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# Immunoelectron Microscopic Identification and Localization of *Streptococcus sanguis* with Peroxidase-Labeled Antibody: Localization of *Streptococcus sanguis* in Intact Dental Plaque

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*Streptococcus sanguis* has been localized ultrastructurally within intact dental plaque by means of an indirect technique which utilizes horseradish peroxidase-labeled antibody. The technique allows for complete diffusion of the reagents to all portions of the plaque specimens. Control procedures can be carried out on serial sections of plaque with a bacterial composition similar to that of the experimental specimen. The 30-μm-thick sections can be examined in the light microscope to localize areas specifically labeled with peroxidase prior to cutting ultra-thin sections for electron microscopy. This study demonstrated that specific bacteria can be localized within intact dental plaque. The results also indicated that *S. sanguis* grows in dental plaque as columnar shaped microcolonies perpendicular to the tooth surfaces. Growth appears to be by cell division rather than deposition of new cells at the surfaces. Despite their relatively good structural preservation, the cells in the deeper (older) layers of plaque appear to have lost some of their antigenic activity in comparison to the cells near the surface.

Dental plaque is considered to be an important etiological factor in the pathogenesis of caries and periodontal disease (7, 8, 27). A number of investigators have studied the morphology of dental plaque at the ultrastructural level (1, 5, 6, 24, 25, 29) and have shown that it is composed of a wide variety of microorganisms. Although it is sometimes possible to distinguish gram-positive from gram-negative bacteria in the electron microscope by the structure of their cell wall (4, 9, 23), it is generally not possible to identify specific species by their morphology alone. Indeed, the morphology of bacteria in plaque may be quite different from the same organisms in pure culture. It is known from existing reports that morphological variations may be induced in certain microorganisms by altered growth conditions in vitro (3, 22). With immunofluorescent markers (26), it has been shown that the morphology of filamentous gram-positive bacteria in plaque may differ widely from the morphology of the same organisms in pure cultures. Recent serological studies of oral bacteria can now serve as a sound basis for developing techniques for the in situ localization of bacteria in plaque (C.-H. Lai, B. Rosan, and M. Listgarten, 51st Int. Ass. Dent. Res. Meet., Abstr. 737, p. 243, 1973).

Preliminary studies demonstrated the feasibility of using horseradish peroxidase (HP)-labeled antibody to identify *Streptococcus sanguis* cells in pure cultures (12). These studies also demonstrated the specificity of the reaction. The purpose of the experiments reported here was to determine whether *S. sanguis* could be identified and localized within intact dental plaque.

## MATERIALS AND METHODS

**Collection of dental plaque.** Well-fitted epoxy resin strips approximately 0.5 to 1 mm thick were custom made on plaster models of the mandibular central and lateral incisors of human volunteers. The resin strips were attached to the lingual surface of the subject's lower incisors by means of 4-0 surgical cotton. Plaque was collected on the strips for periods of up to 7 days. The Epon strips covered with plaque were carefully removed and immediately fixed and processed as described below.

**Antisera.** Rabbit antisera against *S. sanguis*, strain M-5, was produced by immunization with formalinized cells as described previously (11). The M-5 antisera was used at a dilution of 1:20 because preliminary tests indicated that at this dilution the reaction with *S. sanguis* cells was intense, specific, and no cross-reactions occurred with other species tested (12, 21). Preimmune sera were used at the same dilution for controls. These sera had also been shown

to be nonreactive with both homologous and heterologous strains.

Commerical goat anti-rabbit immunoglobulin G (IgG) (Research Division, Miles Laboratories, Kankakee, Ill.) was conjugated with HP (Type II, Sigma Chemical Co.) according to the method of Nakane and Pierce (15).

**Preparation of sections for the localization and identification of bacteria.** The Epon strips covered with plaque were fixed in a 4% paraformaldehyde-picric acid fixative (28) at 4 C for 6 h. The samples were washed overnight with 0.01 M phosphate-buffered saline (PBS) solution at pH 7.2 and dehydrated in graded aqueous ethanol solutions. Before the final dehydration step, the strips were carefully cut along a plane parallel to the vertical axis of the tooth, into 5-mm-long pieces. These pieces were embedded in pure polyethylene glycol in a vacuum oven at 40 C according to the method of Mazurkiewicz and Nakane (14). Blocks were hardened in a refrigerator at 4 C and stored in a desiccator.

Samples of plaque embedded in polyethylene glycol were cut at 30 μm with an A-O Spencer Rotary Microtome and floated on a 5% glycerol solution for 1 h. The sections were transferred to PBS at 4 C to dissolve part of the water-soluble polyethylene glycol. This step allows antisera or HP-labeled antibody to penetrate freely into the deeper layers of dental plaque. To minimize nonspecific reactions caused by "natural" antibodies, plaque sections were preincubated with normal sheep serum for 1 h at room temperature. The sections were washed in PBS for 1 h and treated for another hour with 1:20 diluted rabbit anti-M-5 sera for the experimental group, or similarly diluted normal rabbit serum for the control group. The sections were again washed in PBS and treated with peroxidase-labeled goat anti-rabbit IgG (HP anti-rabbit IgG). After another washing, they were fixed in phosphate-buffered 5% glutaraldehyde at 4 C and washed overnight at 4 C in 0.05 M phosphate buffer containing 4.5% sucrose.

The sections were incubated for 1 h in a solution of 3,3'-diaminobenzidine (80 mg per 100 ml in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 7.6), followed by an incubation of 1 to 5 min in the same solution containing 0.005% hydrogen peroxide to develop the peroxidase label (10). Sections were washed in PBS and transferred to microscope slides. A cover slip was placed temporarily over the section with 90% glycerin in PBS solution for examination by light microscopy.

The cover slips were then carefully removed and the sections were washed in several changes in PBS for 1 h. Sections were treated with 2% osmium tetroxide buffered with veronal acetate (16), washed, and dehydrated in graded ethanols. After two passages through propylene oxide, the sections were infiltrated with graded mixtures of Epon and propylene oxide and embedded in Epon (13). The sections were oriented to permit the cutting of full thickness sections of the plaque layer. Ultra-thin sections were cut at 0.1 μm and examined in a Philips EM-300 electron microscope without additional stain. Some sections were also examined after staining with lead citrate alone (17) and with uranyl acetate followed by lead citrate. In addition to the controls described above, the following controls were also included in these studies: (i) sections of plaque treated with normal sheep sera only; (ii) sections of plaque treated with 1:20 diluted normal rabbit sera only; (iii) sections of plaque treated with HP anti-rabbit IgG only. After washing, the three controls were fixed in phosphate-buffered 5% glutaraldehyde fixative and processed as described earlier.

## RESULTS

A representative area from a sample of dental plaque treated with normal sheep serum, M-5 antisera, followed by HP anti-rabbit IgG is shown in Fig. 1a, b, and c. The darker regions in the light micrographs are due to the brownish color produced by the reaction of the peroxidase with the substrate. The positively reacting organisms were arranged in columnar shaped microcolonies which were oriented more or less perpendicularly to the Epon surface. An electron micrograph of one of these microcolonies is shown in Fig. 1e. These columnar microcolonies were not demonstrable in the control samples of dental plaque treated with normal rabbit serum followed by HP anti-rabbit IgG (Fig. 1d).

A full thickness section of plaque containing labeled cells of *S. sanguis* at various levels in the plaque sample is shown in Fig. 2a. The cells corresponding to the brownish columnar microcolonies were coated with an electron dense layer of peroxidase-labeled antibody similar to that demonstrated in labeled *S. sanguis* cells in pure culture (12). This electron dense layer was detectable both with and without staining, although staining tended to enhance its contrast. At higher magnification (Fig. 2b and 3a), the most well-defined electron dense layer around the cells was observed in the most superficial portion of the plaque; the ultrastructural features of the coated cells in plaque were similar to those of *S. sanguis* in pure culture. Occasionally, a few cells at the surface of the plaque which were ultrastructurally distinct from cells of *S. sanguis* also demonstrated a less well-defined coating layer (Fig. 3b). However, unlike the coating due to specific serological interactions, these layers could not be detected in unstained sections.

A section from the central portion of the plaque is shown in Fig. 4a and b. In comparison to the cells at the more superficial levels (Fig. 2b and 3a), the specific electron dense coating of these cells was somewhat less homogeneous. At this or deeper levels of plaque, no instances were observed of coating around cells which were ultrastructurally distinct from *S. sanguis*.

As shown in Fig. 5a and b, very little coating was present around cells at the deepest levels.

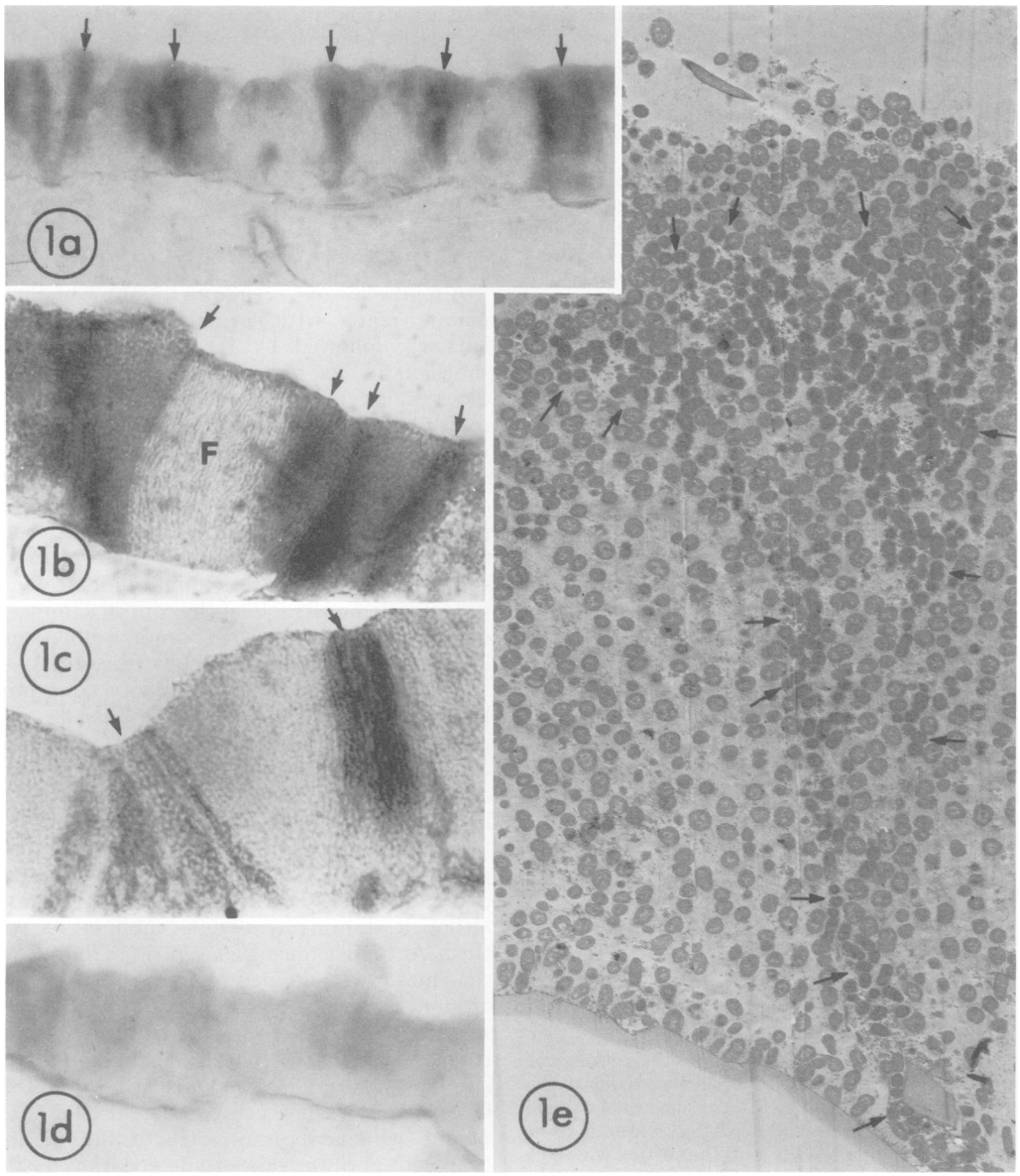


FIG. 1. (*a*) *Light micrograph of 30-μm section of intact dental plaque on epoxy resin strip. Section was incubated with normal sheep sera, 1:20 diluted rabbit anti-M-5 sera, and HP-anti-rabbit IgG, and treated to reveal localization of HP. The dark columnar regions (arrows) indicate the presence of S. sanguis cells.* ×*240.* (*b and c*) *Columnar microcolonies in dental plaque. The label is confined to coccal colonies. F, unlabeled microcolony consisting of predominantly filamentous bacteria.* ×*560.* (*d*) *Light micrograph of serial section of Fig. 1a incubated with normal sheep sera, 1:20 diluted normal rabbit sera, and HP-anti-rabbit IgG, and treated to reveal the localization of HP. There are no detectable labeled colonies.* ×*240.* (*e*) *Electron micrograph of ultra-thin section from specimen shown in Fig. 1a. Labeled cells are coated with an electron dense layer of HP-labeled antibody (arrows). These cells are located in the labeled microcolonies shown in Fig. 1a-c.* ×*2,800.* (*All electron micrographs are of sections treated with uranyl acetate and lead citrate.*)

However, the cells still demonstrated ultrastructural features similar to those of *S. sanguis* and appeared to be continuous with the columns of *S. sanguis* cells at the more superficial levels. These cells were, therefore, considered to be *S. sanguis*.

A representative area from a control sample of plaque treated with normal sheep serum

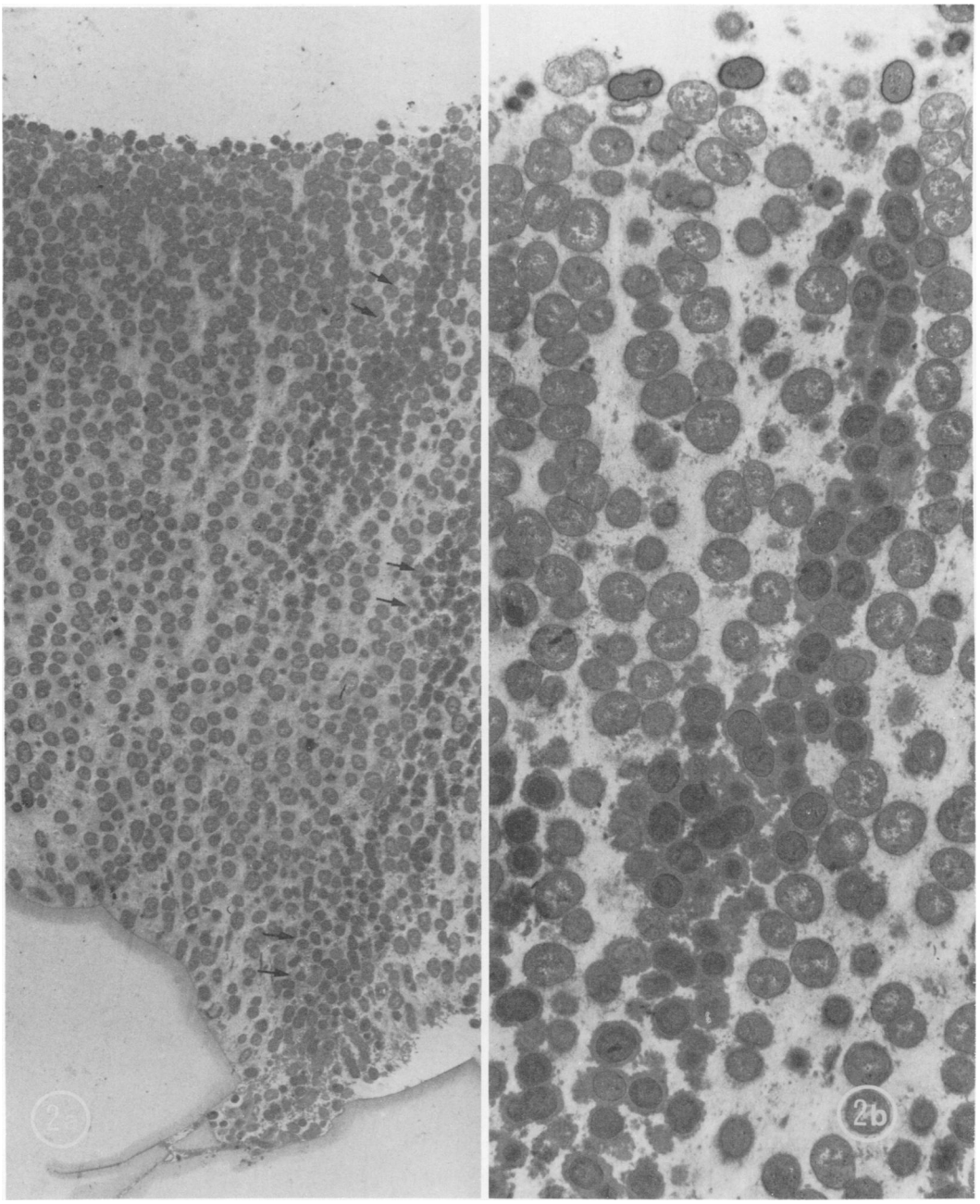


FIG. 2. *(a) Electron micrograph of dental plaque with labeled S. sanguis cells (arrows). Magnified sections of this micrograph appear in Fig. 2b, 3, 4, and 5. ×2,400. (b) Superficial portion of labeled plaque from Fig. 2a. Note the relationship of the labeled cells to one another and their neighbors. ×7,800.*

followed by normal rabbit serum and HP anti-rabbit IgG is shown in Fig. 6. At low magnification, some cells appeared to be electron dense (Fig. 6a). However, at high magnification (Fig. 6b), these cells, many of which were ultrastructurally similar to *S. sanguis*, did not have a coating. On the other hand, some surface cells which were ultrastructurally distinct from *S. sanguis* were coated in these controls (Fig. 6c). These coatings were only present in sections which had been stained with heavy metal salts. They did not appear in unstained sections and

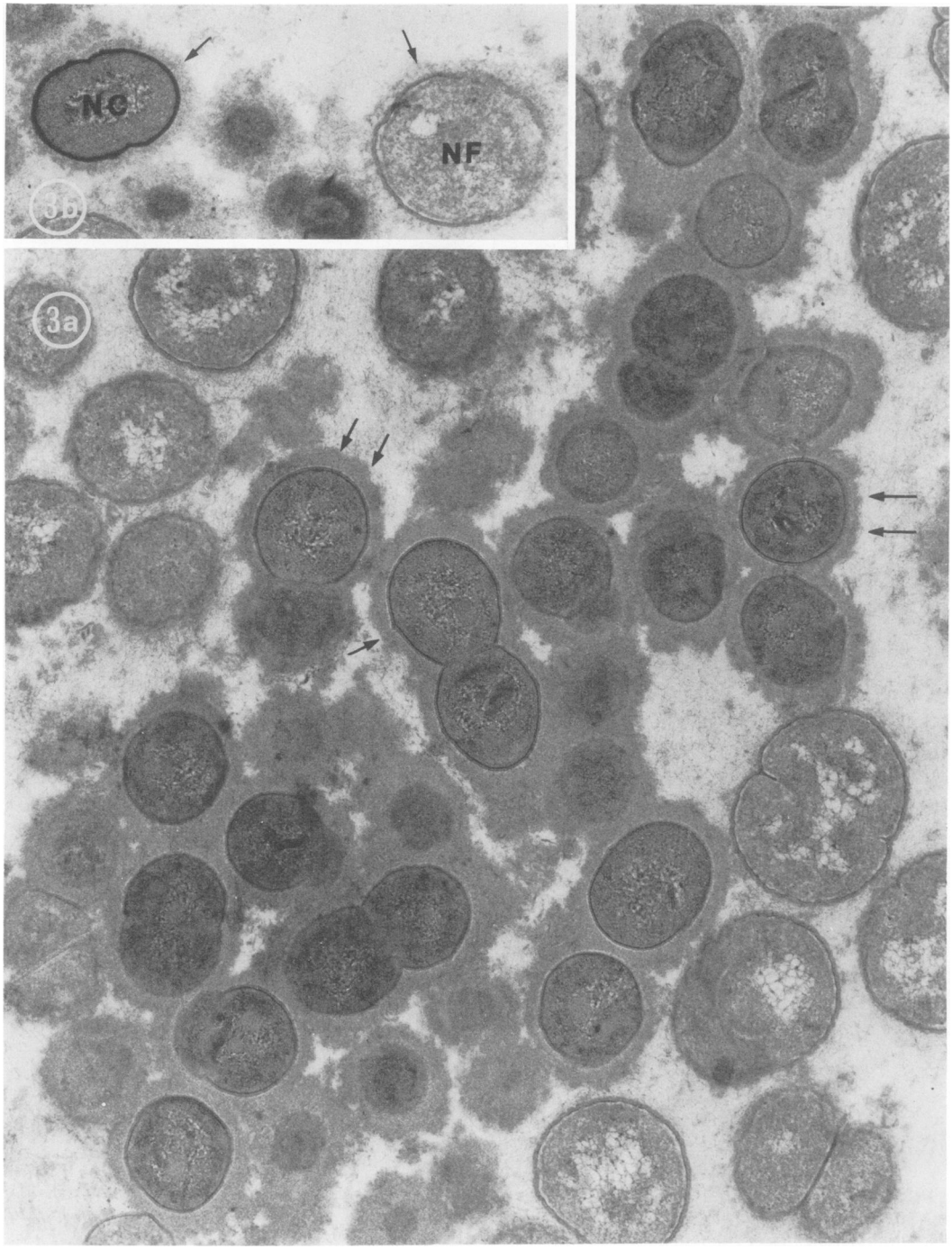


FIG. 3. *Higher magnification of Fig. 2b. The cells specifically labeled with a distinct electron dense coating of HP-labeled antibody (arrows) are structurally similar to S. sanguis cells in pure culture. The label can also be detected in unstained sections. ×29,800. (b) Some surface cells (NC and NF) from Fig. 2a have a nonspecific electron dense coating (arrows) in stained sections. This coating is not detectable in unstained sections. Note that these cells are ultrastructurally distinct from S. sanguis cells. ×29,800.*

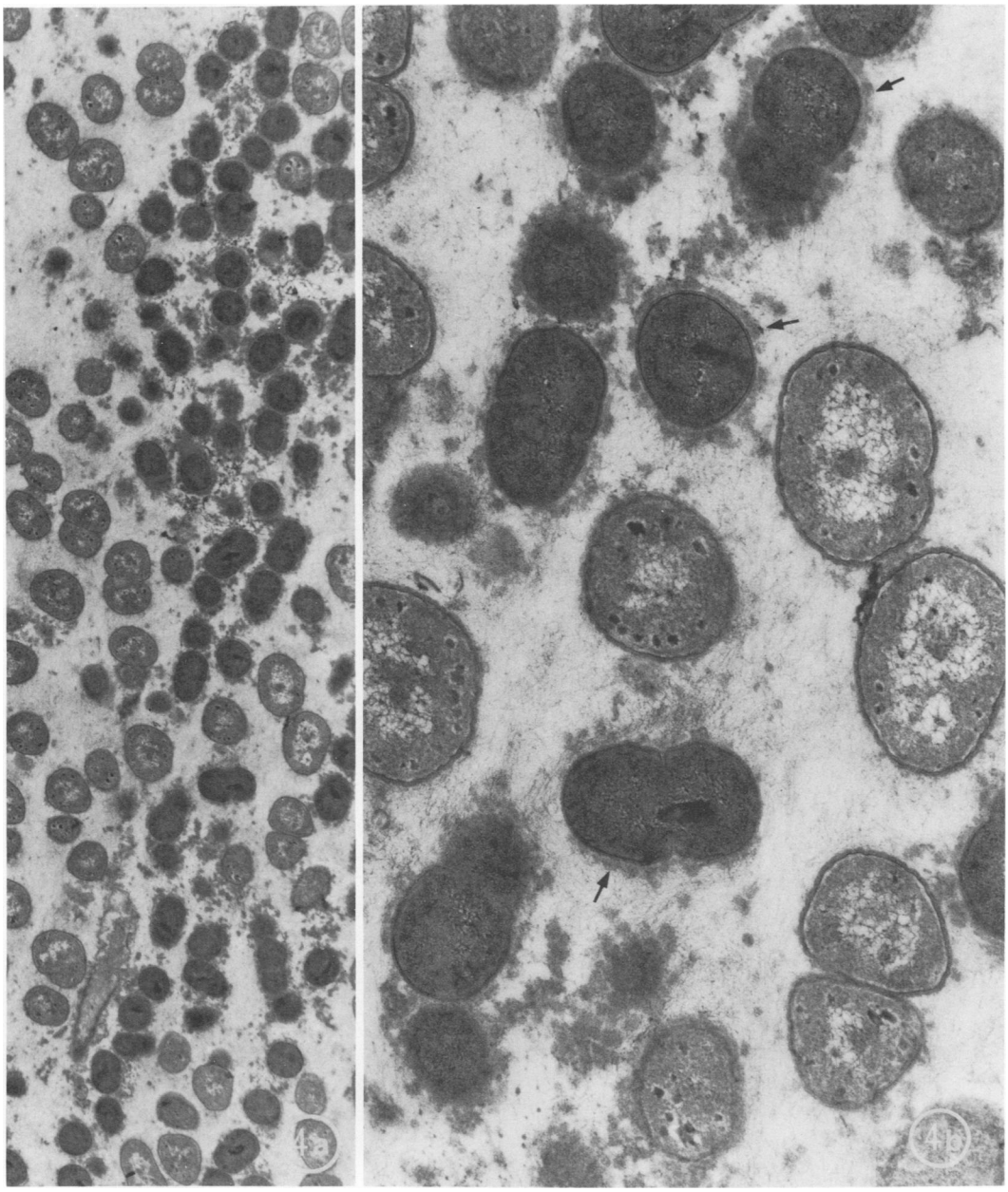

FIG. 4. (a) *Central portion of labeled plaque shown in Fig. 2a.* ×7,800. (b) *Higher magnification of Fig. 4a. The specifically labeled electron dense coating of these cells (arrows) is somewhat less compact and homogeneous than that of the more superficial S. sanguis cells (Fig. 2b and 3a). Despite their lack of homogeneity, these labeled layers could be detected in unstained sections.* ×29,800.

were, therefore, not considered to represent specific labeling.

Such nonspecific reactions were also observed to be associated with both coccal and filamentous organisms in specimens incubated with normal sheep sera only (Fig. 7a, b, and c). As was the case in the previously described specimens, the nonspecific coatings were not detectable in unstained sections. No electron dense coating was demonstrable around cells

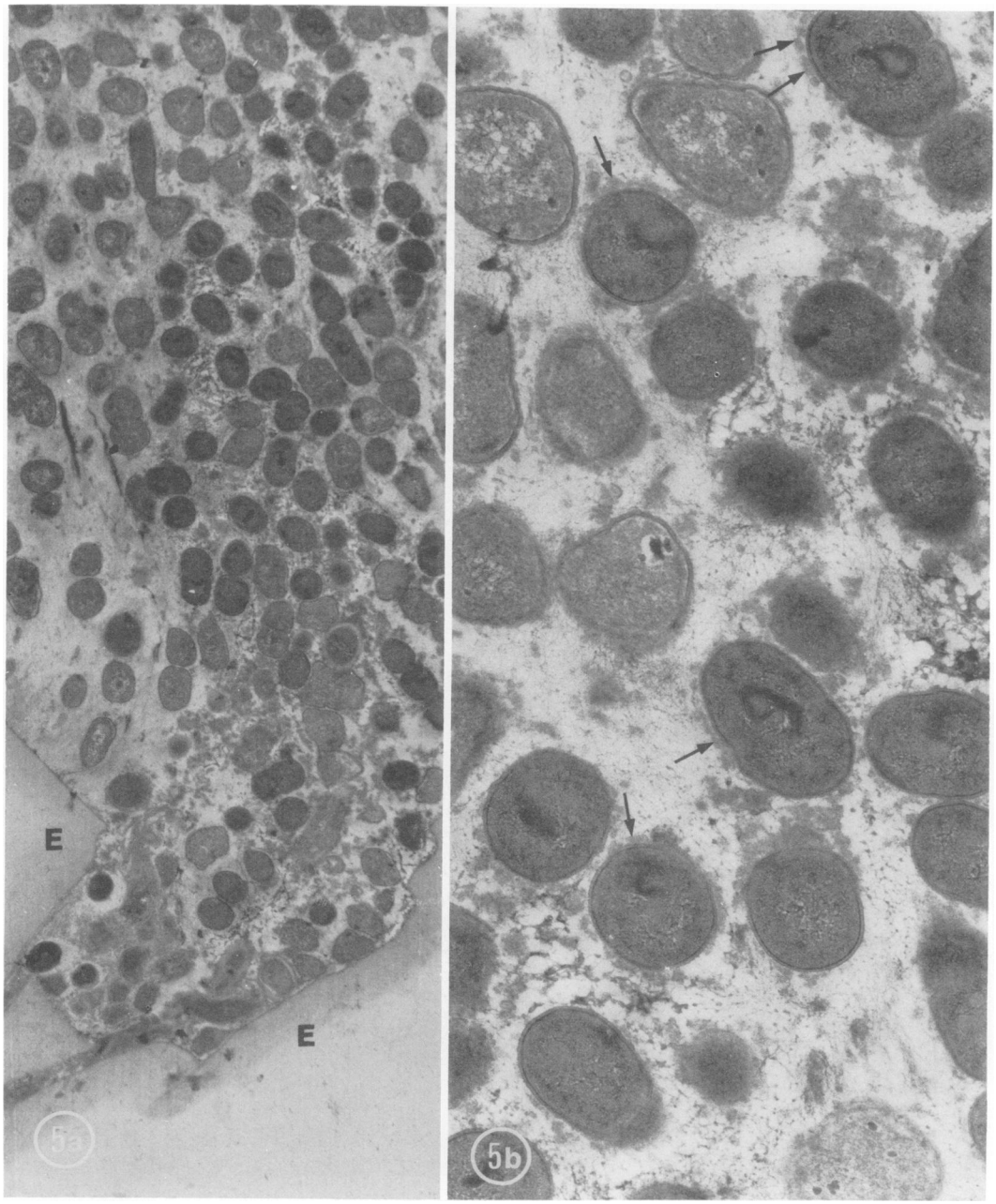


FIG. 5. (a) *Deep portion of labeled plaque. E, Epon strip.* ×7,800. (b) *Higher magnification of Fig. 5a. Relatively little coating (arrows) is present around cells which are ultrastructurally similar to and continuous with the columnar microcolony of S. sanguis cells. This patchy coating was detectable in unstained sections as well.* ×29,800.

from the remaining controls treated with either normal rabbit serum only or HP anti-rabbit IgG only.

## DISCUSSION

Two major problems blocked our initial attempts at the ultrastructural localization of specific bacteria within dental plaque: (i) the failure of antisera to penetrate the deeper layers of plaque when plaque-covered Epon strips were reacted in toto; (ii) the presence of nonspecific labeling.

In the present study, the failure of antibody

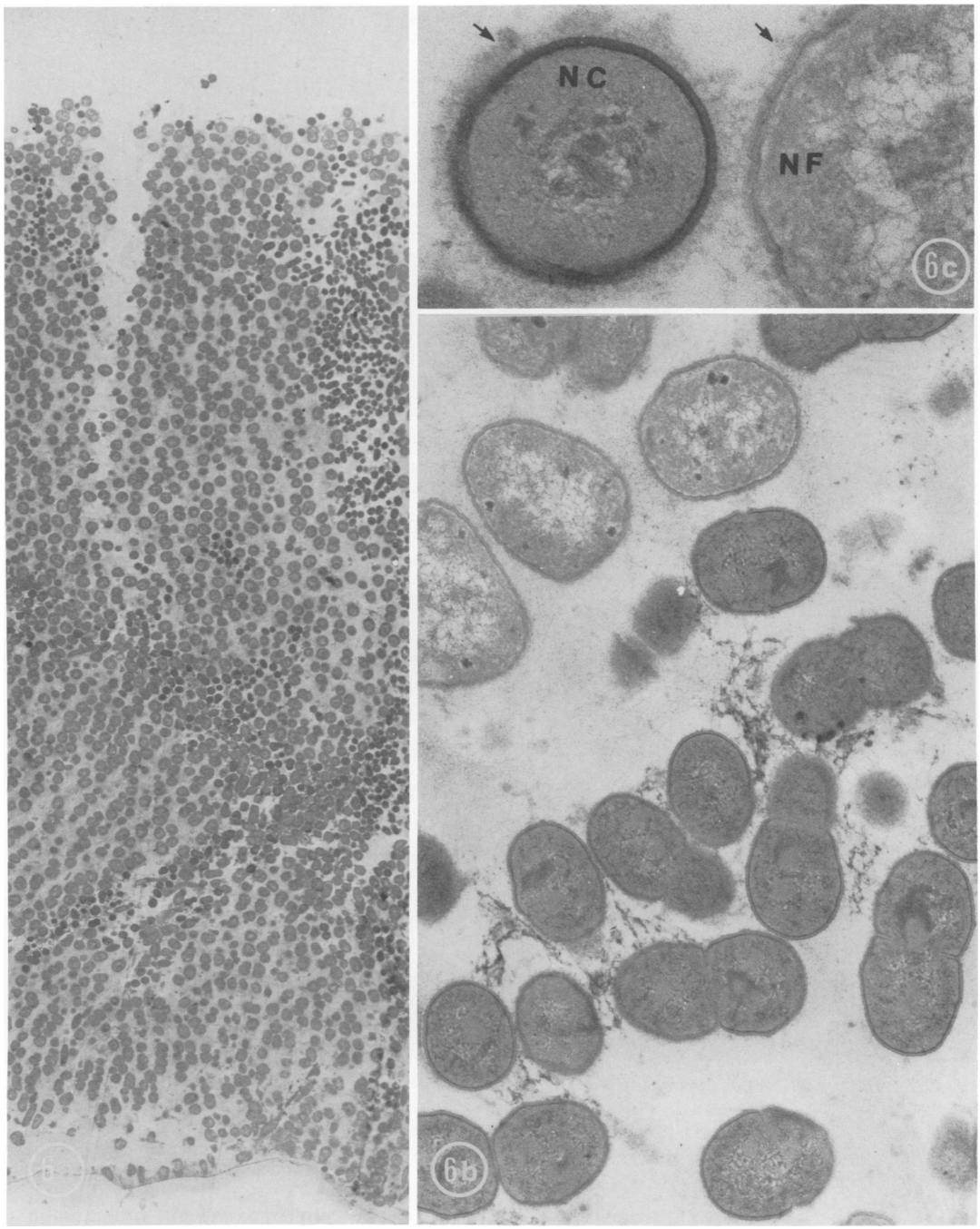


FIG. 6. (a) *Ultra-thin control section from specimen shown in Fig. 1d. No specifically labeled cells are present.* ×*1,900.* (b) *Higher magnification of Fig. 6a. Cells ultrastructurally similar to S. sanguis fail to exhibit an electron dense coating.* ×*29,800.* (c) *Some surface cells in the control shown in Fig. 6a demonstrate a nonspecific electron dense coating (arrow). This coating could not be detected in unstained sections.* ×*86,000.*

to diffuse into the deeper layers was overcome by cutting the plaque-covered Epon strip into 30-μm-thick sections prior to the incubation with labeled antisera. These "thick" sections allowed for complete diffusion of the high molecular weight reagents into the depth of the

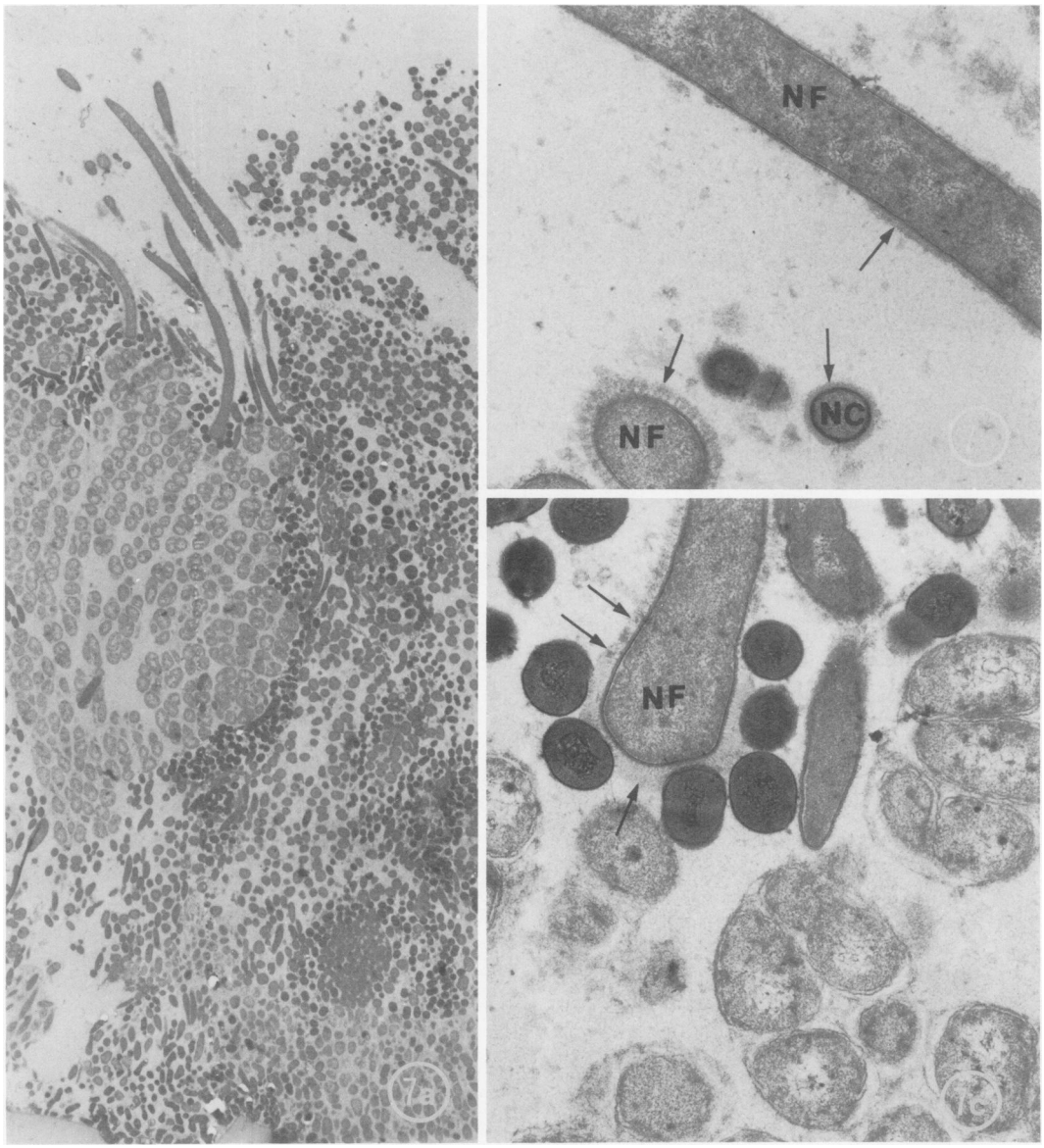


FIG. 7. *(a) Control section incubated with normal sheep serum only. Some surface cells demonstrate a nonspecific electron dense coating.* ×*1,900. (b and c) Higher magnification of Fig. 7a reveals cells near the plaque surface which are non-specifically coated with normal sheep serum (arrow). These coating layers could not be detected in unstained sections. NC, Nonspecifically coated coccus; NF, Nonspecifically coated filamentous organism.* ×*15,000.*

plaque. A further advantage of this method was the possibility it offered of using serial sections for control procedures which form the basis upon which the specificity of the reactions in plaque could be determined. Finally, it was possible to screen the 30-μm slices by light microscopy prior to processing them for electron microscopy.

The use of an indirect labeling procedure in which HP was coupled to the anti-rabbit globulins not only increased the sensitivity of the procedure by increasing the size of the coating layer (60 versus 130 nm), but aided in distinguishing nonspecific from specific labeling. Whereas specific labeling appeared as an electron dense coating detectable even in unstained sections, the nonspecific electron dense coating could only be observed after staining of the

sections with heavy metal salts. The peroxidase label was also helpful in visualizing areas of specific labeling by light microscopy (Fig. 1a-c). Our results indicated that nonspecific coatings may be present on cell types other than *S. sanguis*. The control data suggest that the occurrence of such nonspecific coatings is possibly due to the presence of substances in serum which react with sites which can be blocked by normal sheep serum. Such sites would not be stained by HP-labeled anti-rabbit IgG. Therefore, light microscopy of plaque would not reveal any brownish discoloration in such areas coated with sheep serum, nor would such sites be visible in electron micrographs of unstained sections. In addition, the ultrastructural features of the cells, if they are sufficiently distinct, can serve as a useful adjunct in distinguishing among different cell types. Since nonspecific electron dense coatings were only noted among a few cells confined to the plaque surface of stained sections, such findings should not present a serious problem in the identification of specific bacterial groups in plaque.

The advantages of utilizing epoxy resin to collect samples of dental plaque for this study include: (i) the preservation of the structural integrity of dental plaque during the various procedures; (ii) the avoidance of decalcification which might cause denaturation of bacterial antigens in dental plaque; (iii) the lack of noticeable differences between plaque collected on epoxy resin material and enamel (2). The columnar arrangement of the cells perpendicular to the Epon strip surface confirms the results of a previous report that the increase in plaque mass during early plaque formation is associated with growth by cell division rather than by addition of cells to the surface (M. A. Listgarten et al., J. Periodont., in press).

The fact that cells located nearer to the epoxy resin surface are older might account for the less intense reaction with labeled antibody observed at that level. The older cells may have lost some antigenic determinants whereas the younger cells at the surface are more likely to contain a full complement of undenatured antigen. The loss of antigenic determinants may not be due to aging per se, since no alterations in the degree of labeling were observed between 1-day-old and 7-day-old cultures of *S. sanguis*, even when cells had undergone lysis. Perhaps the exposure of older cells in plaque to the intra-oral environment resulted in alterations of the cell surface, possibly mediated by intra-oral enzymes, which were not present in pure cultures. Such alterations could interfere with the localization of certain microorganisms in plaque, when the identifying antigen is very labile. It is also conceivable, though less likely, that antigenic sites at the surface of older cells may be more extensively blocked by antibody molecules derived from saliva or gingival fluid. Some investigators have previously reported the localization in plaque of various microorganisms labeled with fluorescent antibody (18, 19, 20, 26). However, immuno-electron microscopic techniques provide greater resolution in the localization of individual cells or groups of cells. In addition, it is possible to study the ultrastructural features of labeled as well as unlabeled cells in the same specimens. Where low power orientation is required, it is also possible to prepare sections of labeled cells from the same specimens for light microscopic examination.

Perhaps the most significant aspect of these investigations is that for the first time we can look at the exact localization of specific bacterial cells within intact dental plaque. Thus, it should be possible with the development of specific reagents to determine, more accurately than has heretofore been possible, the localization of certain microorganisms at different time intervals within the developing dental plaque. This could also serve as a useful test system for evaluating the effect of specific antibacterial agents on microbial components of human dental plaque.

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