyde in phosphate buffer pH 7.4 for 5 h. All autopsies were performed on former patients who had no known history of head and neck squamous cell carcinoma and who did not exhibit any post-mortem lesions in the upper digestive tract as evaluated by both gross anatomical and histological examinations.

For obvious ethical reasons this study does not include any normal, fresh human tissue. The autopsies were obtained at the mortuary at the Panum Institute, University of Copenhagen. The tissue was taken from individuals who had previously signed written statements to donate their corpses to science and education. These statements have been accepted by the Committee of the Capital Region of Denmark for Health and Growth.

After fixation, the tissue samples were stored in Tris-buffered saline for 48 h, embedded in paraffin and cut into 4 μm thick sections.

Microscopy

Digital images were recorded in a Zeiss Axioplan 2 microscope equipped with 5×, 10×, 20×, 40× and 60× magnification optics. The microscope was equipped with excitation filters for FITC 475 nm and for Rhodamine 542 nm and a 12 V/100 W halogen rectangular filament.

Histochemical agents

The probes that served to visualize ganglioside GM1 in the sections were: (1) Cholera toxin sub-unit B from *Vibrio Cholera* (Sigma-Aldrich, ST. Louis, MO). This lyophilized FITC-conjugate was dissolved in Tris buffer (Stock solution: 1 mg/ml) 0.05 M pH 7.4 with 0.2 M NaCl, 3 mM NaN_3 and 1 mM EDTA; and (2) Two polyclonal anti-ganglioside GM1 rabbit antibodies. One was produced by Calbiochem (Darmstadt, Germany) and—according to the manufacturer—cross-reacts slightly with asialo-GM1 but not with other carbohydrate epitopes. The other GM1 antibody was produced by Abcam (Cambridge, UK; ab 23943) and has been tested for use in immunocytochemistry. The antibodies were dissolved in Tris buffer (Stock solution: 1 mg/ml) 0.05 M pH 7.4 with 0.2 M NaCl, 3 mM NaN_3 and 1 mM EDTA. The monoclonal antibody 3C9, which recognizes T-antigen (Galβ1-3-GalNac bound α1-3 to Ser/Thr), was a gift from Dr H. Clausen (Copenhagen, Denmark).

Some sections were stained with Alcian blue at pH 2.5, while others were stained with Alcian blue (Alcian blue 8GS, Imperial Chemical Industries Ltd., London, UK) and PAS in order to demonstrate muco-substances in the sections.

Incubation of sections with the biomarkers

The sections were pre-incubated with 1% BSA in TBS pH 7.4 for 1 h prior to incubation with the biomarkers. Titration experiments showed that FITC-conjugated toxin (1 mg/ml) diluted 1:500 and the polyclonal antibodies (1 mg/ml) diluted 1:300 yielded the highest differences between the specific activity and background luminosities.

The luminosities were quantified in Photoshop® according to Kirkeby and Thomsen [17]. The FITC-fluorescence signal was visualized with the TSA Kit #2 from Molecular Probes and the antibody staining was visualized with the Envision+ System (DAKO, Copenhagen, Denmark). All incubations were performed at 4°C for 24 h.

Specificity

Several controls were run in order to ensure the specificity of the biomarkers: (1) some sections were incubated without primary antibody or toxin; (2) Adult male mouse brain tissue samples were fixed in Clark's fixative and were processed as above; (3) The staining pattern of the GM1 antibody was

compared with the pattern obtained after incubation with an antibody against the blood group T-antigen: Galβ1-3GalNAcα1-Ser/Thr (3C9 [18]); and (4) The staining pattern of the anti-ganglioside-GM1 from Calbiochem was compared to the staining pattern after incubation with the anti-ganglioside-GM1 antibody (Abcam; ab 23943).

Results

Sections of formalin- and glutaraldehyde-fixed tissue only revealed traces of specific reaction, whereas a strong-to-moderate fluorescence was detected in sections of tissue fixed in Clark's fixative. Therefore, the following description only refers to tissue fixed in Clark's fixative.

Immunofluorescence microscopy showed that cholera toxin and the antibodies against GM1 were co-localized in some acini but not in others. Figures 1A and B are micrographs of the submandibular gland in which some groups of mucous acini are stained with either cholera toxin or the GM1 antibody, while other groups of acini are detected by both the toxin and the GM1 antibody. There was no reaction in the striated or excretory ducts.

The submandibular gland consists of secretory units (acini), most of which are either purely serous or purely mucous; the gland also has mixed sero-mucous acini. The micrographs shown in Figure 2 are of sections that were incubated with either cholera toxin (Figure 2A) or anti-GM1 (Figure 2C). These sections were subsequently immersed in Tris buffer for 3–4 days in order to remove their coverslips. After a rinse, the sections were stained with Alcian blue, as described in the Materials and methods section (Figures 2B and D).

Most of the mucous acini in the submandibular gland reacted with the toxin and antibodies, although some clusters remained unstained after incubation with the biomarkers, as shown in Figures 2A and C. The cluster of mucous acini marked 'x' in Figure 2A is stained by the FITC-conjugated cholera toxin and with Alcian blue, while the cluster of mucous acini marked 'y' did not react with the toxin but was stained by Alcian blue alone (Figure 2B). Likewise, when the anti-GM1 and Alcian blue reactions—as shown in the section illustrated in Figures 2C and D—are compared, it is evident that only some of the Alcian blue positive mucous acini are stained with anti-GM1.

The localization of the reaction within the mucous cells is illustrated in the double-stained sections shown in Figures 2E and F. The cholera toxin seems mainly to react with the cell membranes, while the antibody against GM1 gives rise to a staining of the cytoplasm. Figure 2F also indicates that clusters of serous acini react with GM1 biomarkers. These clusters are few in number, but were stained as described for the mucous acini, e.g. the toxin reacts with the membrane and the antibody with the cytoplasm.

All the secretory units in the parotid gland are serous. The immunohistochemical results differed from those seen in the submandibular gland. The cytoplasm in some secretory acinar cells was stained by the cholera toxin, whereas only small spots on the plasma membranes reacted with anti-GM1 (Figure 3A). The plasma membranes in the parotid excretory ducts appeared to have reacted to anti-GM1 (Figure 3B) but not to cholera toxin. Not only the large salivary glands, but also the minor salivary palatinal glands of the oral cavity were stained by the cholera toxin and by anti-GM1, as shown in Figure 3C. The secretory units in the human palatinal glands are purely mucous. It appears that some acini are stained by both biomarkers, while others are stained with either toxin or antibody alone.

The procedures for evaluating the specificity of the GM1 biomarkers showed the following: (1) No staining was detected in sections that were incubated in a medium without the toxin or antibody; (2) In sections of mouse brain, the antibody stained the nuclear membrane of the nerve cells in the cerebral cortex while the toxin mainly detected the processes extending from the nerve cell bodies, as shown in Figures 4A and B; (3) Incubation of sections with glandular tissue with the antibody against the blood group T-antigen produced a strong reaction in the luminal content in some ducts and in some

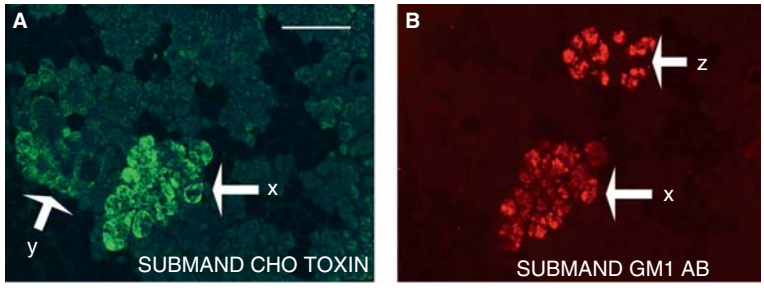

Figure 1. Submandibular gland section incubated with FITC-conjugated cholera toxin (A) and anti-ganglioside GM1-antibody (B). The acini in the cluster marked X are stained by both the toxin and the antibody, while the acini in the cluster marked Z only react to the GM1 antibody. The acini in the cluster marked 'y' only reacted to the toxin. Scale bar: 125 μm.

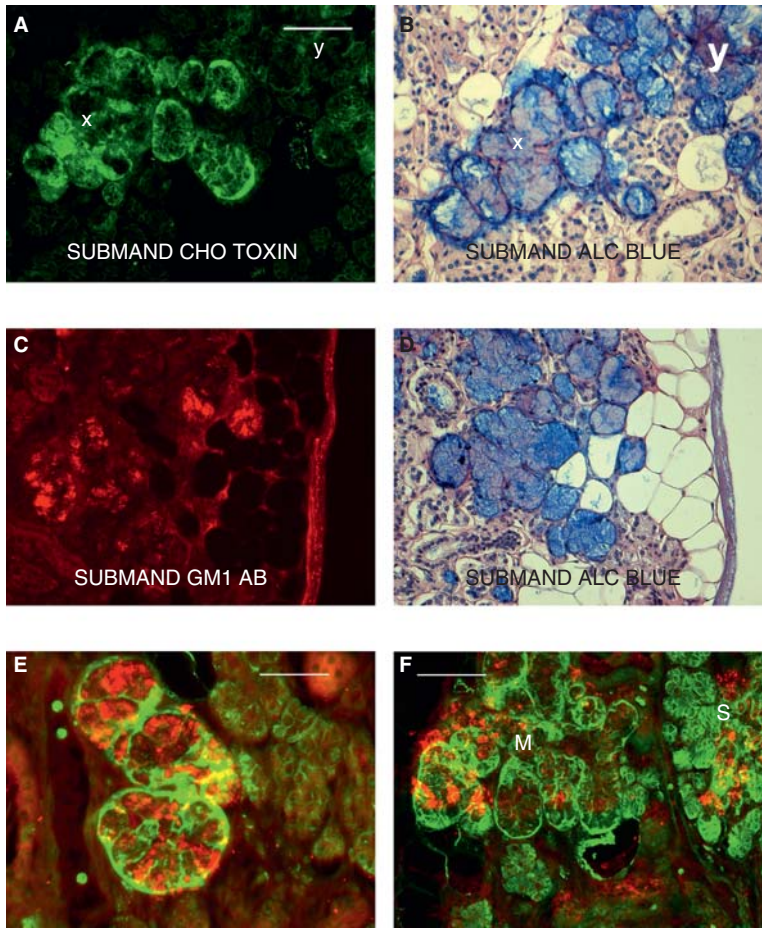

Figure 2. Sections of the submandibular gland. (A and B) A section that was incubated with cholera toxin (A) and thereafter stained with Alcian blue (B). The mucous acini in the cluster marked X are stained by both the toxin and Alcian blue, while the mucous acini in the cluster marked 'y' are only stained by Alcian blue. (C and D) A section that was incubated with anti-GM1 (C) and thereafter stained with Alcian blue (D). Scale bars (A–D): 62.5 μm. (E and F) Double-stained sections of the gland. Green fluorescence: cholera toxin. Red fluorescence: GM1 antibody. In (F) the letter M marks a cluster of mucous acini and the letter S marks a cluster of serous acini. Scale bars in (E) and (F): 31 μm.

connective tissue cells. The glandular epithelial cells did not react with the antibody (Figure 4C); in serial sections, identical cells were stained after incubation with the two different antibodies against ganglioside GM1 (Figures 4D and E).

Discussion

Gangliosides are expressed in most mammalian cells and are by far the most predominant sialoglycans in neural cells. Gangliosides exhibit diverse functionalities, e.g. modulating the activity of membrane-associated enzymes, cations or integrins. Functional interaction between the cross-linking β-galactoside-binding tissue lectin galectin-1 and ganglioside GM1 is the molecular basis for growth regulation of human neuroblastoma [19]. Not only do GM1 gangliosides extend from the plasma membrane of most eukaryotic cells, they also occur in the nuclear envelope where they regulate nuclear calcium through association with a nuclear sodium-calcium exchanger [20].

Cholera toxin attaches itself to many different cell types [9], but its affinity for glycolipids is believed to be very specific since it binds to GM1 but not to GM2, GD2 GD1b gangliosides or non-acid glycosphingolipids, lactoneotetraosylceramide and similar sequences [21]. There are five identical binding sites for the toxin–ganglioside interaction. According to Chaudhuri and Chatterjee [22], the binding is a 'two-fingered grip' in which the terminal Gal-GalNAc of the GM1 pentasaccharides are deeply buried in the toxin and the sialic residue lies along the toxin surface.

The enterotoxin from *vibrio cholera* is often used as a probe to detect GM1 ganglioside because of its high affinity for this glycosphingolipid. Nevertheless, by comparing results obtained with flow cytometry with those obtained by TLC and TLC-overlay assays, Yanagisawa et al. [23] observed that cholera toxin B-binding did not correlate with GM1 expression in mouse embryonic cells. The cholera toxin reacted mainly with the cell membranes of the mucous acini in the submandibular gland, while incubation with the antibody against GM1 gave rise to a staining of the

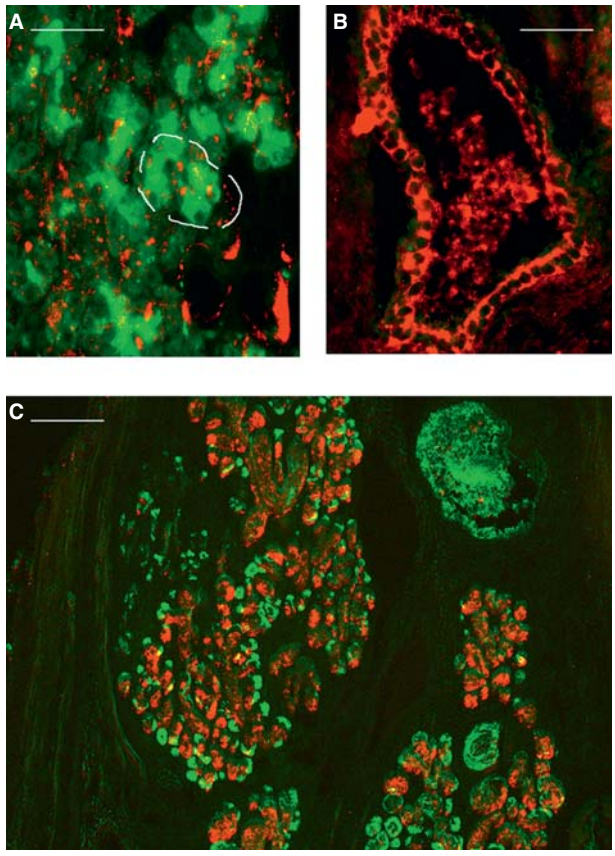

Figure 3. (A and B) Double-stained sections of the parotid gland. Green fluorescence: cholera toxin. Red fluorescence: GM1 antibody. (A) The secretory units of the gland. The encircled structure is an acinus. Scale bar: 27.5 µm. The duct shown in (B) reacts with GM1 antibody but not with the toxin. The weak green color in the figure stems from a non-specific staining of the connective tissue. Scale bar: 27.5 µm. (C) Double-stained section of the palatinal gland. Green fluorescence: cholera toxin. Red fluorescence: GM1 antibody. Scale bar: 160 µm.

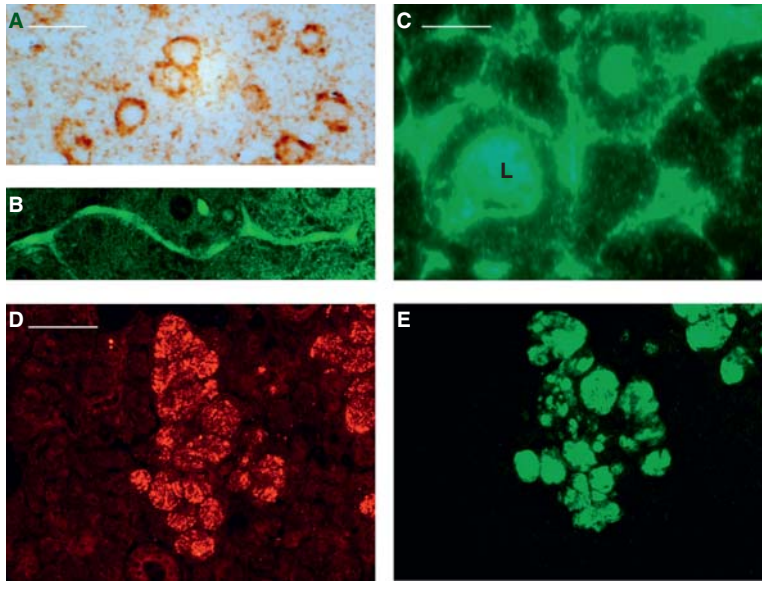

Figure 4. Cortex from mouse brain stained with anti-GM1 (A) and with cholera toxin (B). Scale bar: 31 µm. (C) A section of human submandibular gland incubated with an antibody against the T-antigen; (L) marks the lumen of a duct. Scale bar: 16 µm. (D and E) Serial sections of a human submandibular gland incubated with the two anti-ganglioside antibodies; (D) The antibody from Calbiochem, (E) The antibody from Abcam. Scale bar: 62.5 µm.

cytoplasm. The cytoplasm in some secretory acinar cells in the parotid gland was stained by the cholera toxin, whereas only small spots on the plasma membranes reacted with anti-GM1. The plasma membranes in the parotid excretory ducts appeared to have reacted to anti-GM1, but not to cholera toxin. The results obtained in this study, therefore, imply that adult human salivary glands also exhibit a limited correlation between the *in situ* localization of cholera toxin-binding and the immunohistochemical detection of GM1 glycosphingolipid.

The biomarkers used in this study seem to be specific for ganglioside GM1 for the following reasons: (1) The staining pattern in the mouse brain was in accordance with previous reports: GM1 antibodies stained the nuclear membrane and cholera toxin marked neuronal processes [20]; (2) The two terminal sugar residue of the ganglioside GM1 consist of the T-antigen disaccharide. Moreover, the terminal galactose moiety of the GM1 receptor is more extensively involved in the interaction between the complete GM1 saccharide and cholera toxin than the other four sugar moieties [24]. This could imply that the GM1 biomarkers might react with the T-antigen disaccharide Galβ1-3GalNAc. Since the GM1-antibody and the toxin both stained glandular epithelial cells and the T-antibody stained the luminal content in some of the glandular ducts and connective tissue cells, there does not seem to be any cross-reactivity between the markers for the GM1 glycolipid and the antibody directed against the T-antigen; and (3) In serial sections, identical cells were stained after incubation with the two antibodies against ganglioside GM1.

Nowroozi et al. [12] identified GM1 in the striated ducts and the acinar cells in the human parotid gland. They suggested that this glycolipid plays an important role in the secretory function of the parotid gland. Furthermore, cholera toxin binds to the mucous layer of high-molecular weight-proteins (mucins) at the apical side of the intestinal cells and stimulates mucus secretion from intestinal goblet cells [13].

The chitin-binding protein GbpA of *Vibrio cholera* binds specifically to GlcNAc residues of intestinal mucin, and up-regulates *MUC2* and *MUC5AC* genes [16].

Cholera toxin induces the expression of ion channels and carriers in the small intestine and increases the production of secretory mucins. The basic secretory unit of the mucin-producing salivary gland consists of a cluster of acinar cells and the secretory part of a duct. The cells in both the acini and ducts contain ion channels that modify the final saliva and the glandular cells produce a variety of mucins. The distribution of GM1 glycolipid epitopes and cholera toxin-binding in the human salivary glands is now established. The next step will be to investigate the connection between GM1, cholera toxin and mucin expression in the glands.

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***Declaration of Interest*:** The author reports no conflicts of interest. The author alone is responsible for the content and writing of the paper.

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