Immunoperoxidase labeling of *Streptococcus mutans* for scanning electron microscopy

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Berthold, P. Immunoperoxidase labeling of *Streptococcus mutans* for scanning electron microscopy. *Acta Odontol. Scand.* 1983, 41, 125–128. Oslo. ISSN 0001-6357.

The application of an immunoperoxidase method to labeling of *Streptococcus mutans* subsp. *sobrinus* for scanning electron microscopy is described. Bacteria from four separate cultures were fixed in 2.5% buffered glutaraldehyde solution. The specimens were treated in accordance with an indirect immunolabeling procedure using a rabbit anti-*S. mutans* subsp. *sobrinus* γ-globulin. Labeled bacteria were equipped with a layer of small, distinct globules of horseradish peroxidase reaction product. Bacteria exposed to control incubations were not equipped with this layer. □ *Immunocytochemistry; ultrastructure; horseradish peroxidase; bacteria*

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During the past decade enzyme electron immunocytochemical identification of different cell components and of whole cells has attracted a great deal of interest (18). This technique was originally introduced for transmission electron microscopy (TEM) by Sri Ram et al. (17) and Nakane & Pierce (13). Immunocytochemical methods have also been adapted for scanning electron microscopy (SEM) (12). Among the many different marker substances that have been suggested for use in immuno-SEM are latex spheres (11), colloidal gold granules (7), ferritin (20), and silica spheres (14). Horseradish peroxidase (HRP)-conjugated antibodies have been used for labeling of antigens on lymphocytes (21) and virus-induced antigenic sites on HeLa cells (10). The occurrence of salivary antibodies on the surface of *Streptococcus mutans* cells has been demonstrated with SEM and latex spheres as marker (16).

A TEM immunoperoxidase method was recently applied for identification of *S. mutans* in pure cultures and in mixed cultures (3). In the present report this method was used for immunolabeling of whole cells of *S. mutans* for SEM.

Materials and methods

Bacterial strain and growth conditions. S. mutans subsp. *sobrinus* strain B13 (5) was grown in Trypticase Soy broth (Difco, Detroit, Mich.) on thin, sterile polyester resin foils (3) for 24–36 h at 37 °C. Specimens from four separate cultures were studied.

Immunocytochemical and electron microscopy procedures. The foils with adhering bacteria were harvested, rinsed in phosphate-buffered saline (PBS; pH 7.2–7.4), fixed in 2.5% glutaraldehyde dissolved in a 300-mOsM phosphate buffer (pH 7.2–7.4) for 1 h at 4 °C, and rinsed in the buffer overnight at 4 °C. Preparation of the specific antibodies and the labeling procedures have been described in detail elsewhere (3).

The immunolabeling procedure includes the following main steps: 1) incubation with rabbit anti-*S. mutans* subsp. *sobrinus* γ-globulin (0.03 mg protein/ml), 2) incubation with HRP-conjugated sheep anti-rabbit IgG (1) diluted 1/10 in PBS, and 3) incubation in 3,3′-diaminobenzidine tetrahydrochloride (1/2000 w/v in TRIS-HCl buffer) plus 0.01% hydrogen peroxide for visualization of HRP (6). After step 3 the specimens were treated

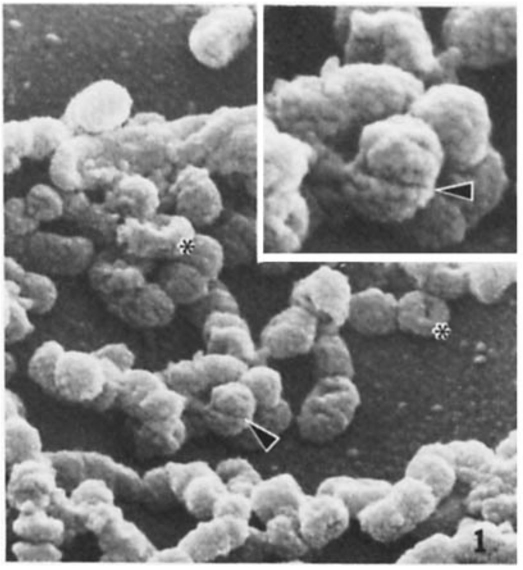

Fig. 1. SEM micrograph. Immunoperoxidase staining of *S. mutans* strain B13 using anti-*S. mutans* subsp. *sobrinus* γ-globulin. All bacteria are covered with a globular HRP reaction product. Several bacteria show a distinct interruption in the HRP reaction product (arrows). A few bacteria show an indentation (asterisk). Note that the supporting foil is covered by the same type of globules that are found on the bacteria. (×10,000; inset ×20,000.)

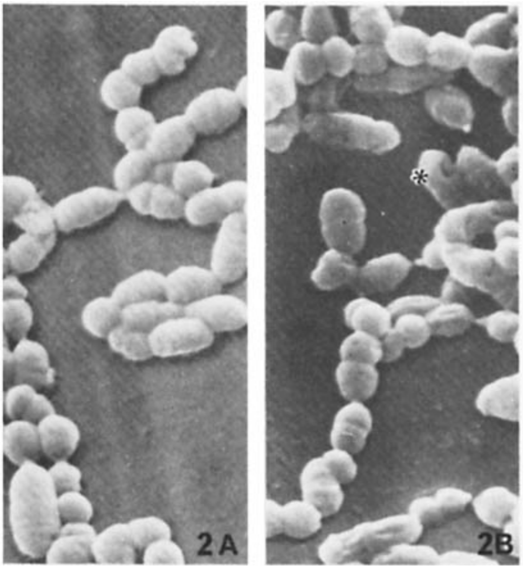

Fig. 2A. SEM micrograph. Control 1. Incubation with PBS only. No HRP reaction product globules can be found on either the bacteria or the foil. (×10,000.) 2B. SEM micrograph. Control 2. Incubation with normal rabbit γ-globulin. All bacteria are free of HRP reaction product. A few bacteria exhibit shallow indentations (asterisk). The foil is free of adhering globules. (×10,000.)

with 2% OsO_4 in distilled water for 30 min at 4°C, rinsed in distilled water, dehydrated in acetone to 100% and rehydrated to 50%, air-dried, and coated with a 100–200-Å-thick layer of gold. The specimens were examined in a Jeol scanning electron microscope (JSM3) operated at 15–20 kV and with an angle to the electron beam of 35°.

Controls. Two controls were used: 1) specimens incubated in steps 1–3 with PBS, and 2) specimens incubated in step 1 with normal rabbit γ-globulin.

Results

Bacteria that had become attached to the foils formed characteristic short chains or small colonies (Figs. 1 and 2). The samples contained many bacteria that were undergoing division. All cells subjected to the immunohistochemical treatment with anti-*S. mutans* subsp. *sobrinus* γ-globulin were covered with a continuous layer of small globules (30–100 nm in diameter), which gave the bacteria a raspberry-like appearance (Fig. 1). A distinct demarcation in this globular layer was noted on several bacteria. A few bacteria had a shallow indentation in the center of the cell. The supporting foil was covered with small globules that were similar in both shape and size to those attached to the bacteria.

Bacteria incubated with PBS only (control 1, Fig. 2A) or with normal rabbit γ-globulin (control 2, Fig. 2B) were smooth and did not have the characteristic globular elevations that were observed on the cell surface after the incubation with specific antibody. The foils were uncovered in both controls.

Discussion

Kumon et al. (8) suggested that a marker substance useful for immuno-SEM should possess the following four properties: 1) morphologically recognizable shape and size, 2) little or no tendency towards non-specific adsorption to the cell surface, 3) chemical stability, and 4) easy purification and acquisition. HRP seemed to fulfill these criteria and was consequently used as marker substance in this study. Moreover, the immunoperoxidase method had earlier been successfully used for immuno-TEM labeling of surface antigens on *S. mutans* in pure and mixed cultures and in dental plaque (3).

Another important criterion is that of immuno-specificity. The specificity of *S. mutans* subsp. *sobrinus* γ-globulin was reported in a previous paper (3). Immunoelectrophoresis and electron immunoperoxidase staining procedures were then used for cross-reaction tests with several oral bacteria. The fully absorbed *S. mutans* subsp. *sobrinus* γ-globulin did not react with any of the tested strains other than those belonging to *S. mutans* subsp. *sobrinus*. However, it cannot be completely ruled out that there exist classified or unclassified bacteria in the complex oral flora which might cause cross-reactions. The use of highly diluted and absorbed γ-globulin fractions and the use of appropriate fixatives can minimize unexpected cross-reactions (4, 15). A clear difference in appearance existed between the positively labeled cells and the controls. Although the globules observed on the labeled cells are distinct, it cannot be assumed that each globule represents one antigenic site or that all antigenic sites are labeled (19).

In an earlier paper by Berthold & Berthold (3), in which the immuno-TEM labeling of *S. mutans* was discussed, the HRP reaction product was described as a structure of densely packed spherical units, 10–40 nm in size, which at certain sites had aggregated into globules 50–80 nm in diameter. It was also observed earlier that labeled bacteria lacked HRP reaction product at sites adjacent to cross-wall formations (2). The globules of HRP reaction product observed on the bacterial surface in this study are most likely the same as those observed after the immuno-TEM procedure and represent a true labeling. The distinct interruption in the layer of HRP reaction product should then represent a site adjacent to a cross-wall.

The preparative procedures of biological samples intended for SEM analysis vary from critical point-drying of fixed specimens (12) to air-drying with or without the use of fixation and dehydration (22). Klainer et al. (9) studied the effect of both critical point-drying and air-drying on different bacteria. These authors demonstrated that different bacteria required different preparative procedures. It was shown that air-drying of gram-positive cocci produced a satisfactory result. However, to improve the preservation of the specimens they had to be rehydrated to 50% acetone before exposure to the air. The indentation of some bacteria is most likely an artifact due to the preparative procedures.

After completion of the immunochemical procedures, the supporting foil was covered by globules that were similar to those observed on the surface of the bacteria. These globules probably represent true labeling of antigenic components derived from the supernatant and attached to the foil. Free antigenic components of different sizes have been shown by immuno-TEM procedures to occur in the broth (2).

It can be concluded that the immunoperoxidase labeling procedure applied for SEM seems useful for the identification purposes of whole bacteria belonging to *S. mutans* subsp. *sobrinus*.

Acknowledgements.—This work was supported by a grant from the Faculty of Odontology, Karolinska Institute, Stockholm, and by USPHS grants DE-02636 and DE-07085 from the National Institute for Dental Research (NIDR). Parts of this paper were presented at the 54th General Session of the International Association for Dental Research (1976).

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Received for publication 16 November 1981