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Active Release of Bound Antibody by *Streptococcus mutans*

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Previous studies have shown that *Streptococcus mutans* is capable of releasing many surface protein antigens, particularly antigen P1. Antigen P1 is immunodominant and has been implicated in adherence of *S. mutans* to the acquired pellicles. The purpose of this study is to investigate the significance of release of this antigen by the cells. *S. mutans* NG8 (serotype c) was incubated with an anti-P1 rabbit immunoglobulin G (IgG) or a human colostral IgA which contains natural anti-P1 activity. Results indicated that the bound antibodies were released by the cells in a pH- and time-dependent manner. The optimal pH for release was between 6 and 8, and the release rate reached a plateau in 1 h at 37°C. The release of bound antibodies was considered an active process, since heat-killed cells remained capable of antibody binding but failed to release the antibodies. The release was also dependent on the age of the culture, with early-exponential-phase cells releasing the maximum amount of bound IgG. The released IgG was isolated by polyethylene glycol precipitation and protein A-Sepharose column chromatography and found to be associated with antigen P1, indicating that the antibodies were released together with the antigen in the form of immune complexes. The binding of *S. mutans* by secretory IgA (SIgA) inhibited the adherence of the cells to salivary agglutinin-coated hydroxylapatite. However, when the SIgA-coated *S. mutans* was allowed to release the bound antibodies, the inhibitory effect of SIgA on adherence was abrogated. These results suggest that *S. mutans* is capable of shedding surface-bound antibodies in the form of antibody-antigen immune complexes. Such an action may be a strategy employed by the cells to counter the neutralizing effect of naturally occurring antibodies in the oral cavity.

Streptococcus mutans is a normal inhabitant of the human oral cavity and is the etiological agent for dental caries (29). This organism expresses a number of proteins located on its cell surface (37). One such surface protein is antigen P1, alternatively termed antigen B (36), antigen I/II (35), IF (19), or Pac (32). Immunogold labelling and molecular studies show that this protein is associated with the fibrillar fuzz on the *S. mutans* cell surface (5, 27). The apparent biological function of antigen P1 is to interact with a high-molecular-weight (ca.-400-kDa) salivary glycoprotein (agglutinin), which is one of the constituents of the acquired pellicle coating the tooth surface (9, 27). Such an interaction may contribute to the adherence of *S. mutans* to saliva-coated tooth surfaces in a sucrose-independent manner (6). The alanine-rich region located near the N terminus of antigen P1 has been shown to be the domain which interacts with the salivary agglutinin (15, 33).

In humans, natural antibodies against *S. mutans* are found in serum, colostrum, milk, tears, and saliva (3, 4, 28, 40). These antibodies have been shown to have specificity for antigen P1, glucosyltransferases, and cell wall carbohydrates (12). Brown and Mestecky (11) demonstrated that the secretory immunoglobulin A (SIgA) in human parotid saliva with specificity for the above antigens is predominantly associated with the SIgA1 subclass.

SIgA antibodies are the host's first line of mucosal defense against adherence and colonization by pathogens (21). The presence of naturally occurring SIgA antibodies in saliva may neutralize the adherence ability of *S. mutans*. It has been demonstrated by others that the binding of *S. mutans* to saliva-coated hydroxylapatite (HA) could be inhibited by naturally occurring human SIgA antibodies (17, 23, 34). One study clearly showed the inhibition of adherence was caused by SIgA with specificity for antigen P1 (17).

Bacteria present in saliva are generally coated with SIgA (10). Furthermore, SIgA has been detected in dental plaques (39) and *Streptococcus sanguis* and *Streptococcus gordonii* obtained from incipient dental plaques have been shown to be coated by the Fab_{α} fragment of SIgA (1). Other bacteria, such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus*, obtained from the nasopharynx are coated by SIgA, IgG, and complement C3b (38). Given the preceding examples, it is conceivable that *S. mutans* in its natural habitat is also coated by anti-*S. mutans* SIgA.

Unlike *S. sanguis* and *Streptococcus oralis*, *S. mutans* is not known to produce IgA proteases (13, 20, 22), which have been implicated as a way for the bacteria to counter the neutralizing effect of SIgA (34). Without the IgA proteases, it is not clear by what means *S. mutans* cells are able to avoid or circumvent neutralization by the salivary antibodies in the oral cavity.

A recent study from this laboratory reported that *S. mutans* is capable of releasing its surface proteins, notably antigen P1, under defined conditions (25). The release was apparently due to an enzymatic activity which can be inactivated by heat, Cu^{2+}, Zn^{2+}, and thiol-blocking reagents and which is most active between pH 5 and 6. The release of surface proteins such as antigen P1 may have significant implications. The process of surface protein release may be a way for the organism to get rid of the bound antibodies. Alternatively, the process may be a means for the bacterium to neutralize the surrounding antibodies, which are literally consumed by the released antigens.

The present study shows that *S. mutans* is capable of liberating antibodies bound to its cell surface. The data demonstrate that the released antibodies exist in the form of an antigen-antibody immune complex. This phenomenon suggests that the antibodies are released as a result of the liberation of the cell surface antigens. When SIgA-coated *S. mutans* was allowed to release the bound antibodies, the inhibitory effect of

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SIgA on the adherence of cells to salivary agglutinin-coated HA (AHA) was abrogated.

MATERIALS AND METHODS

Bacteria and growth conditions. *S. mutans* NG8 (serotype c) and its P1-deficient isogenic mutant 834 have been described previously (27). *Streptococcus pyogenes* type M1 (isolate 40/58) was obtained from C. Schalén (University of Lund, Lund, Sweden). Cells were grown in Todd-Hewitt broth in the presence of air without agitation at 37°C (27).

Antibodies. The rabbit polyclonal anti-P1 antisera used in this study have been described previously (25). The IgG fraction was isolated by passing the sera through a protein A-Sepharose column (Sigma Chemical Co., St. Louis, Mo.) by the method described by Harlow and Lane (18). Briefly, 1 ml of $(NH_4)_2SO_4$-precipitated serum was applied to a column of 1.5 ml of protein A-Sepharose packed in a 53/4-in (14.6-cm) Pasteur pipette. The column was washed with 20 ml of 0.1 M Tris, pH 8.0, and 10 ml of 0.01 M Tris, pH 8.0. IgG was eluted with 0.1 M glycine, pH 3.0, and was collected as 0.4-ml fractions which were neutralized immediately with 50 μl of 1.0 M Tris, pH 8.0. The eluted materials contained 5 mg of IgG per ml, as determined by capture enzyme-linked immunosorbent assay (ELISA), and a total protein concentration of 7 mg/ml. ELISA showed that the IgG had a titer of 6,400 against purified antigen P1. This purified IgG was used in this study as the anti-P1 IgG.

The monoclonal anti-P1 antibody, 4-10A, kindly donated by A. S. Bleiweis (University of Florida, Gainesville, Fla.), was used in the form of an ascitic fluid. This monoclonal antibody recognizes an epitope near the center region of the P1 molecule (8).

Human colostral SIgA (reagent grade) was purchased from Sigma. Each lot of the SIgA was tested for anti-P1 activity by ELISA, and the average titer of these SIgA lots was 75 (range 50 to 100).

ELISA. The procedure for ELISA was followed as described previously (25). For the determination of antibody titers, 96-well polystyrene microtiter plates were coated with 10 ng of antigen P1 (25). Twofold serially diluted antibodies were added, and the reaction was detected by alkaline phosphatase-conjugated goat anti-rabbit IgG (specific for whole IgG, 1/3,000; Sigma) or alkaline phosphatase-conjugated goat anti-human IgA (α-chain specific, 1/3,000; Sigma). The titers of antibodies were expressed as the reciprocal of the dilution which produced an A_{405} reading 0.05 higher than that of the controls (without antigen P1).

For the quantitation of IgG or SIgA, microtiter plates were primed with either mouse anti-rabbit IgG monoclonal antibody (γ-chain specific, 1/1,000, clone RG-96; Sigma) or mouse anti-human IgA monoclonal antibody (α-chain specific, 1/2,000, clone GA-112; Sigma) to capture rabbit IgG or colostral SIgA, respectively. The captured antibodies were detected with the alkaline phosphatase conjugates as described above. Standard curves of rabbit IgG (Sigma; reagent grade) and colostral SIgA were run alongside of each determination.

For the quantitation of antigen P1, microtiter plates were coated with anti-P1 monoclonal antibody 4-10A, which captured the antigen, and the ELISA was performed as described previously (25).

Binding and release of antibodies. *S. mutans* cells were harvested by centrifugation (10,000 × *g*, 4°C, 10 min) and were washed twice with cold phosphate-buffered saline (PBS; 10 mM sodium phosphate [pH 7.6] plus 145 mM NaCl). The cells were resuspended in PBS to an optical density at 600 nm (OD_{600} of 50 U/ml or ca. 6.03×10^{10} CFU/ml. One OD_{600} unit (OD_{600} of 1) was defined as the spectrophotometer reading of 1.000 when a light at 600 nm was passed through a 1-cm path of a cell suspension at room temperature. The spectrophotometer used in this study was an HP8452A Diode-Array Spectrophotometer (Hewlett-Packard, Waldbronn, Germany). The cells were incubated with equal volumes of antibodies (IgG, 50 μg/ml; and SIgA, 400 μg/ml) at 4°C for 1 h in a vertical rotator at 12 rpm (Hematology mixer, model 346; Fisher Scientific, Edmonton, Alberta, Canada). This ratio of cell density to immunoglobulins was used in all the studies except the adherence assay. The suspension was centrifuged (11,750 × *g*, 2 min, room temperature) with a microcentrifuge. The supernatant fluid was saved as the unbound antibody fraction, and the cells were washed twice with cold PBS. The cells were then resuspended in 0.1 M sodium acetate buffer or sodium phosphate buffer and were incubated with rotation at 37°C for 2 h to allow the release of bound antibodies to occur. The cell suspension was centrifuged, and the supernatant fluid was saved.

ELISA was used to determine the total amount of antibodies in the various fractions. The results showed that antibodies in the two PBS washes each accounted for less than 1% of the original antibodies. Therefore, the amount of antibodies bound to the cells was estimated as the difference between the initial amount of antibodies and the amount found in the unbound antibody fraction. The total amount of antibodies bound was calculated as nanograms of antibody bound to cells per milliliter/total CFU per milliliter.

To determine the proportion of antibody specific for P1 bound to the cells, the fractions obtained as described above were assayed on microtiter plates coated with purified P1 (10 ng per well). Titration curves of the fractions against the purified P1 were obtained by serial twofold dilution of the fractions in ELISA. The A_{405} readings from the linear part of the curves were used for the following calculation. The percentage of the total anti-P1 activity bound was calculated as follows: {[(A_{405} reading of initial antibody × dilution factor) − (A_{405} reading of unbound antibody fraction × dilution factor)]/A_{405} reading of initial antibody × dilution factor} × 100.

Growth of culture and release of proteins and bound IgG. One liter of prewarmed Todd-Hewitt broth was inoculated with 40 ml of an 18-h-old *S. mutans* NG8 culture before incubation at 37°C. The culture was sampled six times over the entire growth period. Each sample was processed immediately after sampling. The cells from the samples were washed twice with PBS and were resuspended in 0.4-ml aliquots at an OD_{600} of 50 U/ml. For the release of surface proteins by the cells, 0.2 ml of the cell suspension was pelleted by centrifugation (11,750 × *g*, 2 min), and the cells were resuspended in 0.2 ml of 0.1 M sodium phosphate buffer, pH 6.0, and incubated at 37°C for 2 h. The cells were then pelleted, and the supernatant fluid was boiled with the sample buffer of Laemmli (24) before being analyzed on a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel electrophoresis (PAGE) gel. Antigen P1 in the supernatant fluid was quantitated by capture ELISA as described above.

To check whether any of the proteins released were derived from the cytoplasm as a result of cell lysis, the supernatant fluid was assayed for a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase activity (30). The results showed that the supernatant fluid contained no detectable glucose-6-phosphate dehydrogenase activity, while an equal amount (50 μg) of proteins from an *S. mutans* NG8 cell sonicate yielded 14 U (in micromoles of NADPH per minute) of glucose-6-phosphate dehydrogenase activity. Similar findings were reported previously (25).

For the release of bound antibody, 0.2 ml of the cell suspension was mixed with an equal volume of anti-P1 IgG (50 μg/ml) prior to incubation on ice for 1 h. The cells were then pelleted and washed twice with PBS before incubation in 0.2 ml of sodium phosphate (0.1 M, pH 6.0) for 2 h at 37°C. The cell suspension was centrifuged, and the released IgG in the supernatant fluid was quantitated by capture ELISA.

Tests for IgG Fc receptor and IgA receptor of *S. mutans*. Late-exponential-phase *S. mutans* NG8 cells were washed three times in PBS and were resuspended at an OD_{600} of 0.4 U/ml. The cell suspensions (100-μl aliquots) were used to coat a polystyrene microtiter plate at room temperature for 1 h. The plate was blocked with 1% gelatin in PBS, and half of the plate was incubated with 100-μl aliquots of a rabbit IgG Fc fragment (10 μg/ml; Bio/Can Scientific, Mississauga, Ontario, Canada). The other half of the plate was used for a reaction with PBS as a control. The plate was incubated at room temperature for 1 h. Alkaline phosphatase-conjugated $F(ab')_2$ fragment of a goat anti-rabbit IgG (Fc specific, 1/5,000; Bio/Can) was then added to the plate. After a 1-h incubation, Sigma 104 substrate (Sigma) was added and A_{405} was measured at 1-h intervals over 5 h of incubation at room temperature.

For the IgA receptor assay, an experiment similar to that described above was performed. The polystyrene microtiter plate was coated with *S. mutans* NG8 cells. After the plate was blocked, a human serum IgA (10 μg/ml, polyclonal; Bio/Can) was used for a reaction with half of the samples. The binding of the IgA by the bacteria was detected by an alkaline phosphatase-conjugated F(ab′)2 fragment of a goat anti-human IgA (α-chain specific, 1/10,000; Sigma).

In both assays, *S. pyogenes* (type M1) cells adjusted to the same cell density as *S. mutans* cells were used as a positive control.

Isolation of immune complexes. Cultures (twelve 100-ml aliquots) of *S. mutans* NG8 at the exponential phase of growth (OD_{600}, 0.35) were centrifuged (10,000 × *g*, 4°C, 10 min), and the cells were washed twice with cold PBS and resuspended in PBS (twelve 1.4-ml portions) in 1.5-ml microcentrifuge tubes. Anti-P1 IgG (7 μl containing 35 μg of IgG) was added to each of the 12 cell suspensions prior to incubation on ice for 1 h, with occasional mixing. The cells were pelleted (11,750 × *g*, 10 min) and were washed three times with 20 mM Tris, pH 8.0, to wash away any unbound IgG. The cells were allowed to release the bound IgG by resuspension in 0.1 M sodium acetate buffer, pH 5.0 (0.7 ml per tube) and were incubated at 37°C for 90 min with occasional mixing. The cells were again pelleted, and polyethylene glycol (PEG; average molecular weight of 8,000; Sigma) in 0.1 M sodium borate buffer, pH 8.5, was added to the supernatant fluid to a final concentration of 5% (wt/vol). The solutions were incubated at 4°C for 16 h. Under these conditions, less than 1% of the monomeric IgG was precipitated. This result is consistent with studies by Creighton et al. (14) which showed that PEG at this concentration preferentially precipitates soluble immune complexes while monomeric IgG remains in solution. Precipitates were collected at 11,750 × *g* (10 min) and were washed once with 5% cold PEG in the borate buffer. Each pellet was dissolved in 50 μl of 50 mM Tris, pH 8.0, and the resulting solution was pooled and recentrifuged. The clarified supernatant fluid was applied to a protein A-Sepharose column. The immune complexes were eluted from the column with 0.1 M glycine, pH 3.0, and were neutralized immediately with 1.0 M Tris, pH 8.0, as described above.

To locate the immune complexes, 10-μl samples from each fraction were dot blotted onto a piece of nitrocellulose membrane. The membrane was blocked with 1% (wt/vol) gelatin, used for a reaction with the rabbit anti-P1 antiserum (1/200), and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (1/3,000) and Sigma 104 substrate. The fractions that showed reactivity (fractions 3 to 10) were pooled. The pooled fractions contained a total of 22 μg of protein.

For a control, the experiment was repeated exactly as described, except that the step of anti-P1 IgG coating of cells was omitted. Immunodot blotting of samples collected from the protein A-Sepharose column indicated that none of

the fractions showed reactivity with the anti-P1 antiserum, suggesting that the released P1 alone did not interact with protein A-Sepharose. In a separate control experiment to test whether antigen P1 was precipitated by PEG, the materials released from cells which were not exposed to antibodies and which contained antigen P1 were treated with 5% PEG as described above. Antigen P1 was not detected in the PEG pellet by ELISA, indicating that P1 was not precipitated by 5% PEG.

Preparation of salivary agglutinin. Agglutinin was isolated from clarified whole saliva by a method described previously (27). The yield of agglutinin was 2 to 3 μg/ml of saliva, and the preparation contained 0.4 to 0.8% IgA, which are values comparable to those previously reported (27). SDS-PAGE analysis showed that the agglutinin migrated as a diffused band of an estimated 400 kDa (data not shown).

Adherence assay. Adherence of *S. mutans* cells to AHA was assayed as described previously, with modifications (27). *S. mutans* cells were radiolabelled by growing in 10 ml of Todd-Hewitt broth containing 50 μCi of [*methyl*-^{3}H]thymidine (specific activity, 82.9 Ci/mmol; Du Pont Canada Inc., Markham, Ontario, Canada) per ml. Cells were harvested at the mid-exponential phase of growth (OD_{600}, 0.4), were washed once with the adherence buffer (50 mM KCl, 1 mM $CaCl_2 \cdot H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$, 0.78 mM KH_2PO_4, 1.22 mM K_2HPO_4; pH 7.2), and were resuspended in 3 ml of adherence buffer. Preliminary results indicated that several batches of cells harvested at the same growth phase (OD_{600}, 0.4) were radiolabelled at a fairly reproducible (standard deviation of ±5%) level of specific activity (31,000 cpm/10^8 CFU). The cells were dechained by rapid passages (15 times) through a 27-gauge half-inch (1.27-cm) needle. Microscopic examinations showed that after this treatment cells were found mainly in a single or double conformation, and fewer than 5% were in short chains (three to five cells).

The cells (0.3 ml) were incubated with either SIgA (0.3 ml at 4 μg/ml) or anti-P1 IgG (0.3 ml at 40 μg/ml) on ice for 1 h. After this treatment, cells remained in single or double conformation when examined under a microscope, suggesting that antibody-induced aggregation did not occur. Results from preliminary testings showed that this ratio of cell density to antibody was desirable because antibody-induced aggregation was absent and a reasonable level of adherence inhibition could be obtained. The cells were pelleted (16,000 × *g*, 5 min) before being washed two times with adherence buffer and resuspended in 0.6 ml of either 0.1 M sodium acetate buffer (pH 4.0), sodium phosphate buffer (pH 6.0), or adherence buffer. The cell suspensions were incubated at 37°C for the appropriate intervals, and the cells were then pelleted and washed once with adherence buffer. The cells were diluted in adherence buffer to yield an activity of 300,000 cpm/ml.

In the adherence assay, the granulated form of HA (Sigma; type III) was used in place of the conventional bead form. The granulated HA consisted of spherical aggregates (diameter, 200 to 250 μm) of HA crystals and contained about 1% silicic acid as the aggregating agent (31).

The granulated HA (dry weight, 18 mg) was equilibrated overnight in 7.65 ml of adherence buffer at 4°C. The buffer was removed by aspiration, and salivary agglutinin was added to a final concentration of 20 μg/ml. The suspension was constantly mixed by a vertical rotator at 12 rpm for 1 h at 37°C. The AHA was allowed to settle under gravity for 10 min, was washed once with adherence buffer, and then was blocked with 1% (wt/vol) bovine serum albumin in adherence buffer for 1 h at 37°C. The AHA suspension was divided into aliquots (200 μl) with a cut-off pipette tip in 0.5-ml microcentrifuge tubes. The HA in each tube represented ca. 0.47 mg of HA (dry weight). Preliminary experiments indicated that this amount of HA produced the maximum level of cell binding with the amounts of agglutinin and cells used. After the AHA had settled (10 min), the liquid was removed carefully with a pipette and 200 μl of ^{3}H-labelled (ca. 60,000 cpm) *S. mutans* cells was added. The cells and AHA were mixed by rotation prior to incubation at 37°C. After 1 h, the AHA was allowed to settle under gravity for 10 min. During this period, the tubes were very gently tapped two or three times to ensure that fine particles settled on the inner wall had fallen to the bottom of the tube. The liquid above the AHA (150 μl) was removed, and activity was counted in a liquid scintillation counter. Percent adherence of cells to HA was calculated as follows: [(control counts − test counts)/control counts] × 100, where control counts were counts from tubes from which HA was omitted.

SDS-PAGE and Western immunoblotting. Proteins were analyzed on SDS-PAGE gels by the method of Laemmli (24). Western immunoblotting was performed as described previously (26).

Protein concentration determination. The protein concentrations in samples were estimated by the method of Bradford, with a rabbit IgG as the standard (7).

RESULTS

Binding of antibodies to *S. mutans* cells. To obtain some insight into the amount of antibody binding to *S. mutans* NG8 cells under our experimental conditions, cells were harvested, washed in phosphate-buffered saline, and used for reactions with rabbit anti-P1 IgG or human colostral SIgA. The amount of unbound antibodies was determined by capture ELISA, and the amounts of bound antibodies were calculated. The average amounts of IgG bound to live and heat-inactivated (100°C, 15 min) *S. mutans* cells were 5.87×10^{-7} and 5.01×10^{-7} ng/CFU, respectively. The average amounts of SIgA bound by live and heat-inactivated *S. mutans* cells were 2.42×10^{-6} and 2.01×10^{-6} ng/CFU, respectively. A nonimmune rabbit IgG which has an anti-P1 titer of 10 also bound to *S. mutans* cells but to a lesser extent (5.8×10^{-9} ng/CFU).

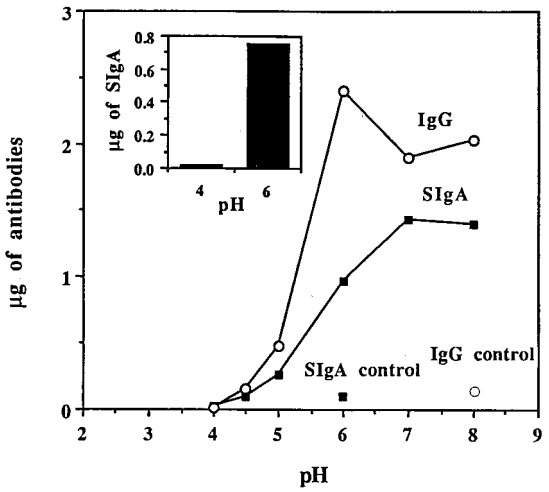

FIG. 1. Effect of pH on the release of bound antibodies by *S. mutans* NG8. Late-exponential-phase cells (OD_{600}, 0.9) were used in reactions with antibodies, washed, and incubated in 0.1 M sodium acetate buffer (pH 4, 4.5, and 5) or 0.1 M sodium phosphate buffer (pH 6, 7, and 8) at 37°C for 2 h. The amounts of released antibodies were estimated by capture ELISA. Cells that were heat inactivated before antibody binding and release are shown as SIgA control and IgG control. The data in the inset show that cells incubated at pH 4 which were then resuspended in pH 6 buffer resumed the release of bound-SIgA.

The live and heat-inactivated *S. mutans* cells were found to bind 84 and 63% of the total anti-P1 activity in the anti-P1 IgG preparation, respectively. The proportions of the total anti-P1 activity in the SIgA bound by the live and heat-inactivated cells were 68 and 57%, respectively.

When *S. mutans* NG8 was tested for IgG Fc receptor and IgA receptor in an ELISA, the bacterium did not show any reactivity (A_{405}, <0.02) after 5 h of incubation. In contrast, *S. pyogenes* (type M1) yielded A_{405} readings of 0.203 and 0.382 for the IgG Fc fragment and serum IgA, respectively, after 1 h of incubation.

Release of bound antibodies by *S. mutans*. During initial testing in the laboratory, we noted that antibodies bound to *S. mutans* cells were released by the cells. To study this phenomenon further, *S. mutans* cells were coated with antibodies, washed, and resuspended in buffers at various pH. The released antibodies were quantitated. As shown in Fig. 1, the amount of bound antibodies released increased with pH. The maximum liberation of bound IgG occurred at pH 6 to 8. The maximum release of bound SIgA occurred at pH 7 and 8. Heat-inactivated cells, which still bound significant amounts of antibodies as indicated above, showed very little release of either IgG or SIgA (Fig. 1, IgG control and SIgA control). These results suggest that the release of the bound antibodies was not due to the simple dissociation of antibodies from the cell surface antigens when cells were incubated in buffer at near neutrality.

Cells that had been incubated at pH 4 which released a minimal amount of bound SIgA were pelleted by centrifugation and resuspended in pH 6 buffer. After incubation (1 h), a

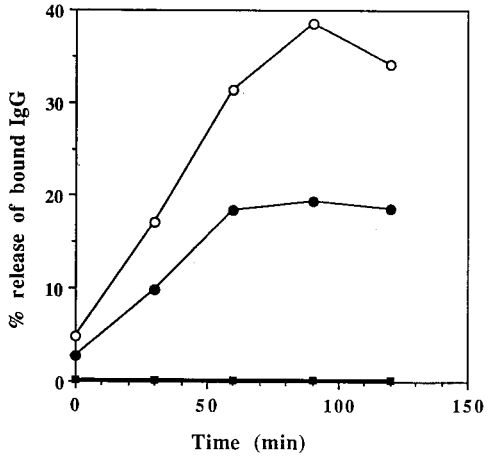

FIG. 2. Release of bound IgG by *S. mutans* NG8 over incubation time. Live cells were used in reactions with anti-P1 IgG (○) or a nonimmune rabbit IgG (●). Heat-inactivated cells were used in reactions with anti-P1 IgG (■). The release was performed in 0.1 M sodium phosphate buffer, pH 6.0. Percent release of bound IgG was calculated as (micrograms of released IgG/micrograms of bound IgG) × 100.

significant amount of SIgA was released by these cells (Fig. 1, insert). This result confirms that *S. mutans* cells were not able to release the bound antibodies at a low pH but could do so at pH 6 or above.

When the release of bound antibodies by *S. mutans* was examined over time, a rapid and linear release of anti-P1 IgG was observed over the first 60 min of incubation (Fig. 2). After this period, the amount of released IgG appeared to level off. The release of nonimmune rabbit IgG over time occurred similarly. Again, heat-inactivated cells showed no release of bound anti-P1 IgG. The release of bound SIgA over the incubation time by *S. mutans* occurred similarly to the release of bound-IgG (data not shown).

Growth of culture and release of proteins and bound IgG. To further study the characteristic of the release of bound antibodies by *S. mutans*, the bacterial culture was sampled over its entire growth period. The cells were harvested, were washed to free them of culture medium, and were assayed for the release of the bound anti-P1 IgG as well as surface proteins. Figure 3A shows the growth curve of *S. mutans* and the rate of release of bound IgG and antigen P1 by the cells. The results showed that as the culture aged, the ability of the cells to release the bound IgG diminished. Cells from the early exponential phase of growth have the best ability to release bound IgG. The release of antigen P1 by the cells, however, did not show such a trend. Cells obtained during the early part of growth released an increasing amount of antigen P1, and cells from the mid-exponential phase of growth released the largest quantity of P1. Cells in the late exponential and stationary phase of growth released less P1.

Examination of the released cell surface proteins by SDS-PAGE showed the complex nature of the released proteins (Fig. 3B). This complex nature of proteins was also observed previously (25). The relative amounts of non-P1 proteins released by cells during the different growth periods displayed a trend similar to that of antigen P1, with the highest concentration occurring for cells obtained from mid-exponential-phase growth.

Nature of released materials. The above results clearly showed that *S. mutans* could release the antibodies bound to its cell surface. To gain some insight into the nature of the released antibodies, some of the released materials were isolated by PEG precipitation followed by protein A-Sepharose column chromatography. Immunodot blotting showed that the isolated materials in their native state reacted with a goat anti-rabbit IgG, confirming the presence of rabbit IgG in the samples (Fig. 4A). Attempts to have the isolated materials react with mouse anti-P1 monoclonal antibody in the dot blot assay were unsuccessful. However, when the isolated materials were denatured

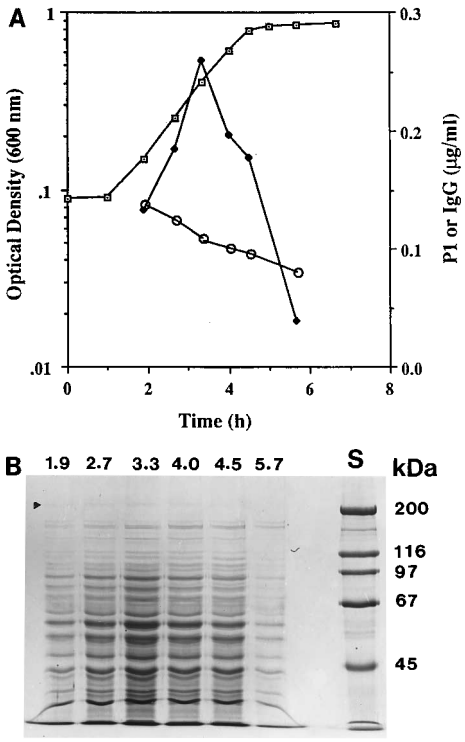

FIG. 3. Growth and release of surface proteins and bound IgG by *S. mutans* NG8. (A) Growth of cells in Todd-Hewitt broth (⊡) and release of antigen P1 (◆) and bound anti-P1 IgG (○). (B) SDS-PAGE of cell surface proteins released by *S. mutans* NG8. The sampling time (hours) of each sample is indicated above the lane. Each lane represents 10 μl of a 200-μl sample. The arrowhead indicates antigen P1. S, protein markers.

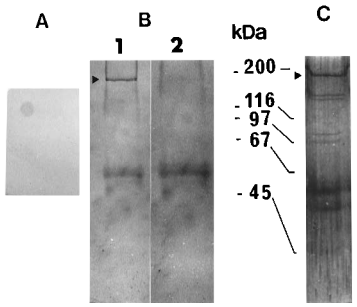

FIG. 4. Immune complexes released by *S. mutans* NG8. The immune complexes were isolated by PEG precipitation and protein A-Sepharose column chromatography. (A) Immunodot blotting showing the isolated immune complexes in their native state reacting with the goat anti-rabbit alkaline phosphatase conjugates. (B) Western blots showing the denatured immune complexes (1 μg) containing two bands reacting with rabbit polyclonal anti-P1 antisera and goat anti-rabbit alkaline phosphatase conjugates (lane 1) and a rabbit IgG (0.2 μg) reacting with goat anti-rabbit alkaline phosphatase conjugates (lane 2). (C) Components of the immune complexes (1 μg) separated on an SDS-PAGE gel and revealed by silver staining. The arrowheads indicate antigen P1.

in hot SDS and separated by SDS-PAGE and then were used for reactions with the anti-P1 IgG, a strong band corresponding to the size of antigen P1 was observed (Fig. 4B, lane 1). These data show that antigen P1 was a component of the isolated materials. On the same immunoblot, an additional band of ca. 55 kDa was observed. This 55-kDa reactive band is likely to be the heavy chain of IgG, since a rabbit IgG produced a similar reactive band with the goat anti-rabbit IgG alkaline phosphatase conjugates (Fig. 4B, lane 2). Silver staining of the SDS-PAGE gel revealed that the released materials contained 10 polypeptides, and antigen P1 appeared to be a prominent component (Fig. 4C).

When the released materials were obtained from the control experiment in which *S. mutans* had not been bound with IgG, none of the above-described reactions were observed. These results strongly suggested that some of the IgGs released by *S. mutans* were found in a complex with antigen P1. The stoichiometry of the IgG-antigen P1 complex is not known at this time.

Release of bound antibodies and adherence of *S. mutans*. Saliva contains a high level of SIgA, some of which displays specificity for antigen P1 (11, 12). Therefore, it is conceivable that in the oral cavity *S. mutans* is coated with SIgA and, as indicated earlier, SIgA has an inhibitory effect on the adherence of *S. mutans* to surfaces. If the bacterium is able to release these bound SIgAs, it may enhance its ability to adhere to the surface. To test this hypothesis, colostral SIgA-coated *S. mutans* cells were tested for their ability to adhere to salivary AHA. As shown in Table 1, the level of adherence of *S. mutans* NG8 (not coated with SIgA) to AHA was about 64% and the level of adherence to HA not coated with agglutinin was 28%. The adherence level of *S. mutans* NG8 to AHA decreased to 41% when cells were bound by SIgA (SIgA control). However, when the cells were incubated in pH 6 buffer to allow the release of bound SIgA and antigen P1 to occur, the level of adherence to AHA increased with the time of incubation. After a 1-h incubation, the percent adherence of *S. mutans* NG8 to AHA was equivalent to that of cells that had not been exposed to SIgA. In contrast, when NG8 cells were incubated in pH 4 buffer, a condition in which bound SIgA would not occur, the adherence of cells to AHA remained at a level similar to that of the SIgA control.

S. mutans 834, the isogenic P1-deficient mutant of *S. mutans* NG8, showed very little difference between the levels of adherence to AHA and to HA not coated with agglutinin (no agglutinin control). This observation is consistent with previous findings that antigen P1 reacted specifically with salivary agglutinin and accounted for the adherence of *S. mutans* to AHA (27). The treatment of SIgA had no apparent effect on the adherence of mutant 834 to AHA. Incubation in pH 6 buffer, however, did result in increased adherence, as in the case of *S. mutans* NG8.

The inhibition of adherence was also studied, with anti-P1 IgG used as the inhibitory antibody (Table 1). The levels of adherence of *S. mutans* NG8 to HA and AHA in the control experiments were 23 and 70%, respectively. The level of adherence of *S. mutans* NG8 to AHA was decreased to 45% when the cells were coated with 20 μg of anti-P1 IgG per ml. When a nonimmune IgG was used, no apparent inhibition was observed, indicating the specific inhibition of adherence by anti-P1 IgG. Similarly to the SIgA inhibition experiment, when the anti-P1 IgG-coated cells were treated by incubation at pH 6, the percent adherence of cells increased with the time of incubation.

TABLE 1. Adherence of antibody-coated *S. mutans* NG8 and its isogenic P1-deficient mutant, 834, to human salivary AHA

Inhibitory antibody	Treatment	% Adherence[a] of *S. mutans*:	
		NG8	834
SIgA	No agglutinin control	27.9 ± 2.0	28.2 ± 3.5
	No SIgA control	64.2 ± 3.2	24.0 ± 6.3
	SIgA control	40.8 ± 2.9	30.0 ± 11.2
	pH 6.0, 0 min[b]	49.9 ± 2.9	12.4 ± 9.1
	pH 6.0, 30 min	59.3 ± 0.4	25.7 ± 3.6
	pH 6.0, 60 min	66.2 ± 1.6	32.7 ± 3.7
	pH 4.0, 0 min	42.1 ± 3.1	ND[c]
	pH 4.0, 30 min	45.8 ± 5.2	ND
	pH 4.0, 60 min	40.3 ± 2.2	ND
IgG	No agglutinin control	23.2 ± 1.2	ND
	No anti-P1 IgG control	69.5 ± 5.2	ND
	Anti-P1 IgG control	45.2 ± 3.9	ND
	Nonimmune IgG control[d]	65.7 ± 4.1	ND
	pH 6.0, 0 min[e]	48.2 ± 2.1	ND
	pH 6.0, 30 min	55.4 ± 6.9	ND
	pH 6.0, 60 min	63.2 ± 1.3	ND

[a] Results are expressed as the means ± standard deviations of triplicate samples.

[b] Cells were coated with 2 μg of SIgA per ml before being washed in the adherence buffer and resuspended in 0.1 M sodium phosphate buffer (pH 6.0) or sodium acetate buffer (pH 4.0) for the time indicated at 37°C. The cells were then washed in adherence buffer before use in the adherence assay.

[c] ND, not determined.

[d] The nonimmune IgG used was a rabbit IgG at 20 μg/ml.

[e] Cells were coated with 20 μg of anti-P1 IgG per ml before being washed and resuspended in 0.1 M sodium phosphate buffer (pH 6.0) at 37°C. The cells were then washed in adherence buffer before use in the adherence assay.

DISCUSSION

The results presented in this study clearly demonstrate that *S. mutans* is capable of releasing antibodies bound to its cell surface. The release was pH and incubation time dependent and was not carried out by heat-killed cells. The rate of release was dependent on the physiological state (age) of the cells. These findings indicate that the release was an active process, even though the exact nature of the activity which causes the release is not known.

In this laboratory, it was previously demonstrated that *S. mutans* has an enzymatic activity that releases its cell surface antigens (25). For this activity, the term surface protein-releasing enzymatic (SPRE) activity was coined. It is quite possible that the cell-bound antibodies were released by the action of SPRE. The strong evidence to support this notion is the discovery that some of the released IgGs were associated with proteins from *S. mutans* (Fig. 4). One of these proteins was identified as surface antigen P1. In other words, some of the liberated IgGs were in fact coming off the cells as a result of the release of surface protein antigens by SPRE. Hence, this SPRE activity may be responsible for part, if not all, of the release of the bound antibodies. However, the possibility of other unknown activities contributing to the process of antibody release cannot be ruled out.

In previous studies, this worker found that the SPRE activity which releases P1 has a pH optimum of 5 to 6 (25). The release rate of bound IgG and SIgA was at its maximum at pH 6 to 8. This difference in optimal pH may be a reflection of the heterogeneous nature of the antibodies used in the present study. It is quite conceivable that these antibodies contain activity for other surface proteins, carbohydrates, and lipoteichoic acids from *S. mutans*. The condition for the release of carbohydrates and lipoteichoic acids by the cells is not known. The release of these components together with the bound antibodies may

occur at a higher pH than the optimal pH of SPRE. The preceding argument may also be valid for the growth study (Fig. 3), in which maximum release of bound IgG did not coincide with the release of P1. Early-exponential-phase cells may release an increased amount of nonproteinaceous materials, and the bulk of the released antibodies may be those with specificity for these nonproteinaceous materials. However, the exact reason(s) for the lack of correlation between the rates of release of antibody and P1 is not known. It may become clear in further experiments in which affinity-purified anti-P1 IgG is available for similar growth studies.

For the following reasons, it appears that the released IgG is an intact molecule: (i) IgG was isolated by protein A, which is known to bind to the Fc part of IgG; (ii) the Fab or antigen binding site appears to be intact, since the IgG was isolated as an immune complex with antigen P1; and (iii) Western blotting (immunoblotting) showed a 55-kDa immunoreactive band which is the full size of the heavy chain of IgG. The light chain of the released IgG was not detected in the Western blot. The fact that a pure IgG was used in the control lane (Fig. 4B, lane 2) in the blot and its light chain was also not detected by the goat anti-rabbit antibody suggests that the goat antibody used had no specificity for this part of the IgG. According to the manufacturer's (Sigma) information, the goat antibody was raised with the intact or whole molecule of rabbit IgG.

On the basis of the results from the IgG studies and the fact that *S. mutans* is not known to possess IgA proteases (20), some of the SIgAs liberated are likely to be in a complex with antigen P1 and other *S. mutans* surface components. The SIgA released is also likely to be an intact molecule.

S. mutans NG8 apparently does not possess IgG or IgA receptors on its cell surface, as indicated by the results. Thus, the binding of rabbit IgG to the *S. mutans* cell is probably due to the specific recognition of cell surface antigens by the antibodies. These antigens could be antigen P1, cell wall carbohydrates, lipoteichoic acids, and other surface components. Since antigen P1 is an abundant protein on the *S. mutans* cell surface (5, 36), when the immune rabbit IgG is used, anti-P1 molecules may be a more predominant IgG bound to the cells. Thus, it is logical that a larger amount of IgG was bound by the cells when immune IgG was used than when nonimmune IgG was used. For human SIgA, a higher degree of binding than that for immune IgG was observed. This difference could be due to the cells being incubated with 10-fold more SIgA than IgG. However, it is worth noting that the amount of SIgA binding to *S. mutans* cells in this study is comparable to the amounts reported for other oral streptococci (2). It is logical that a reduced amount of antibodies was bound by heat-killed cells, since some of the antigenic determinants were destroyed by heat.

The binding of SIgA and anti-P1 IgG to *S. mutans* cells inhibited the adherence of cells to AHA (Table 1). This result is consistent with that reported by other workers (16, 17, 23, 34). It is interesting that about a 10-fold greater amount of anti-P1 IgG than SIgA was required to achieve similar levels of inhibition. This observation confirms that SIgA is a much more effective antiadherence antibody than IgG (21).

When the SIgA-coated *S. mutans* NG8 cells were allowed to release bound SIgA (at pH 6), the inhibitory effect of SIgA on adherence was abrogated. Such an effect was not observed when the cells were incubated at pH 4, a condition in which bound SIgA was not released by the cells. Similar abrogation of adherence inhibition was observed when anti-P1 IgG-coated cells were incubated at pH 6. This result suggests the involvement of P1-related release in the abrogation process. It is worth noting that when isogenic P1-deficient mutant 834 was incubated at pH 6, an increase in adherence was also observed. This finding suggests that there is a non-P1-related mechanism of release contributing to the increasing adherence observed for *S. mutans* NG8. In other words, the abrogation of adherence inhibition observed for *S. mutans* NG8 is likely due to at least two mechanisms, one being P1 related and the other being non-P1 related.

In conclusion, the results of this study strongly suggest that *S. mutans* has the ability to eliminate bound antibodies and, by doing so, the cells may abrogate the inhibitory effect of SIgA to colonization. Such an ability may be a mechanism employed by the bacterium to counter the inhibitory effect of salivary antibodies in the oral cavity.

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