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Effects of antibodies against a fusion protein consisting of parts of cell surface protein antigen and glucosyltransferase of *Streptococcus sobrinus* on cell adhesion of mutans streptococci

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Background/aims: The cell surface protein antigen (PAg) and glucosyltransferases (GTFs) produced by *Streptococcus sobrinus* are considered to be major colonization factors of the organism.
Methods: We constructed a fusion gene encoding a protein composed of the alanine-rich region of PAg (PAgA) and the glucan-binding domain (GB) of GTF-I, which catalyzes the synthesis of water-insoluble glucan in *S. sobrinus*. The fusion protein PAgA-GB was purified from cell extracts of *Escherichia coli* harboring the fusion gene, and antibodies against the fusion protein were prepared in rabbits.
Results: In the presence of sucrose, the antibody against PAgA-GB significantly inhibited the adhesion of both *S. sobrinus* MT8145 and *Streptococcus mutans* Xc to saliva-coated hydroxyapatite beads, and the inhibitory effect on *S. sobrinus* was stronger than that on *S. mutans*. In the absence of sucrose, the antibody against PAgA-GB significantly inhibited the adhesion of both *S. sobrinus* and *S. mutans*, however the inhibitory effect on *S. sobrinus* was unexpectedly weaker than that on *S. mutans*. A similar result was observed with the antibody against the intact recombinant PAg protein (rPAg), while the same antibody reacted more strongly against *S. sobrinus* than against *S. mutans* cells.
Conclusion: Taken together, these results show that the antibody against *S. sobrinus* GTF-I may be useful for effective inhibition of the sucrose-dependent adhesion of *S. sobrinus*. However, PAg of *S. sobrinus* may not function primarily as a receptor for acquired pellicles, and other cell surface proteins may be involved in the sucrose-independent adhesion of *S. sobrinus*.

Key words: adhesion; cell surface protein antigen; glucosyltransferase; mutans streptococci; protein antigen

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Dental caries is perhaps the most common bacterial infection in humans. Among the oral bacteria, mutans streptococci are considered to be causative agents of dental caries (8) and among the mutans streptococci, *Streptococcus sobrinus* and *Streptococcus mutans* are frequently isolated from human dental plaque (20). Colonization of tooth surfaces by these microorganisms is the first important step in the induction of dental caries. The colonization process is mediated by sucrose-independent and

sucrose-dependent mechanisms (12, 15). The former involves an interaction between bacterial cells and acquired pellicles on tooth surfaces (14). The latter is attributable to the synthesis of water-insoluble glucan from sucrose, catalyzed by glucosyltransferases (16).

Both *S. sobrinus* and *S. mutans* possess high-molecular-weight protein antigens, known as SpaA (9) or PAg (23) and B (27), I/II (30), or PAc (24), on their cell surfaces. PAc of *S. mutans* is believed to participate in the attachment of the streptococcal cell to acquired pellicles on tooth surfaces (14, 19). The PAc protein possesses two internal repeating amino acid sequences: one alanine-rich sequence (the A region) in the N terminus and one proline-rich sequence (the P region) located in the middle portion (11, 24). The N-terminal portion, including the A region, is thought to be one of the important domains that interacts with salivary receptors on tooth surfaces (3, 4, 21). PAg of *S. sobrinus* cross-reacts serologically with PAc of *S. mutans* (2). The amino acid sequences of PAg and PAc are 66% similar (33). In addition, the proteins share similar structural features; PAg of *S. sobrinus* also has an alanine-rich N-terminal region and proline-rich middle portion (35). The biological function of PAg is not well known. However, the extensive sequence similarity between PAg and PAc suggests that the proteins may have similar functions.

S. sobrinus and *S. mutans* produce both water-soluble and water-insoluble glucans from sucrose, by the combined action of glucosyltransferases (8, 20). The *de novo* synthesis of the water-insoluble glucan is necessary for the accumulation of these cells on the tooth surface and the induction of dental caries (12, 36). *S. sobrinus* and *S. mutans* both have a water-insoluble glucan-synthesizing enzyme (GTF-I). The GTF-I protein consists of two functional domains: an N-terminal sucrose-binding domain (SB) and a C-terminal glucan-binding domain (GB) (1, 5, 16). The activities of GTF-I are mediated through both catalytic and glucan-binding functions (13, 32).

The important role of cell surface protein antigen and GTF-I in the cariogenicity of mutans streptococci makes them rational targets for the development of an anticaries vaccine. Yu et al. (37) constructed a fusion protein that contained both the alanine-rich region of *S. mutans* PAc and the glucan-binding domain of *S. mutans* GTF-I and demonstrated that rabbit antibody raised against the fusion protein strongly suppresses both sucrose-dependent and sucrose-independent adhesion of *S. mutans* in *in vitro* study. In this study, we constructed a fusion protein, PAgA-GB, composed of the A region of *S. sobrinus* PAg fused with the GB domain of *S. sobrinus* GTF-I for the purpose of simultaneous inhibition of these colonization factors. We then examined the inhibitory effects of antibody against this fusion protein on the *in vitro* colonization of the organism on saliva-coated hydroxyapatite (S-HA) in the presence and absence of sucrose.

Materials and methods

Bacteria

Escherichia coli DH5α, *S. sobrinus* (MT8145, OMZ176, OU8, AHT) and *S. mutans* (Xc, LM7, MT6219) were used. All strains of *S. sobrinus* and *S. mutans* used in this study were from the Department of Preventive Dentistry, Kyushu University Faculty of Dental Science, Fukuoka, Japan. The media used were 2× YT broth (containing yeast and tryptone; Difco, Detroit, MI) for the *E. coli* DH5α and Todd–Hewitt broth (Difco) for the streptococcal strains.

Construction and purification of the PAgA-GB fusion protein derived from *S. sobrinus*

Chromosomal DNA was isolated from *S. sobrinus* MT8145 as described previously (14). DNA fragments of *pagA* [base pairs (bp) 913–1650, associated amino acid residues 222–467, GenBank accession no. D90354] and *gtf-IGB* (bp 3457–4956, associated amino acid residues 1093–1592, GenBank accession no. D90213) were amplified by polymerase chain reaction (PCR), using the primer pairs listed in Table 1. The PCR mixture (50 μl) consisted of 0.2 mM each deoxyribonucleoside triphosphate, 10 mM Tris–HCl buffer (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1 U Pyrobest DNA polymerase (Takara Bio Inc., Tokyo, Japan), a 0.5 μM concentration of each primer, and 1 μl template DNA. The reactions were carried out for 30 cycles under the following conditions: denaturation at 98°C for 10 s, annealing at 60°C (for *pagA*) or 65°C (for *gtf-IGB*) for 30 s, and extension at 72°C for 90 s. The *pagA* amplicon was digested with *Bam*HI and *Sac*I and then cloned in-frame into the pQE-80 L plasmid (QIAGEN, Hilden, Germany), to produce pQE-PAgA. The *gtfI-GB* amplicon was digested with *Sac*I and *Hin*dIII and then inserted in-frame, immediately downstream of the *pagA* sequence in plasmid pQE-PAgA. The resultant expression vector pQE-PAgA-GB thus contained a DNA fragment encoding the amino acid sequence of a six-histidine tag and the PAgA-GB fusion protein (Fig. 1). *E. coli* DH5α cells were transformed with the

Table 1. PCR primers

Target	Sequence (5′ → 3′)
PAgA	ATATGGATCCGCTAATAATGACAGTCAAGCA
	ATATGAGCTCCTTCTTGTACTGAGCAAGC
GTFI-GB	ATATGAGCTCCTATACTACTTCGGTAAAGAC
	TTTTAAGCTTAGTTCCAGCCACGGTAGAT
PAg	ATATAGCCCGGGTGAAGAAACAAGTACCACT
	ATATCTCGAGGATTCTTTAGTCCTGCTTCCG
GTF-I	ATATCCCGGGGATGGAGAAGAATGTACGTTTT
	ATATCTCGAGATATTAGTTCCAGCCACGGTA

Underlined sequences are restriction enzyme sites.

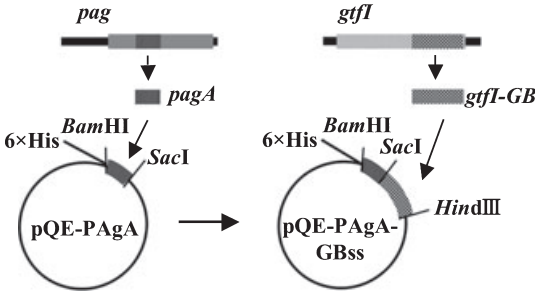

Fig. 1. Construction of PAgA-GBss fusion protein. The *pagA* (bp 913–1650 of the *pag* gene) and *gtfI-GB* (bp 3457–4956 of the *gtfI* gene) sequences were amplified by PCR. The *pagA* amplicon was digested with *Bam*HI and *Sac*I and then cloned in-frame into pQE-80L vector to generate pQE-PAgA. The *gtfI-GB* amplicon was digested with *Sac*I and *Hin*dIII and was then inserted in-frame into plasmid pQE-PAgA. The resultant expression vector, pQE-PAgA-GBss, contained a DNA fragment encoding a six-histidine tag and the PAgA-GBss fusion protein.

expression plasmid pQE-PAgA-GB. A transformant was grown at 37°C until an absorbance at 550 nm (A_{550}) of 0.5 was attained. Expression was induced with 1 mM, isopropyl-β-D-thiogalactopyranoside. The cells were harvested, and the fusion protein was purified using a Ni-nitrilotriacetic acid resin column (QIAGEN), according to the manufacturer's protocol. The purity of the protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Construction and purification of recombinant PAg and GTF-I proteins derived from *S. sobrinus*

The sequences of *pag* (bp 364–4947 of the *pag* gene, associated amino acid residues 39–1567) and *gtf-I* (bp 181–4956 of the *gtf-I* gene, associated amino acid residues 1–1592) were amplified by PCR, using chromosomal DNA of MT8145 (as template) and the primer pairs listed in Table 1. The PCR mix was as described above. Reactions were carried out for 30 cycles under the following conditions: denaturation at 98°C for 10 s, annealing at 67°C for 30 s, and extension at 72°C for 5 min. The amplicons were digested with *Sma*I and *Xho*I and then ligated into pGEX-6P-1 vector (Amersham Biosciences, Little Chalfont, UK), which had been predigested with the same restriction enzyme combination, to produce pGX-PAg and pGX-GTFI. The *E. coli* DH5α cells containing each expression plasmid were grown at 30°C in 2× YT broth containing ampicillin (100 μg/ml), to an A_{550} value of 0.5. Expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The cells were then harvested, and the recombinant PAg (rPAg) and GTF-I (rGTF-I) proteins fused with glutathione *S*-transferase (GST) were purified from each cell lysate using a glutathione–Sepharose 4B column (Amersham Biosciences), according to the manufacturer's protocol. The rPAg and rGTF-I proteins were obtained by cleavage of GST with PreScission protease (Amersham Biosciences).

Preparation of antibodies

Antisera to the purified recombinant proteins were raised in female Japanese White rabbits. Rabbits were immunized intradermally with 0.15 mg of each purified protein emulsified in Freund's complete adjuvant, followed 2 weeks later by an intradermal injection of 0.3 mg of the same protein, also emulsified in Freund's complete adjuvant. Serum was collected 2 weeks after the last immunization. Two different rabbits were immunized with PAgA-GB, rPAg or rGTF-I. Rabbit serum against PAcA-GB derived from *S. mutans* MT8148, a fusion protein of the A region (PAcA, amino acid residues 200–481) of PAc with the glucan binding-domain (GB, amino acid residues 1083–1457) of *S. mutans* GTF-I, was produced as described previously (37). To distinguish the GB domain of *S. sobrinus* from that of *S. mutans*, the former is referred to as GBss and the latter as GBsm. Antibodies were purified from the rabbit sera using an Affi-Gel protein A MAPS II kit (Bio-Rad Laboratories, Hercules, CA).

SDS–PAGE

SDS–PAGE was performed according to Laemmli (18). Proteins were stained with Coomassie brilliant blue. Cell lysate prepared from *E. coli* DH5α cells harboring the pQE-PAgA-GBss plasmid contained the fusion protein PAgA-GBss, which migrated at ∼80 kDa on SDS–PAGE gels, and a single protein band was obtained from the cell lysate using Ni-nitrilotriacetic acid resin (Fig. 2). The rPAg and rGTF-I proteins were purified from cell lysates prepared from *E. coli* DH5α harboring each expression plasmid. The purified rPAg and rGTF-I proteins were observed as protein bands of ∼210 kDa and ∼170 kDa, respectively, on SDS–PAGE (Fig. 3).

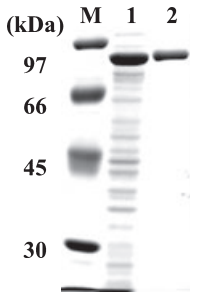

Fig. 2. Purification of the PAgA-GBss protein. Samples of crude cell lysates of *E. coli* DH5α harboring the expression plasmid pQE-PAgA-GBss and purified fusion protein were suspended in SDS–PAGE loading buffer and heated at 100°C for 5 min. The samples were then subjected to SDS–PAGE (10% polyacrylamide), and the gel was stained with Coomassie brilliant blue. Lane M, molecular mass marker; lane 1, cell lysate (20 μg) of *E. coli* harboring pQE-PAgA-GBss; lane 2, purified PAgA-GBss (5 μg).

Immunoblotting

The immunoreactivity of the anti-PAgA-GBss antibody with recombinant PAg and GTF-I proteins and with acetone precipitates of whole cultures of *S. sobrinus* (MT8145) and *S. mutans* (Xc) was analyzed by immunoblotting. The recombinant proteins and precipitated samples were subjected to SDS–PAGE as described above and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were treated with the rabbit antibody raised against PAgA-GBss. The antibodies bound to proteins immobilized on the membrane

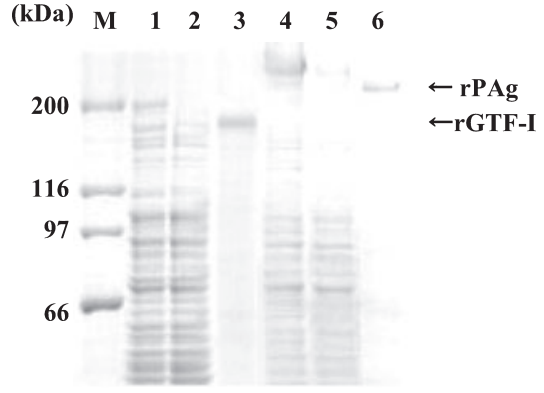

Fig. 3. Purification of the rPAg and rGTF-I proteins. Samples of crude cell lysates of *Escherichia coli* DH5α harboring each expression plasmid and purified recombinant protein were suspended in SDS–PAGE loading buffer and heated at 100°C for 5 min. The samples were then subjected to SDS–PAGE (8% polyacrylamide), and the gel was stained with Coomassie brilliant blue. Lane M, molecular mass marker; lane 1, IPTG-induced *E. coli* harboring pGX-GTFI; lane 2, *E. coli* harboring pGX-GTFI (no IPTG); lane 3, purified rGTF-I; lane 4, IPTG-induced *E. coli* harboring pGX-PAg; lane 5, *E. coli* harboring pGX-PAg (no IPTG); lane 6, purified rPAg.

were detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins.

Sucrose-independent and sucrose-dependent adhesion of *S. sobrinus* and *S. mutans* cells to S-HA

Whole unstimulated saliva was collected from a healthy adult subject and clarified by centrifugation (12,000 ***g***, 20 min). Hydroxyapatite beads (5 mg, MP Biomedicals, Inc., Aurora, OH) were incubated with 120 µl clarified human whole saliva for 1 h at 37°C and washed three times with buffered KCl (6). The S-HA beads were incubated with 0.2% [weight/volume (W/V)] bovine serum albumin in buffered KCl for 1 h at 37°C to block nonspecific binding. The cells of *S. sobrinus* (MT8145, OMZ176, OU8, AHT) and *S. mutans* (Xc, LM7, MT6219) were radiolabeled with [^{3}H]thymidine as previously described (37). The [^{3}H]thymidine-labeled bacteria (2×10^7) were allowed to react with the S-HA beads (5 mg) in the presence or absence of 10 mM sucrose in 120 µl buffered KCl. After incubation at 37°C for 3 h, the beads were washed three times with buffered KCl, and the radioactivity associated with the beads was determined. The number of bacteria adsorbed was determined from the calculated radioactivity of the bacteria. To evaluate the inhibitory effects of antibodies on the adherence of cells to S-HA beads, [^{3}H]thymidine-labeled bacteria (2×10^7) were allowed to react with S-HA beads (5 mg) in 120 µl buffered KCl containing preimmune antibody or antibody against each protein antigen (0 or 100 µg) and sucrose (0 or 10 mM) at 37°C for 3 h. The differences in the inhibition of the adhesion of streptococcal cells to S-HA were compared between preimmune antibody (as control) and test samples, using Bonferroni multiple comparisons. The percentage inhibition was calculated as $100 \times [(a–b)/a]$, where a is the mean value with preimmune antibody, and b is the mean value with antibodies against each protein antigen.

Analysis of expression of cell surface protein antigen in MT8145 and Xc

Whole cells (2×10^8) of *S. sobrinus* MT8145 and *S. mutans* Xc were suspended in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBST) and 0.1% (w/v) bovine serum albumin. Anti-rPAc antibody or preimmune antibody was mixed with the cell suspensions and incubated for 2 h at room temperature. The cells were collected by centrifugation, washed three times with PBST, and resuspended in PBST. The bound antibodies were detected with an alkaline phosphatase-conjugated anti-rabbit antibody, followed by the addition of *p*-nitrophenylphosphate substrate solution (1 mg/ml). The A_{405} was measured with a microplate reader.

Results

Immunoreactivity of rabbit antibodies

Rabbit antibodies raised against PAgA-GBss reacted with both rPAg and rGTF-I proteins (Fig. 4). Rabbit antibodies raised against rPAg and rGTF-I both reacted with the PAgA-GBss fusion protein (data not shown). The immunoreactivity of the rabbit antibodies against PAgA-GBss with acetone precipitates of whole cultures of *S. sobrinus* and *S. mutans* is shown in Fig. 5. The 210-kDa and 170-kDa protein bands strongly reacted in the case of *S. sobrinus* MT8145 (lane 1, Fig. 5). In the case of *S. mutans* Xc, the 190-kDa protein band was dominant, and the 160-kDa protein band reacted weakly (lane 2, Fig. 5).

Inhibition of bacterial adhesion to S-HA

The effects of the rabbit antibodies on the sucrose-independent and sucrose-dependent adhesion of *S. sobrinus* MT8145 and *S. mutans* Xc to S-HA beads are shown in Table 2. The antibody against rPAg significantly inhibited the adhesion of both *S. sobrinus* MT8145 and *S. mutans* Xc in the absence of sucrose but not in its presence. The antibody against rGTF-I significantly inhibited only sucrose-dependent adhesion. Significant inhibition of both sucrose-dependent and sucrose-independent adhesion of MT8145 and Xc cells to S-HA was observed on the addition of anti-PAgA-GBss or anti-PAcA-GBsm antibodies. Similar results were obtained with the antibody from another rabbit immunized with the fusion protein PAgA-GB (data not shown).

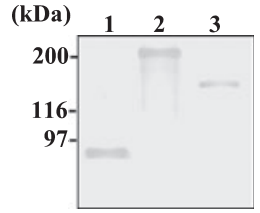

Fig. 4. Immunoreactivity of anti-PAgA-GBss antibody with rPAg and rGTF-I proteins. Purified samples (0.25 µg each) were subjected to SDS–PAGE (10% polyacrylamide) and then electrophoretically transferred to PVDF membranes. The membranes were reacted with the anti-PAgA-GBss antibody. Lane 1, purified PAgA-GBss; lane 2, purified rPAg; lane 3, purified rGTF-I.

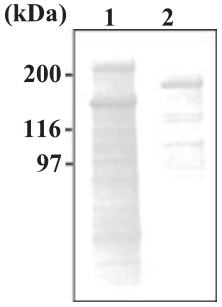

Fig. 5. Immunoreactivity of anti-PAgA-GBss antibody with acetone precipitates of whole cultures of *Streptococcus sobrinus* and *Streptococcus mutans*. Cultures of MT8145 and Xc were precipitated by adding ice-cold acetone. The precipitated proteins (1.0 µg each) were subjected to SDS–PAGE (7.5% polyacrylamide) and then electrophoretically transferred to PVDF membranes. Lane 1, MT8145; lane 2, Xc.

Table 2. Inhibition of sucrose-independent and sucrose-dependent adhesion of *Streptococcus sobrinus* (MT8145) and *Streptococuus mutans* (Xc) to S-HA beads by rabbit IgG antibodies[1]

	No. of cells adhering to S-HA (10^6)[2]			
	Without sucrose		With sucrose	
Antibody	MT8145	Xc	MT8145	Xc
Preimmune IgG	1.09 ± 0.09	2.17 ± 0.12	13.46 ± 1.02	12.06 ± 0.82
Without IgG	1.13 ± 0.03	2.19 ± 0.10	13.92 ± 1.43	10.58 ± 1.32
Anti-rPAg	0.83 ± 0.03*	1.20 ± 0.05*	11.29 ± 2.35	12.01 ± 1.48
Anti-rGTF-I	0.99 ± 0.03	2.09 ± 0.06	2.67 ± 0.11*	6.04 ± 0.22*
Anti-PAgA-GBss	0.81 ± 0.03*	1.28 ± 0.07*	2.43 ± 0.37*	7.26 ± 0.55*
Anti-PAcA-GBsm	0.91 ± 0.05*	1.09 ± 0.04*	9.11 ± 0.50*	1.49 ± 0.16*

[1][^{3}H]thymidine-labeled cells (2×10^7) were allowed to react with 5 mg S-HA beads with IgG antibodies (100 µg protein) in the presence and absence of 10 mM sucrose in 120 µl of buffered KCl.
[2]Values reported are means ± standard deviations of triplicate assays.
The differences in the inhibition of the adhesion of MT8145 and Xc to S-HA were compared between preimmune IgG (as control) and test samples, using Bonferroni multiple comparisons.
*$P < 0.05$.

The percentage inhibition of each rabbit antibody on the adhesion of MT8145 and Xc cells to S-HA revealed the result that the anti-PAgA-GBss inhibited the adhesion of *S. mutans* Xc (41%) more strongly than that of *S. sobrinus* MT8145 (26%) in the absence of sucrose. The anti-rPAg also inhibited *S. mutans* Xc (45%) more strongly than that of *S. sobrinus* MT8145 (24%). Similar results were observed for the inhibition of anti-PAgA-GBss antibody on sucrose-independent adhesion of three other strains of *S. sobrinus* and two other strains of *S. mutans* (data not shown). In contrast, in the presence of sucrose, the anti-PAgA-GBss antibody inhibited the adhesion of *S. sobrinus* MT8145 more strongly (82%) than that of *S. mutans* Xc (40%), as did the anti-rGTF-I antibody (80% and 50% in *S. sobrinus* and *S. mutans*, respectively). In addition, the anti-PAcA-GBsm antibody also inhibited the adhesion of Xc (88%) more strongly than the adhesion of MT8145 (32%), in the presence of sucrose.

Analysis of the expression of cell surface protein antigen in MT8145 and Xc cells

The expression of PAg and PAc on the surface of intact bacterial cells was confirmed by the immunoreactivity of the anti-rPAg antibody. The anti-rPAg antibody reacted more strongly with PAg of MT8145 than with PAc of Xc, whereas the reaction of the preimmune antibody with PAg was equally as weak as with PAc, suggesting that a large amount of PAg was expressed on the cell surface of MT8145 (Fig. 6).

Discussion

Both *S. sobrinus* and *S. mutans* produce water-soluble and water-insoluble glucans from sucrose. These glucose polymers provide scaffolding for the aggregation of mutans and other oral streptococci. GTF enzymes, which catalyze the synthesis of these glucans, have been closely associated with pathogenicity (36) and have been considered as vaccine targets (13, 32). Taubman et al. have shown that a rat serum antibody against GLU (a 22-mer peptide corresponding to the amino acid sequence of the GB domain of the *Streptococcus downei* GTF enzyme) suppressed the synthesis of water-soluble and water-insoluble glucans catalyzed by a crude GTF enzyme preparation from *S. sobrinus* (34). Although the inhibition of glucan synthesis by the rabbit antibodies was not examined in the present study, antibodies against both PAgA-GBss and rGTF-I of *S. sobrinus* showed strong inhibition of the sucrose-dependent adhesion of *S. sobrinus* cells to S-HA. It is believed that these antibodies bind the glucan-binding site of *S. sobrinus* GTF-I and suppress the synthesis of water-insoluble glucan. The inhibitory effect of the anti-PAgA-GBss antibody on cellular adhesion to S-HA in the presence of sucrose was weaker with *S. mutans* Xc than with *S. sobrinus* MT8145. A similar result was also observed with anti-rGTF-I antibody. In contrast, anti-PAcA-GBsm inhibited the adhesion of Xc to S-HA more strongly than the adhesion of MT8145 in the presence of sucrose. Jespersgaard et al. reported that an antibody against the glucan-binding domain of *S. mutans* GTF-I inhibited insoluble glucan synthesis in *S. mutans* but not in *S. sobrinus* (10). These findings may reflect differences in the GTF-I amino acid sequence between *S. sobrinus* and *S. mutans*. Alternatively, Taubman et al. have shown that serum against GTF-I of *S. sobrinus* inhibited water-soluble, but not water-insoluble, glucan synthesis by GTF-I of *S. mutans* (34). Thus, it is possible that the antibodies against PAgA-GBss and rGTF-I of *S. sobrinus* can suppress only water-soluble glucan synthesis in *S. mutans*.

S. sobrinus and *S. mutans* express structurally similar major cell-surface proteins, designated PAg and PAc, respectively. Both proteins are highly immunogenic and have been studied as candidates in the development of vaccines against caries and as virulence factors in these microorganisms (32). PAc of *S. mutans* has been demonstrated to bind salivary components in experimental tooth pellicles (14). An antibody directed against intact PAc or its N-terminal portion, including the A region, blocked the adherence of *S. mutans* to S-HA (7, 22, 37). In addition, immunization with PAc (I/II or B) of *S. mutans* protected monkeys against dental caries (28, 29). The biological functions of *S. sobrinus* PAg are not well known, but Redman et al. have reported that immunization with *S. sobrinus* SpaA (PAg) constructs protected rats from caries caused by *S. sobrinus* infection (25, 26). Protection in those experiments could result from antibody blockade of initial colonization events or antibody-mediated agglutination (13, 31, 32).

In our study, the anti-PAgA-GBss antibody inhibited the sucrose-independent adhesion of both *S. sobrinus* and *S. mutans* to S-HA, although the inhibition of *S. sobrinus* was unexpectedly lower than that of *S. mutans*. The anti-rPAg antibody also inhibited the adhesion of *S. mutans* Xc to S-HA more strongly than the adhesion of *S. sobrinus* MT8145, even though the anti-rPAg antibody reacted more strongly with PAg expressed on *S. sobrinus* MT8145 than with PAc on *S. mutans* Xc as shown in Fig. 6. Similar results were obtained with the antibody from another rabbit immunized with the rPAg (data not shown). It is likely that these phenomena reflect the difference of function of PAg and PAc in sucrose-dependent adhesion, although it could not exclude the possibility that the present results reflect the individual response of the immunized rabbits to different epitopes. In the present study, the antibody against PAgA-GBss also reacted with the

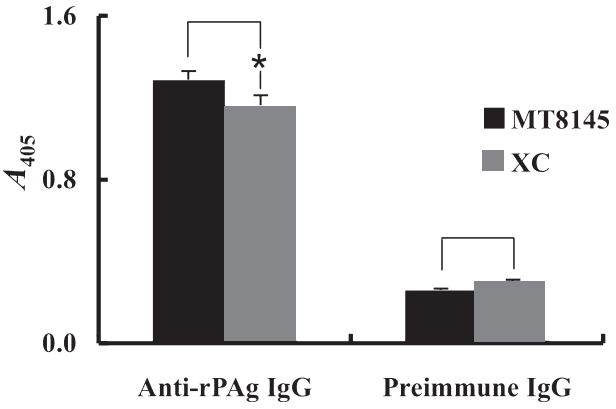

Fig. 6. Analysis of expression of cell surface protein antigen in MT8145 and Xc. The antibodies that bound to each cell were detected with an alkaline phosphatase-conjugated anti-rabbit antibody followed by the addition of *p*-nitrophenylphosphate substrate solution (1 mg/ml). The A_{405} was measured with a microplate reader. Values reported are means ± standard deviations of triplicate assays. The statistical difference between MT8145 and Xc was assessed using Student's *t*-test. $^*P < 0.05$.

PAc of *S. mutans* Xc on Western blots and inhibited the sucrose-independent adhesion of *S. mutans* to S-HA equally as well as the anti-PAcA-GBsm antibody did. Yu et al. reported that an antibody directed against the A region of PAc could block the adhesion of *S. mutans* to S-HA (37). In the present study, the anti-PAgA-GBss antibody may also have interfered by blocking the epitopes for the adhesion of *S. mutans.* It is likely that PAg of *S. sobrinus* does not function primarily as a receptor for acquired pellicles, contrary to our expectation based on the results of Redman et al. (25, 26). Kuykindoll et al. (17) expressed *S. sobrinus* SpaA protein (PAg) in the P1 (PAc)-deficient strain *S. mutans* 834, which is ordinarily defective in the ability to adhere to S-HA surfaces. Their analysis showed that the expression of PAg protein on *S. mutans* 834 cells restored the ability of these cells to aggregate in the presence of saliva or salivary agglutinin, but not the ability to adhere to S-HA. In our preliminary study, we prepared S-HA coated with clarified unstimulated or paraffin-stimulated saliva from three different donors and examined the inhibition of anti-PAgA-GBss antibody on sucrose-independent adhesion of *S. sobrinus* (OMZ176 and MT8145) and *S. mutans* (Xc and LM7) cells that were labeled with fluorescent agent BCECF-AM. The inhibitory effects of both of OMZ176 and MT8145 were weaker than those of Xc and LM7 in all condition of S-HA (unpublished data). *S. sobrinus* may possess another mechanism of initial attachment on artificial acquired pellicles.

The finding that the addition of both anti-PAgA-GBss and anti-PAcA-GBsm antibodies inhibited the adhesion of *S. sobrinus* and *S. mutans* to S-HA suggests two possible explanations. First, the antibodies against the GTF-Is of both *S. sobrinus* and *S. mutans* may be necessary for effective inhibition of the sucrose-dependent adhesion of mutans streptococci, because the inhibitory effects produced by anti-PAgA-GBss and anti-PAcA-GBsm antibodies were specific for *S. sobrinus* and *S. mutans*, respectively. This is consistent with previous studies (10, 34, 37), which examined the inhibition by antibodies against the GB domain of mutans streptococcal GTF-I on insoluble glucan synthesis by *S. sobrinus* and *S. mutans.* Second, the functions of *S. sobrinus* PAg and *S. mutans* PAc may not be the same in sucrose-independent adhesion, because not only anti-PAcA-GBsm antibody but also anti-PAgA-GBss antibody inhibited the sucrose-independent adhesion of *S. mutans* more strongly than that of *S. sobrinus.* In addition, the inhibitory effect of the anti-rPAg antibody was the same as that of the anti-PAgA-GBss antibody in the absence of sucrose, whereas anti-rPAg antibody reacted more strongly with *S. sobrinus* than with *S. mutans* cells (Fig. 6). Although a preventive effect of vaccination against the cell surface protein antigen of *S. sobrinus* has been suggested (25, 26), there may be not enough evidence to support that PAg is a main factor in the colonization of *S. sobrinus* on acquired pellicles, as PAc is in *S. mutans*. Further research is necessary to examine the mechanism of sucrose-independent adhesion of *S. sobrinus* to acquired pellicles.

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