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Characterization of Preparations Enriched for *Streptococcus mutans* Fimbriae: Salivary Immunoglobulin A Antibodies in Caries-Free and Caries-Active Subjects

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The ability of bacteria to adhere to salivary pellicle-coated enamel tooth surfaces is a critical step in oral bacterial colonization. Oral bacteria adhere to receptors of host origin in salivary pellicle. *Streptococcus mutans* has been identified as the major etiological agent of human dental caries and composes a significant proportion of the oral streptococci in carious lesions. Bacterial fimbriae are small (100 to 300 nm) hairlike appendages emanating from the cell surface. Preparations enriched for *S. mutans* fimbriae were isolated by a shearing technique and alternating high- and low-speed centrifugations. A representative fimbrial preparation had two distinct double bands comprising four proteins of approximately 100 to 200 kDa and one faint band at 40 kDa on reducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunoblots and had demonstrable glucosyltransferase activity. Rabbit antisera raised against the preparation specifically stained the fuzzy coat of *S. mutans*, demonstrating short fimbria-like structures protruding 100 to 200 nm from the cell surface. Controls without antifimbria antibody did not exhibit this staining. There were significantly higher ($P \leq 0.05$) levels of salivary immunoglobulin A, but not serum immunoglobulin G, antibodies to the enriched *S. mutans* fimbria preparation by enzyme-linked immunosorbent assay from caries-free subjects than from caries-active subjects. The results suggest that *S. mutans* fimbriae may be an important adherence factor to which caries-free subjects mount a protective salivary immune response.

The first step for a pathogenic bacterium to initiate infection is attachment to a suitable receptor. Extensive studies have shown that, regardless of the site of infection, adhesins located on the cell surface of microorganisms are implicated in this attachment process (22, 32, 35, 37, 38). Oral microorganisms employ a variety of adhesins to bind to each other and to the oral epithelium or salivary pellicle on tooth surfaces. Until recently, the adhesin was believed to be an integral part of the fimbrillar subunit; however, immunoelectron microscopy studies with *Bacteroides loeschei* have shown that the adhesins are usually located in a random pattern on the distal portion of the fimbriae (37). Bacterial fimbriae are defined as small (100 to 300 nm) nonflagellar filamentous proteinaceous surface appendages that do not participate in the transfer of bacterial or viral nucleic acids (3). Fimbriae have been identified on numerous gram-negative microorganisms as long fibrillar structures but have been reported on only a limited range of gram-positive microorganisms, including some streptococci, in which they appear as a much shorter fuzzy coat (8, 30).

Streptococcus mutans has been identified as the major etiological agent of human dental caries and composes a significant proportion of the oral streptococci in carious lesions (26). It is our belief that fimbriae are important virulence factors responsible for *S. mutans* adherence to enamel surfaces. A number of studies with experimental animals and humans have shown that active and passive immunization with *S. mutans*, either with whole cells or with different cellular components, inhibits *S. mutans* colonization and subsequent dental caries formation (10, 17, 20, 24, 27–29, 34). Several laboratories have also shown that naturally occurring and induced salivary immunoglobulin A (IgA) and serum antibodies to *S. mutans* antigens are important in preventing dental caries in humans and animals (4, 7, 18, 25). The purposes of this study were to confirm the existence of fimbriae on *S. mutans*, isolate preparations enriched for the fimbriae, and assess the relationship between fimbriae and the anti-*S. mutans* response in saliva and serum of caries-active and caries-free individuals.

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MATERIALS AND METHODS

Bacteria. *S. mutans* TH16 (serotype *c*) was used in this study. This strain was originally isolated from a human carious lesion and has been shown to be cariogenic in a rat caries model (16). *S. mutans* cells were grown in 30 liters of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 1% glucose at 37°C in 5% CO_2 and 95% air for 24 h.

Enrichment of fimbriae. Fimbrial antigens from *S. mutans* were isolated by the method used by McBride and colleagues (30). Briefly, the fimbriae were removed from the cells by a shearing technique. Cells from 30 liters of culture were harvested by centrifuging at 10,000 × *g* for 15 min at 4°C, washed once in fimbrial buffer (20 mM Tris, 1 mM $MgCl_2$, and 0.02% NaN_3 [pH 6.8]) and frozen as a pellet at −20°C overnight. Frozen cells were thawed, suspended in buffer, and blended in a Waring blender for two 1-min cycles at high speed. Intact cells and cell debris were removed by a slow centrifugation (10,000 × *g*, 4°C, 10 min), and the supernatant, containing the fimbriae, was retained and centrifuged at 110,000 × *g* for 2 h. The resulting fimbrial pellet was resuspended in the same buffer and centrifuged a second time at 10,000 × *g* for 10 min to further remove cell debris and aggregated fimbriae. The supernatant containing the enriched fimbrial preparation and the second 10,000 × *g* pellet containing cell debris and aggregated fimbriae were divided in aliquots and frozen at −80°C until used. Protein and carbohydrate concentrations were determined by the Bio-Rad microprotein assay (Bio-Rad Laboratories, Hercules, Calif.) and the phenol-sulfuric acid assay (2), respectively.

Antifimbria antibody preparation. New Zealand White rabbits were immunized with an enriched *S. mutans* fimbrial preparation (0.377 mg of protein per ml) with the RIBI adjuvant system (monophosphoryl lipid A plus synthetic trehalose dicorynomycolate plus cell wall skeleton emulsion; RIBI ImmunoChem Research, Inc., Hamilton, Mont.). Injection of fimbriae and RIBI adjuvant was done on day 0 and boosted on day 28. A total dose (0.377 mg of protein per ml) of 1.0 ml was administered as follows: 0.3 ml intradermally (0.05 ml in each

of six different sites), 0.4 ml intramuscularly (0.2 ml into each hind leg), 0.1 ml subcutaneously in the neck region, and 0.2 ml intraperitoneally. Blood was collected by cardiac puncture on day 45, and serum was separated as described below.

Collection of saliva and serum samples. Whole saliva and serum samples from caries-active and caries-free subjects were obtained from a previous study (19). Briefly, healthy individuals were screened for the number of decayed, missing, and filled surfaces and for the number of unfilled and active lesions by using a fine-tipped dental explorer and transillumination. Volunteers who had no detectable caries or restorations were classified as caries free. Volunteers who had greater than five restored surfaces and at least five active unrestored carious lesions in the past 5 years represented the caries-active group. Whole saliva samples were collected as described earlier (18). *S. mutans* was detected in whole saliva samples (>1,000 CFU/ml) from all volunteers. Blood samples were obtained by venipuncture and collected in glass tubes. Serum was separated from the clot by centrifugation (5,000 × *g*, 10 min). The saliva and serum samples were stored at −20°C until used for antibody analysis.

Electrophoretic techniques. Fimbrial preparations and the cell debris-aggregated fimbrial pellet were electrophoresed by reducing sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS-PAGE) (National Diagnostics, Atlanta, Ga.). Molecular weight standards were included in every gel (Rainbow colored protein molecular weight markers; Amersham, Arlington Heights, Ill.). Gels were stained for proteins with Coomassie blue and silver nitrate dual staining. Proteins were transferred electrophoretically to nitrocellulose paper for immunoblotting. Blots were probed with rabbit anti-*S. mutans* fimbria serum. Proteins which reacted with alkaline phosphatase-labeled anti-rabbit IgG heavy-chain-specific reagent (Sigma Chemical Company, St. Louis, Mo.) were visualized on nitrocellulose by nitroblue tetrazolium–5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad). Molecular weights were determined by comparison with protein standards with an UltroScan XL laser densitometer and GelScan XL software (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Enzyme-linked immunosorbent assay (ELISA). Saliva and serum samples were assayed for IgA and IgG antibody activity, respectively, to the *S. mutans* fimbrial preparation by a modification of a previously described ELISA (19). Polystyrene microtiter plates (enzyme immunoassay; Linbro; Flow Laboratories, Inc., McLean, Va.) were coated (100 μl per well) with the enriched fimbrillar preparation (1 μg/ml diluted in carbonate-bicarbonate buffer, pH 9.6) and incubated at 37°C for 3 h. Coated plates were washed three times in Tween saline (0.9% NaCl containing 0.05% Tween 20) to remove unbound antigen. Free sites on the plates were blocked by reaction with 200 μl of a solution containing 10 μg of globulin-free human serum albumin per ml (Sigma) for 1 h at 25°C. Fifty clarified (centrifuged at 10,000 × *g* for 10 min) human saliva (diluted 1:4 in Tween saline) and 50 serum (1:100) samples from caries-active and caries-free individuals (25 saliva and 25 serum samples from each group), in triplicate, were added to the wells (100 μl per well) and incubated for 2 h at 37°C. The plates were washed three times with Tween saline and incubated for 3 h at 37°C with 100 μl of horseradish peroxidase-labeled anti-human IgA (for saliva samples) or IgG (for serum samples) heavy-chain-specific reagents (Sigma; 1:1,000). After washing three times with Tween saline, orthophenylenediamine dihydrochloride in citrate buffer containing H_2O_2 was added (100 μl per well). Color development was monitored between 10 and 30 min, and the reaction was stopped with 2 N H_2SO_4. The amount of color that developed was measured at 405 nm in the microtiter plate with a Titertek Multiscan spectrophotometer (Flow). The data were reduced by computing the means and standard errors of the mean of the absorbances of triplicate determinations per sample. The results were analyzed by the paired *t* test, and significant differences were defined as $P \leq 0.05$.

Transmission electron microscopy. Whole *S. mutans* TH16 cells grown overnight in Todd-Hewitt broth and washed once in saline were fixed for 2 h with 3% glutaraldehyde and were stained for 1 h with a 2% osmium tetraoxide solution. The cells were then dehydrated by washing two times with 50% ethanol for 10 min, followed by washing two times with 75% ethanol for 30 min. Cells were embedded in L. R. White resin (Polysciences, Inc., Warrington, Pa.), sectioned for electron microscopy with an ultramicrotome, and placed on Formvar-coated electron microscopy grids.

Sectioned *S. mutans* TH16 bacterial cells were stained either with rabbit anti-*S. mutans* fimbria serum (undiluted) or with rabbit normal serum (undiluted) by adding one drop of the serum on the section for 10 min. The grids were then rinsed three times in deionized glass-distilled water. Colloidal gold-Affinipure goat anti-rabbit IgG beads (6 nm; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) were diluted in sterile saline solution (1:20) and incubated on the coated grids for 10 min. Negative control grids containing *S. mutans* cells without rabbit antifimbrial antibodies or rabbit normal serum were incubated only with goat anti-rabbit IgG-labeled gold beads.

Sectioned and immunogold bead-labeled *S. mutans* TH16 bacterial cells were counterstained by placing one drop of uranyl acetate on the sections for 10 min and then staining for 2 min with lead citrate.

Glucosyltransferase (GTF) assay. The presence of GTF enzyme activity in the enriched *S. mutans* fimbrial preparation and the ability of rabbit anti-*S. mutans* fimbria antibody to neutralize GTF enzyme activity were determined by a previously described method (19).

TABLE 1. GTF enzyme activity of a representative enriched *S. mutans* fimbria preparation and enzyme neutralization by anti-*S. mutans* fimbria antisera

Reagent	cpm[a]	% Neutralization
Background	41.9 ± 18.4	
Fimbriae	145.0 ± 9.8	
Antifimbriae plus fimbriae	134.0 ± 0.9	10.6

[a] Mean ± standard error of the mean.

RESULTS

A representative enriched *S. mutans* fimbria preparation was composed of 85.0% protein and 15.0% carbohydrate and had demonstrable GTF enzyme activity equal to 3.5 times background that was only partially inhibitable by rabbit anti-*S. mutans* fimbria serum (Table 1). Reducing SDS-PAGE analysis of enriched fimbrial preparations of *S. mutans* stained with Coomassie blue and silver stains demonstrated the presence of two distinct double bands (composed of four proteins) ranging from approximately 100 to 200 kDa and one faint band at 40 kDa, with little contaminating material (Fig. 1). The faint 40-kDa band, although visible on the original gel, did not photograph well. *S. mutans* cell debris and aggregated fimbriae exhibited several additional protein bands. The same three sets of bands seen after Coomassie blue and silver nitrate dual staining of the fimbria preparations were present with immunoblots probed with rabbit anti-*S. mutans* fimbria antisera (Fig. 2).

The presence of fimbriae on *S. mutans* whole cells was confirmed by transmission electron microscopy of uranyl acetate- and lead citrate-stained, and immunogold-labeled, *S. mutans* whole cells (Fig. 3). Gold bead-labeled goat antibody against rabbit IgG bound to rabbit anti-*S. mutans* fimbria antibody attached to the periphery of the cells and in some cases demonstrated very small hairlike projections (100 to 200 nm long) on the surface of the cells. Control *S. mutans* cells without rabbit antifimbria antibodies did not exhibit binding to the

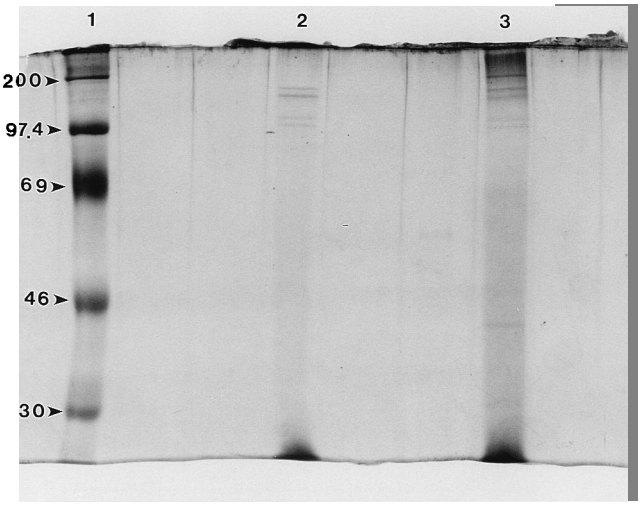

FIG. 1. Representative reducing SDS–10% PAGE gel of enriched *S. mutans* fimbria preparation double stained with Coomassie blue and silver stain. Lane 1, molecular weight standards (molecular weights of 200,000, 97,400, 69,000, 46,000, and 30,000); lane 2, enriched *S. mutans* fimbria preparation (9.5 μg of protein); lane 3, *S. mutans* cell debris and aggregated fimbrial pellet remaining after second centrifugation at 10,000 × *g*.

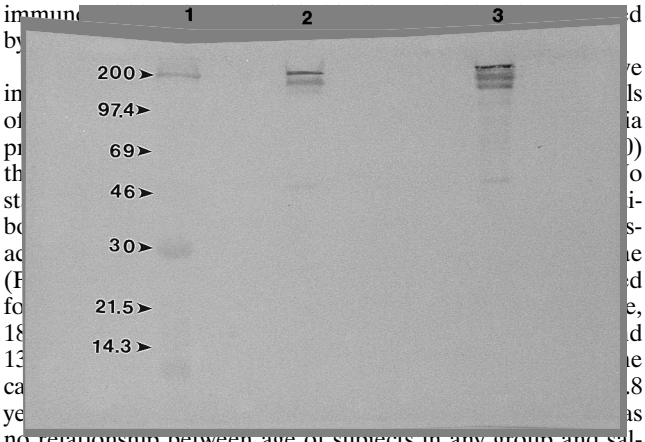

FIG. 12. Representative immunoblot of an enriched *S. mutans* fimbria preparation stained with rabbit anti-*S. mutans* fimbria antisera. Lane 1, molecular weight standards (molecular weights of 200,000, 97,400, 69,000, 46,000, 30,000, 21,500, and 14,300); lane 2, enriched *S. mutans* fimbria preparation (9.5 μg of protein); lane 3, *S. mutans* cell debris and aggregated fimbrial pellet remaining after second centrifugation at 10,000 × *g*.

no relationship between age of subjects in any group and salivary IgA or serum IgG antibody activity to the *S. mutans* fimbria preparation by ELISA (correlation coefficients ranged from $r^2 = 0.0061$ to 0.187).

DISCUSSION

Over the past decade, the adhesive characteristics of bacteria and other prokaryotic and eukaryotic cells have attracted a great deal of attention, and their significance in microbial ecology and pathogenesis has become increasingly apparent (23). Adhesion to the host surface is a necessary first step in the pathogenicity of microorganisms involved in the development of many diseases, including dental caries. Bacterial fimbria-mediated interactions have been postulated as a mechanism of adhesion of oral bacteria to the tooth surface (6, 8, 12, 13). Bacterial adhesion is a highly specific phenomenon. The remarkable specificity exhibited during interaction of bacterial adhesins and host tissues has been compared with antibody-antigen-specific recognition (5) and explains why *S. mutans* is more abundant in dental plaque than in other sites in the oral cavity (14). The fimbria-mediated adhesive properties of gram-negative bacteria have been extensively studied in the medical (e.g., *Escherichia coli* infections [33]) and dental (e.g., *Porphyromonas gingivalis* and periodontal disease [31]) fields. Few fimbria-related studies have been conducted on oral gram-positive microorganisms. However, studies conducted on *Streptococcus parasanguis* FW213 (a member of the group of microorganisms formerly classified as *Streptococcus sanguis*), which is one of the primary colonizers of dental plaque, have demonstrated that attachment to saliva-coated hydroxyapatite is mediated by a 36-kDa adhesin protein (FimA) which is a component of fimbriae of the bacterium and which is able to displace bound FW213 cells (8, 32). It has also been concluded that *S. parasanguis* fimbriae are essential for the microorganism to attach since wild-type fimbriated cells bind well to saliva-coated hydroxyapatite in an in vitro tooth model, whereas afimbriated mutants do not (12). There has been no study conducted to investigate the fimbriae of *S. mutans*, the major etiological agent of human dental caries (26). Therefore, the present study constitutes the first attempt to characterize partially purified *S. mutans* fimbriae and to assess the relationship between fimbriae and the anti-*S. mutans* antibody response in caries-active and caries-free individuals.

The shearing technique used in this study, which was originally described by McBride and colleagues (30) for *S. sanguis*, was successful in enriching an *S. mutans* fimbria preparation. This was corroborated by transmission electron microscopy after immunogold bead staining with rabbit antibody raised against the fimbria preparation. No cross-reactivity occurred between the immunogold beads alone and *S. mutans* whole cells or fimbriae. The surface of *S. mutans* cells exhibited the presence of a fuzzy coat or fringe of constant width and density around the bacterial cell from which short fimbria-like structures protruded 100 to 200 nm. This type of surface fimbrillar structure has been described previously for a majority of *S. sanguis* biotype I and II strains and is referred to as a peritrichous structure. On some *S. sanguis* strains, asymmetric tufts of fimbriae were detected alone or in combination with the peritrichous structure (21). None of the *S. mutans* TH16 cells examined in this study demonstrated the presence of fimbria tufts.

Our experiments concluded that the enriched *S. mutans* fimbria preparation contained 85% protein and 15% polysaccharide and had demonstrable GTF enzyme activity that was not completely inhibitable by antibody to the *S. mutans* fimbria preparation. Very little is known about the biochemical nature of gram-positive bacterial fimbriae, other than for *S. sanguis* and closely related species; however, glycoproteins in the form of fimbriae have been shown to be adhesins on *Streptococcus salivarius* (36). Part of the difficulty has arisen from the complications in obtaining purified material because of the extremely hydrophobic nature of fimbriae (11). In addition, methods that involve dissociation and depolymerization of fimbriae from gram-negative organisms are ineffective with gram-positive fimbriae. Thus, cloning techniques have become a useful way to characterize in detail the fimbrial subunits and precursors in order to increase understanding of the biochemical and regulatory characteristics of these structures (9, 11). Further studies will be done in order to purify the fimbria preparation obtained in this study and further biochemically characterize the fimbriae.

Pathogenic microorganisms must overcome the host nonspecific defense barriers (e.g., cleansing mechanisms such as coughing, swallowing, and fluid flow) and must also escape recognition by soluble immune or nonimmune host molecules in host secretions (e.g., secretory IgA antibodies may bind to surface antigens of microorganisms in saliva, causing them to agglutinate, thereby facilitating their rapid elimination). Establishment of disease depends on the relative incapacity of the host to provide effective specific and nonspecific protective barriers and on the ability of the microorganism to adhere and

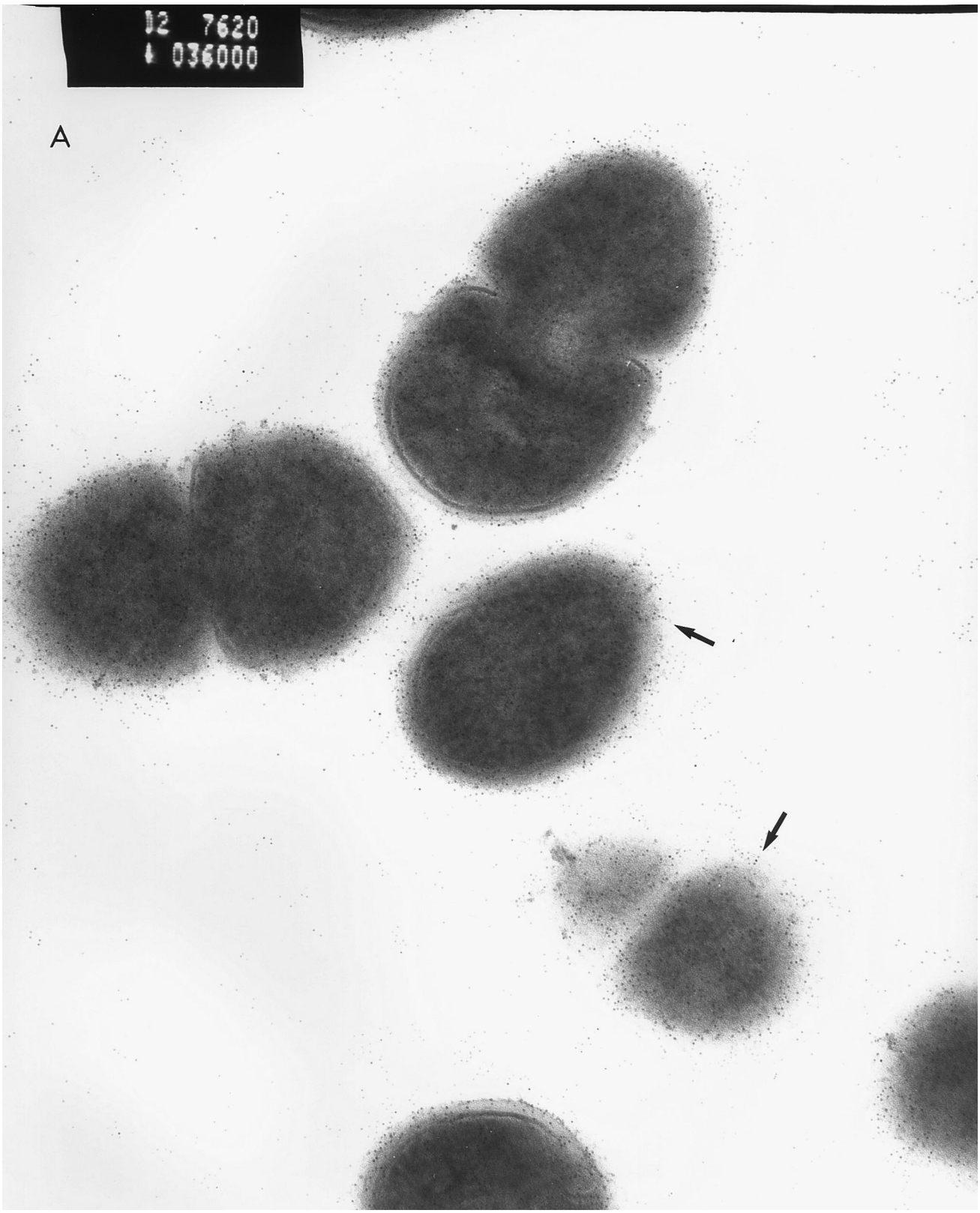

FIG. 3. Transmission electron micrographs of representative *S. mutans* whole cells. (A) *S. mutans* whole cells stained with rabbit anti-*S. mutans* fimbriae and immunogold beads (magnification, ca. ×85,870). (B) *S. mutans* whole cells incubated with rabbit normal serum and immunogold beads (magnification, ca. ×85,870). Arrows indicate *S. mutans* linear arrangement of fimbriae.

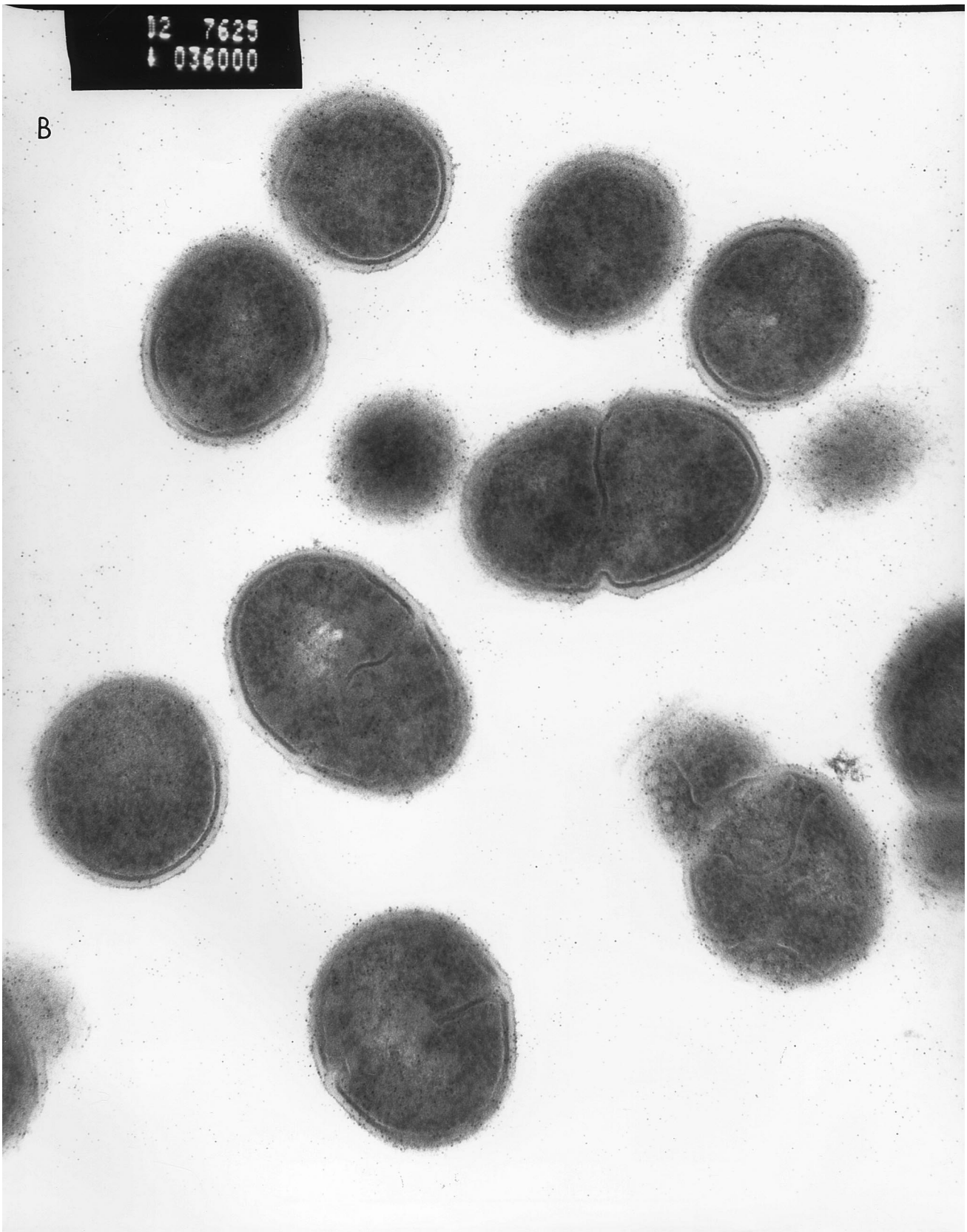

FIG. 3–*Continued.*

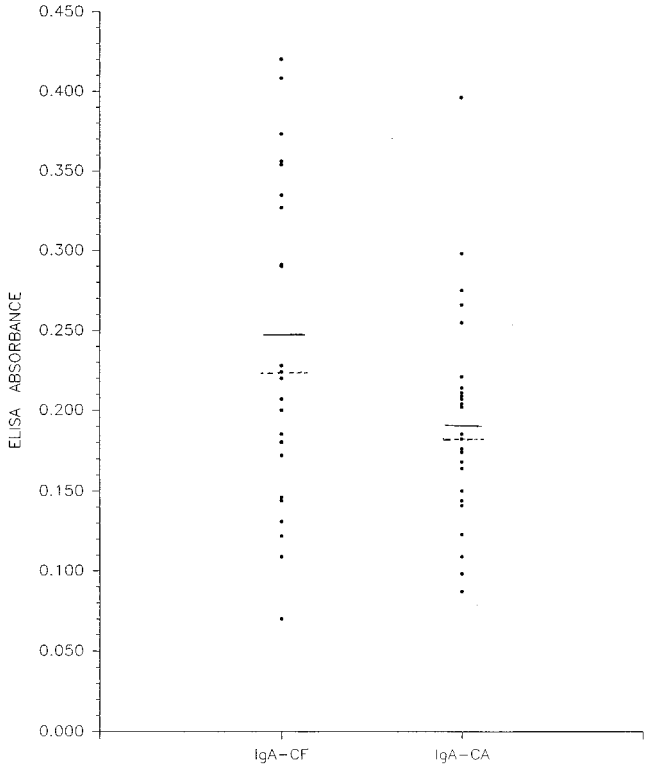

FIG. 4. Scattergram showing salivary IgA antibody levels to a representative enriched *S. mutans* fimbria preparation from 25 dental caries-free (CF) and 25 caries-active (CA) subjects by ELISA. Solid and dashed lines indicate mean and median values, respectively.

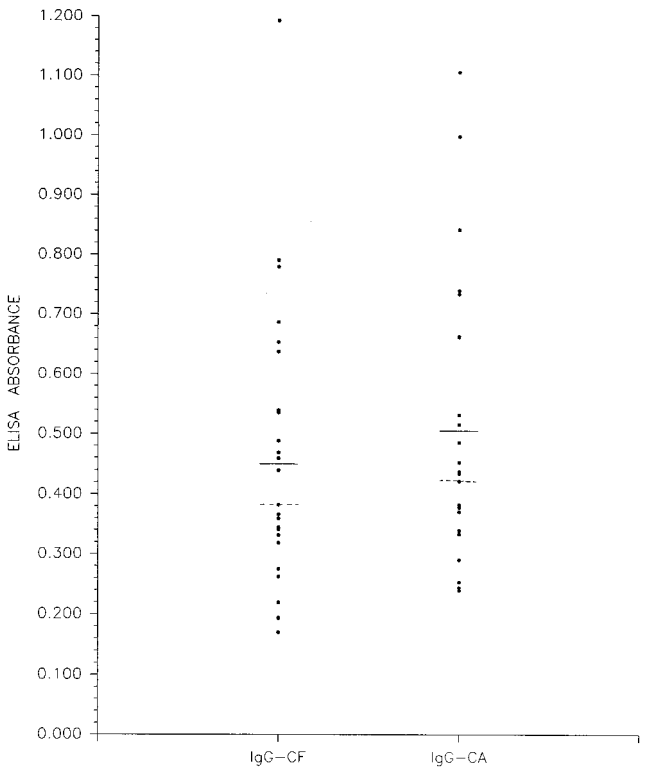

FIG. 5. Scattergram showing serum IgG antibody levels to a representative enriched *S. mutans* fimbria preparation from 25 dental caries-free (CF) and 25 caries-active (CA) subjects by ELISA. Solid and dashed lines indicate mean and median values, respectively.

to overcome these barriers. Previous reports have indicated that a lower susceptibility to dental caries is associated with high levels of salivary IgA antibody to *S. mutans* whole cells, and no association was found with serum antibodies (25). However, Aaltonen et al. (1) reported a significant association of high serum IgG antibody levels to *S. mutans* with low counts of *S. mutans* in plaque and reduced caries activity. Our laboratory has previously reported higher levels of salivary IgA and serum IgG antibodies to several *S. mutans* surface antigens in caries-free individuals than in caries-active individuals (19). It would be of great interest to identify the specific *S. mutans* antigens responsible for the induction of protective salivary and/or serum antibodies. The results from the present study indicate that caries-free individuals have higher levels of salivary IgA antibodies (and no difference in serum IgG antibodies) to an enriched fimbria preparation of *S. mutans* than do caries-active individuals. We found that the *S. mutans* fimbria preparation had demonstrable GTF activity. Bammann and Gibbons (4) have demonstrated that a significant amount of human salivary IgA antibody activity to *S. mutans* whole cells is directed against glucan and GTF determinants. Gregory and Filler (17) concluded that induced salivary IgA antibodies can mediate *S. mutans* colonization. If GTF enzyme is bound to *S. mutans* fimbriae, enzyme neutralization by IgA antibody may inhibit *S. mutans* enzyme activities and, therefore, cariogenicity by reducing both the colonization by *S. mutans* and the virulence of the organism (19). These results suggest that caries-free subjects may be protected immunologically from dental caries by salivary IgA antibody against *S. mutans* fimbrial antigens.

The ultimate goal in the prevention of bacterial adhesion is long-lasting protection conferred by an appropriate vaccine. The ideal candidate for a vaccine against bacterial adhesion would be the isolated and purified fimbrial adhesin itself. Purified fimbriae may thus be useful as a primary immunogen. Two essential elements of fimbria vaccine development are the detection of common fimbrial antigens occurring among most pathogenic isolates and the ability to induce antibodies that block bacterial adhesion (15). In addition, the efficacy of live vaccines at mucosal surfaces could be improved by the molecular introduction of fimbriae or recombinant hybrid fimbriae into an avirulent bacterium (23). It is clearly apparent that there is a need for further studies on *S. mutans* fimbriae.

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