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Differentiation of Salivary Agglutinin-Mediated Adherence and Aggregation of Mutans Streptococci by Use of Monoclonal Antibodies against the Major Surface Adhesin P1

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The ability to adhere to salivary agglutinin-coated hydroxyapatite beads and to aggregate in the presence of fluid-phase salivary agglutinin was tested by using 25 isolates of mutans streptococci representing eight serotypes. Both adherence and aggregation activity correlated with expression of the M_r-185,000 cell surface antigen P1 on *Streptococcus mutans* serotype c, e, and f strains. In addition, it was shown that the P1 molecule itself served as the adhesin of *S. mutans* serotype c, since adherence was significantly inhibited by the presence of recombinant-specified M_r-150,000 P1. The ability of *S. sobrinus* strains to adhere or aggregate did not correlate with expression of the P1 cross-reactive antigen SpaA. There was also evidence for interaction with salivary agglutinin, as manifested by aggregation but not adherence of *S. rattus* serotype b, which does not express a P1 cross-reactive antigen. To understand the interaction of P1 with salivary agglutinin at the molecular level, a panel of 11 anti-P1 monoclonal antibodies was tested for inhibitory activity in adherence and aggregation inhibition assays. Overlapping, but not identical, subsets of monoclonal antibodies were found to inhibit adherence and aggregation, indicating that the interactions of P1 with salivary agglutinin which mediate these two phenomena are different. The localization of functional domains of P1 which may mediate the aggregation and adherence reactions is discussed.

The major etiological agents of dental caries are organisms within the mutans group of streptococci (28, 44), with the species *Streptococcus mutans* and *S. sobrinus* being of particular importance (17). An early step in the pathogenesis of any invading microorganism is its ability to adhere to and colonize host tissue. The ability of oral streptococci to interact with constituents of saliva, i.e., mucins (63), proline-rich proteins (26), fibronectin (4), and secretory immunoglobulin A (IgA) (56), has been the focus of numerous studies. A high-molecular-weight mucinlike glycoprotein, known as salivary agglutinin, has been reported to interact with a major cell surface protein expressed by a number of oral streptococci (14–16, 49, 55). The M_r-185,000 protein expressed by *S. mutans* serotype c strains was first identified as antigen I/II (57) and has been referred to variously in the literature as antigen B (58, 59), IF (31), P1 (3, 22), and PAc (51–53). We will refer to this molecule as P1. Immunologically related proteins expressed by *S. mutans* serotype f, *S. sobrinus*, and *S. sanguis* are called antigen SR (2), SpaA (12, 27, 30), and SSP-5 (14, 15), respectively.

The interaction of cell-associated P1 and P1-like molecules with fluid-phase salivary agglutinin has been reported to mediate aggregation of the bacteria (13, 19, 35, 49). Such an interaction would facilitate the removal of these aggregated streptococci from the oral cavity by a nonimmune mechanism. Alternatively, the salivary agglutinin can be adsorbed onto a solid support such as hydroxyapatite beads to simulate a tooth surface coated by a salivary pellicle (18, 21, 34, 39, 40). In this situation, the streptococcal cell surface protein molecules have been reported to function as adhesins. Salivary components have also been reported to promote intergeneric interactions between bacteria (37), and cell wall proteins have been reported to promote adherence not only to tooth surfaces but to other plaque microorganisms as well (10, 29, 36). Therefore, the fate of any given bacterium would depend on many conditions affecting the oral microenvironment. A better understanding of the interaction of P1 with salivary agglutinin at the molecular level is of potential therapeutic value. The ability to distinguish between the binding of P1 to immobilized agglutinin, which results in adherence, and the binding of P1 to fluid-phase agglutinin, which results in aggregation, may uncover a mechanism which could be used to protect the host against colonization by cariogenic organisms without interfering with the nonimmune protection afforded by bacterial aggregation.

The gene encoding P1, designated *spaP*, has been cloned and sequenced (33, 39), as have several similar genes encoding the same protein from other strains of *S. mutans* (16, 50, 52, 53, 61) and immunologically cross-reactive proteins from *S. sobrinus* (1, 30, 38, 62, 64) and *S. sanguis* (14, 15). Comparison of predicted amino acid sequences of this family of related proteins has shown them to possess a number of common features. These include a 38- to 39-residue amino-terminal signal sequence; a series of three 82-residue, alanine-rich amino-terminal tandem repeats exhibiting a 7-residue periodicity which is predicted to form an alpha-helical structure typical of coiled-coil proteins; a series of two to three 39-residue, proline-rich tandem repeats within the central portions of the molecules which are predicted to form an extended structure; and a proline-rich carboxy-terminal region believed to span the cell wall. A charged cytoplasmic tail is also seen in P1 from *S. mutans* serotype c isolates (33, 53). The functional domains of these molecules which interact specifically with salivary agglutinin have not yet been identified. We have a panel of 11 anti-P1 monoclonal antibodies (MAbs) available in our laboratory which have re-

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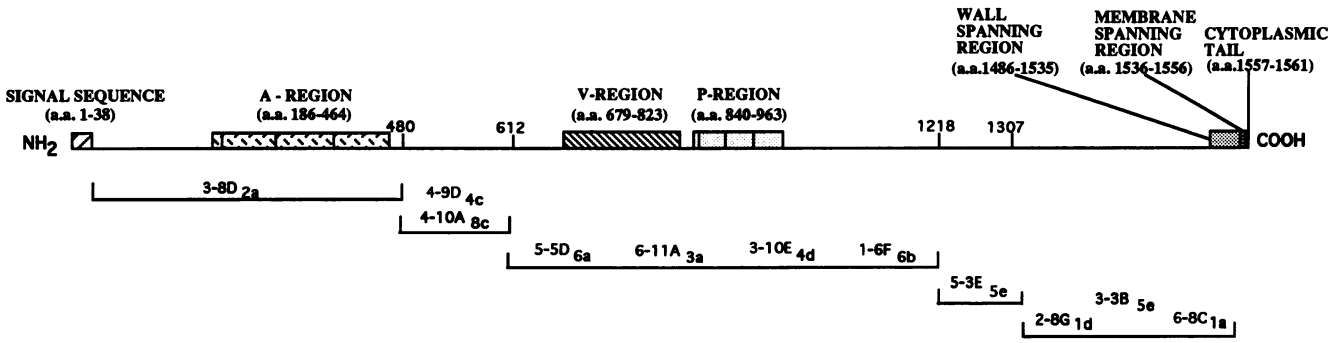

FIG. 1. Schematic representation of the P1 molecule from *S. mutans* serotype c strain NG5. Numbers refer to the amino acid (a.a.) positions deduced from the nucleotide sequence of the cloned *spaP* gene (33). The A-region and P-region designations identify the alanine-rich and proline-rich tandem repeats, respectively. The V-region designation indicates the variable region identified by restriction fragment length polymorphism analysis of the *spaP* gene (5). The lower bars indicate the putative binding domains of a panel of 11 anti-P1 MAbs (6). The order of antibodies within each segment is arbitrary and does not reflect their locations of binding on the P1 molecule.

cently been characterized with regard to their cross-reactivity profiles with P1-like molecules, their approximate locations of reactivity on the P1 molecule, and their relative abilities to bind to P1 in its native configuration on intact cells (6). A schematic diagram of P1 deduced from the sequence of the cloned *spaP* gene and a map of the putative binding domains of the anti-P1 MAbs is shown in Fig. 1. The purpose of the present study was to define further the relationship between expression of P1 (or related proteins) by various strains of mutans streptococci and their involvement in the agglutinin-mediated phenomena of aggregation and adherence. In addition, inhibition studies using our anti-P1 MAbs has enabled us to begin to differentiate between aggregation and adherence at the molecular level.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Streptococcal strains used in this study were *S. cricetus* serotype a AHT and E49; *S. rattus* serotype b BHT and FA-1; *S. mutans* serotype c Ingbritt 175, Ingbritt 162, NG5, NG7, and NG8 from K. Knox, Institute of Dental Research, Sydney, Australia; MT8148-SM and MT8148-SH from S. Michalek, University of Alabama, Birmingham; DP5, DP6, DP7, and DP8, which are fresh isolates from this laboratory; GS5, from our laboratory collection; and 834, a P1-deficient mutant derived by insertional inactivation of the *spaP* gene in NG8 (40). We also used *S. mutans* serotype e V100 from R. McKinney, Centers for Disease Control, Atlanta, Ga.; *S. mutans* serotype f OMZ175; *S. sobrinus* serotype d SL-1; *S. sobrinus* serotype g strains K1-R and 6715-13-WT from R. J. Fitzgerald, Veterans Administration Medical Center, Miami, Fla., 6715-RH from R. G. Holt, Meharry Medical College, Nashville, Tenn., and 6715 (ATCC 27352) from the American Type Culture Collection; *S. downeii* serotype h ATCC 33738; *S. pyogenes* 647 (M protein type 49) from M. D. P. Boyle, Medical College of Ohio, Toledo; and *S. pyogenes* M type 5 from M. Cunningham, University of Oklahoma, Oklahoma City. All streptococcal isolates were grown aerobically for 16 h at 37°C in Todd-Hewitt broth (BBL, Cockeysville, Md.) supplemented with 0.3% yeast extract or in the chemically defined medium (TDM) described by Terleckyj et al. (65). *Escherichia coli* JM109 (International Biotechnologies, Inc., New Haven, Conn.) containing recombinant plasmid pSM2949 (39) from our laboratory was grown aerobically at 37°C with vigorous shaking in LB medium (1% [wt/vol] tryptone, 0.5% [wt/vol] yeast extract, 1% [wt/vol] NaCl, pH 7.0) supplemented with ampicillin at 50 μg/ml (wt/vol) (Sigma Chemical Co., St. Louis, Mo.).

Preparation of salivary agglutinin. Salivary agglutinin was prepared by a modification of the technique of Rundegren and Arnold (56). Unstimulated whole saliva was collected on ice from a single individual and clarified by centrifugation at 7,500 × *g* for 20 min at 4°C. One volume of clarified saliva was diluted with an equal volume of KPBS (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 6.5 mM Na_2HPO_4, pH 7.2) and mixed with 1 volume of a suspension of *S. mutans* serotype c strain NG8 cells. The bacteria, grown overnight in Todd-Hewitt broth supplemented with 0.3% yeast extract, were washed twice with KPBS and resuspended to a Klett reading of 300 (Klett-Summerson photoelectric colorimeter; Klett Mfg. Co., Inc., New York, N.Y.). The saliva and bacteria were incubated on a rotator (Roto-Torque model 7637; Cole-Parmer Instruments Co., Chicago, Ill.) for 30 min at 37°C. The cells were removed from the mixture by centrifugation at 2,000 × *g* for 15 min and washed once with KPBS. Adsorbed agglutinin was eluted from the cells with 1 volume of KPBS containing 1 mM EDTA (Sigma). The agglutinin preparation was filtered through a 0.2-μm-pore-size Acrodisc (Gelman Sciences, Ann Arbor, Mich.), concentrated 10-fold by using a YM10 Diaflo ultrafiltration membrane (Amicon Corp., Danvers, Mass.), dialyzed against KPBS containing 0.02% sodium azide, and stored in aliquots at −20°C. Agglutinin preparations routinely contained 40 to 50 μg of protein per ml when estimated by the bicinchoninic acid protein assay (Sigma) with bovine serum albumin (BSA) as the standard.

Adherence assays. Adherence of mutans streptococci to salivary agglutinin-coated hydroxyapatite was assayed by a modification of the method of Clark et al. (8). Bacteria were radiolabeled by growing to stationary phase in 10 ml of Todd-Hewitt broth supplemented with 0.3% yeast extract and 70 μCi of [*methyl*-^{3}H]thymidine per ml (specific activity, 43 Ci/mmol; Amersham Corp.). The cells were harvested by centrifugation at 1,00 × *g*, washed once with adherence buffer (50 mM KCl, 1 mM $CaCl_2 \cdot H_2O$, 38.3 mM $MgCl_2 \cdot 6H_2O$, 0.78 mM KH_2PO_4, 1.22 mM K_2HPO_4, pH 7.2), and resuspended in 3 ml of adherence buffer. The cell suspension was sonicated for 10 s with a Microson ultrasonic cell disruptor (Heat Systems-Ultrasonics, Inc., Farmingdale, N.Y.) at 20% output power and adjusted to a final Klett value of 150.

Approximately 5 mg of hydroxyapatite beads (BDH Biochemicals Ltd., Poole, England) was rehydrated in 200 μl of adherence buffer overnight in a 250-μl polyethylene tube (Centaur West Inc., Sparks, Nev.). The buffer was removed by aspiration, and 200 μl of salivary agglutinin was added to the beads. The suspension was mixed on a vertical rotator at 7 rpm for 1 h at ambient temperature. The beads were washed once with adherence buffer and blocked with 200 μl of 0.01% BSA in adherence buffer for 30 min. Albumin has been shown to block uncoated or free binding sites on hydroxyapatite preventing nonspecific adherence of bacteria to the beads, while the albumin itself does not promote bacterial adhesion (25). The beads were washed with adherence buffer to remove excess albumin, then incubated with 200 μl of a 1:10 dilution (in adherence buffer) of the bacterial cell suspension, and rotated for 1 h at ambient temperature. Beads were allowed to settle by gravity for 5 min, 100 μl of liquid was removed and counted in a liquid scintillation counter (model L3801; Beckman Instruments, Inc., Fullerton, Calif.), and percent adherence was calculated as [(control counts − test counts)/control counts] × 100. Control counts were obtained from tubes in which hydroxyapatite beads had been omitted. All assays were performed in quadruplicate. No difference in percent adherence was observed when bacteria were grown in TDM, Todd-Hewitt broth containing 0.3% yeast extract, or Todd-Hewitt broth lacking the yeast extract supplement.

Monoclonal and polyclonal antibodies. Polyclonal anti-P1 antiserum was generated by immunizing a female New Zealand White rabbit with P1 isolated by ion-exchange and gel filtration chromatography from *S. mutans* serotype c strain Ingbritt 175. The immunogen was prepared from a mutanolysin cell wall extract according to the procedure of Russell et al. (60). P1 was purified from the extract by passage over a DEAE-Sepharose column (CL-6B; Pharmacia, Piscataway, N.J.). Elution was accomplished by using a 0 to 0.5 M linear gradient of NaCl in 50 mM Tris (pH 7.0). Fractions were analyzed for the presence of P1 by Western immunoblotting with anti-P1 MAb 4-10A (3) as the probe. P1-containing fractions were pooled and dialyzed into phosphate-buffered saline (PBS; pH 7.2), and P1 (M_r=185,000) was separated from lower-molecular-weight material by passage over a column of Sephacryl S-200 (superfine; Pharmacia). A subcutaneous injection of 50 μg of protein in Freund's incomplete adjuvant (Difco, Detroit, Mich.) was given once weekly for 3 weeks, and serum was collected. The immunization schedule was repeated after a 4-month rest, and the serum was collected, frozen, and stored at −20°C. Rabbit anti-SpaA polyclonal antiserum was kindly provided by R. G. Holt, Meharry Medical College, Nashville, Tenn.

Murine hybridoma lines producing anti-P1 MAbs were generated as previously described (3, 6). Murine hybridoma ascites fluids served as the source of MAbs.

IgG was purified from rabbit antiserum and murine ascites fluids by using a column of protein A-agarose (Bio-Rad, Richmond, Calif.) with 3 M glycine–1.5 M NaCl (pH 8.9) as the binding buffer and 0.1 M citrate buffer (pH 3.0) as the elution buffer. Protein levels were quantitated by measurement of UV absorbance at 280 nm.

Preparation of recombinant-specified P1. Recombinant-specified M_r-150,000 P1 was purified by resuspending *E. coli* harboring plasmid pSM2949 (39) to 1/50 the original culture volume in 10 mM Tris (pH 8.0) containing 25% (wt/vol) sucrose. Phenylmethylsulfonyl fluoride (Sigma) and EDTA were each added to a final concentration of 1 mM, and the mixture was incubated at ambient temperature for 10 min. Cells were harvested by centrifugation at 7,000 × *g* for 15 min, resuspended in 1/33 the original culture volume with ice-cold water containing 1 mM each EDTA and phenylmethylsulfonyl fluoride, and incubated on ice for 15 min. Tris (pH 8.0) was added to a final concentration of 10 mM, and the periplasmic preparation was filtered through a 0.2-μm-pore-size Acrodisc (Gelman Sciences). The M_r-150,000 recombinant-specified P1 was separated from other periplasmic proteins by passage over a gel filtration column of Sepharose CL-4B (Pharmacia) equilibrated with 50 mM Tris (pH 8.0). Fractions containing P1 were assayed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% gel. Fractions containing a single M_r-150,000 band were pooled and stored at −20°C. Protein levels were measured by using the bicinchoninic acid protein assay (Sigma) with BSA as the standard.

Source of PepM5. Lyophilized PepM5 from *S. pyogenes* was kindly provided by M. W. Cunningham, University of Oklahoma, Oklahoma City. The purified protein was resuspended to a final concentration of 1 mg/ml in PBS (pH 7.2).

Adherence inhibition assays. All adherence inhibition assays were performed by using *S. mutans* serotype c NG8 as the test strain. For determination of inhibition by anti-P1 MAbs and by polyclonal antisera, the assay was performed as described above except that the suspension of ^{3}H-labeled NG8 cells was diluted 1:10 in adherence buffer containing the stated concentration of protein A-purified IgG and preincubated for 30 min before being added to the BSA-blocked hydroxyapatite beads. For the competitive inhibition assay with fluid-phase inhibitor, the ^{3}H-labeled NG8 suspension was diluted 1:10 in adherence buffer containing the stated concentration of recombinant-specified P1 (or PepM5 as a negative control) and added immediately to the BSA-blocked beads with no preincubation. Percent inhibition of adherence was calculated as [(percent adherence without inhibitor − percent adherence with inhibitor)/percent adherence without inhibitor] × 100. All assays were performed in quadruplicate.

Aggregation assays. Bacterial aggregation was measured by using a modification of the method of Magnusson and Ericson (48). Mutans streptococci were grown overnight in Todd-Hewitt broth containing 0.3% yeast extract. Aggregation assays were found to work much better when the bacteria were grown in Todd-Hewitt broth rather than in TDM. The bacteria were harvested by centrifugation at 1,000 × *g* for 15 min, washed twice with PBS, and resuspended in PBS to a Klett value of 250 (corresponding to an optical density at 700 nm [OD_{700}] of approximately 1.0). Four hundred microliters of bacterial suspension, 100 μl of PBS, 100 μl of salivary agglutinin, and 6 μl of 0.1 M $CaCl_2$ were mixed in a test tube, vortexed, and transferred to cuvettes (1-cm light path). The cuvettes were equilibrated for 5 min at 37°C in a Shimadzu UV 160 spectrophotometer equipped with a temperature-controlled multicuvette positioner (Shimadzu Scientific Instruments, Inc., Columbia, Md.), the samples were automatically positioned, and the OD_{700} was read at 5-min intervals for 1 h. Percent aggregation (percent decrease in OD_{700}) was calculated as [(OD_{700} at 0 min − OD_{700} at 60 min)/OD_{700} at 0 min] × 100. A mock agglutinin preparation was made as described above except that KPBS was substituted for clarified human saliva. This preparation demonstrated no aggregating activity when reacted with *S. mutans* serotype c strain NG8 cells. This negative control ensured that no aggregation-promoting material was eluted directly from the bacteria but was a

constituent of the saliva itself. All strains of mutans streptococci were also tested for self-aggregation activity with 100 μl of PBS substituted for the salivary agglutinin. All assays were performed in triplicate.

Aggregation inhibition assays. All aggregation inhibition assays were performed with *S. mutans* serotype c NG8 as the test strain. Murine ascites fluids served as the source of anti-P1 MAbs. The assays were performed as described above with the following exceptions. A total of six cuvettes were included in each assay. One cuvette contained 400 μl of bacterial suspension, 200 μl of PBS, and 6 μl of 0.1 M $CaCl_2$. The percent decrease in OD_{700} in this cuvette was measured to determine the degree of self-aggregation in the absence of agglutinin. This background value was subtracted from all agglutinin-mediated aggregations calculated in each assay. The second cuvette contained 400 μl of bacterial suspension, 150 μl of PBS, 50 μl of salivary agglutinin, and 6 μl of $CaCl_2$. The amount of salivary agglutinin used was decreased to 50 μl to increase the sensitivity of the inhibition assays. The four remaining cuvettes contained 400 μl of bacterial suspension, 50 μl of salivary agglutinin, 6 μl of 0.1 M $CaCl_2$, and 150, 100, 50, or 25 μl of murine ascites fluid plus 0, 50, 100, or 125 μl of PBS, respectively. All reagents but the salivary agglutinin were preincubated for 30 min at ambient temperature. The agglutinin was then added, the mixture was vortexed, transferred to the cuvette, and allowed to equilibrate at 37°C for 5 min, and the OD_{700} was read at 5-min intervals for 1 h. Percent inhibition of aggregation after 60 min for each of the four volumes of ascites fluid tested was calculated as [(percent aggregation without antibody − percent aggregation with antibody)/percent aggregation without antibody] × 100.

RESULTS

Adherence of mutans streptococci to salivary agglutinin-coated hydroxyapatite beads. Adherence of streptococci to agglutinin-coated hydroxyapatite was assayed by using a modification of the method of Clark et al. (8). These results are summarized in Table 1. All species tested except *S. rattus* serotype b express P1 or immunologically related molecules. Also included in this analysis were P1 nonretainer isolates of *S. mutans* serotype c (3) and a P1-deficient mutant (40). P1 retainer strains express the molecule on their surfaces, while nonretainer strains release P1 primarily into the culture supernatant. Extensive characterization of the P1-deficient mutant 834 has revealed that this strain, derived from the P1 retainer strain NG8, expresses a truncated form of P1 corresponding to the amino acid residues 1 to 612 of the parent P1 protein (6). Two group A (*S. pyogenes*) isolates were also tested in light of recent studies of this species' oral adherence and aggregation properties (7, 9).

All mutans species and serotypes except *S. rattus* serotype b adhered well (35 to 67%) to the agglutinin-coated beads. P1 retainer strains of *S. mutans* serotype c adhered well (40 to 55%), while P1 nonretainer strains did not (<11%). The P1-deficient mutant strain derived from NG8, 834 (40), was also unable to adhere (5%). Two *S. sobrinus* serotype g isolates, 6715 (ATCC 27352) and 6715-RH, were adherent (>34%), while two others, KI-R and 6715-13-WT, were not. The reason for this difference is unclear. All four isolates were equally reactive with serial dilutions of polyclonal anti-SpaA antiserum (kindly provided by R. Holt) in a radioimmunoassay using intact bacteria (data not shown).

Both strains of *S. pyogenes* tested were strongly adherent to salivary agglutinin-coated hydroxyapatite beads (>52%).

TABLE 1. Adherence of mutans streptococci to agglutinin-coated hydroxyapatite beads

Species (serotype)	Isolate	% Adherence
S. cricetus (a)	AHT	61.32 ± 9.72
	E49	34.01 ± 3.12
S. rattus (b)	BHT	6.79 ± 0.85
	FA-1	9.23 ± 1.85
S. mutans (c)	Ingbritt 175	35.53 ± 1.43
	NG7	48.16 ± 3.48
	NG8	45.70 ± 3.57
	DP8	41.40 ± 1.39
	DP7	46.30 ± 2.09
P1 retainers	DP6	48.20 ± 2.48
	DP5	48.32 ± 4.41
	MT8148-SM	48.44 ± 3.28
	MT8148-SH	55.51 ± 4.22
P1 nonretainers	Ingbritt 162	10.34 ± 3.31
	NG5	3.11 ± 1.33
	GS5	1.35 ± 1.33
P1-deficient mutant	834	5.44 ± 1.80
S. mutans (e)	V100	40.18 ± 6.33
S. mutans (f)	OMZ175	41.30 ± 3.44
S. sobrinus (d)	SL-1	60.40 ± 4.04
S. sobrinus (g)	6715 (ATCC 27352)	34.41 ± 2.78
	6715-13-WT	12.11 ± 2.33
	6715-RH	45.34 ± 2.11
	K1-R	7.07 ± 1.78
S. downeii (h)	ATCC 33748	30.28 ± 1.81
S. pyogenes	647	52.57 ± 4.74
	Type 5	64.15 ± 2.39

Inhibition of adherence by fluid-phase recombinant P1. To determine whether the P1 molecule itself, and not an accessory molecule associated with P1, functions as the agglutinin-binding adhesin, the ability of fluid-phase P1 to compete with bacterium associated P1 for binding to immobilized agglutinin was determined by using a competitive inhibition modification of the salivary agglutinin-coated hydroxyapatite adherence assay. Recombinant-specified P1 (M_r = 150,000) encoded by plasmid pSM2949 (37) lacks the 3′-terminal 763 bp of the *spaP* gene and corresponds to amino acid residues 1 to 1307 of the parent P1 molecule. The purified recombinant-specified protein was an effective competitive inhibitor of P1 retainer strain NG8's adherence to agglutinin-coated beads (Fig. 2), with approximately 85% inhibition of adherence observed at a protein concentration of 0.5 mg/ml. PepM5, an amino-terminal product of type 5 *S. pyogenes* M protein produced by pepsin digestion of intact bacteria (11), was used as a negative control. M protein is an alpha-helically coiled-coil protein (20) and was chosen because of its structural similarity to that predicted for the amino-terminal third of the P1 molecule. Adherence of NG8 was not inhibited by PepM5 at any of the concentrations tested.

Aggregation of mutans streptococci in the presence of fluid-phase agglutinin. The same panel of bacterial strains which had been tested for adherence to immobilized agglutinin on hydroxyapatite beads was also tested in a spectrophotometric assay for the ability to aggregate in the presence of fluid-phase agglutinin. These results are summarized in Table 2. A number of strains, *S. cricetus* serotype a E49 and AHT, *S. mutans* serotype c NG7 and MT8148-SM, *S. sobrinus* 6715 (ATCC 27352), *S. downeii* serotype h ATCC 33748, and *S. pyogenes* 647, demonstrated significant self-aggregation (>20% decrease in OD_{700}) in the absence of agglutinin. All but three strains tested, including *S. rattus*

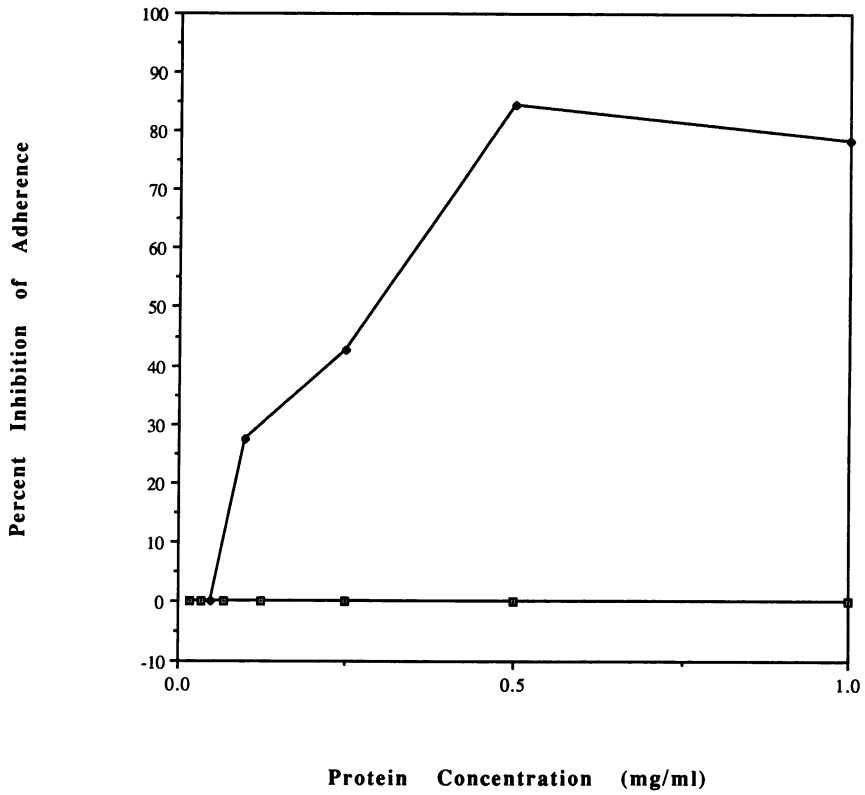

FIG. 2. Percent inhibition of adherence of *S. mutans* serotype c strain NG8 to salivary agglutinin-coated hydroxyapatite beads. Symbols: ◆, inhibition by M_r-150,000 recombinant-specified P1; ⊡, inhibition by PepM5 from *S. pyogenes* M protein type 5.

serotype b FA-1 and BHT, aggregated in the presence of salivary agglutinin; the exceptions were the P1 nonretainer strains of *S. mutans* serotype c and the two nonadherent *S. sobrinus* serotype g strains (KI-R and 6715-13-WT).

Inhibition of adherence to agglutinin-coated hydroxyapatite by anti-P1 MAbs and anti-P1 and anti-SpaA polyclonal antisera. Eleven anti-P1 MAbs, as well as anti-P1 and anti-SpaA rabbit polyclonal antisera, were tested for the ability to inhibit binding of the P1 retainer strain NG8 to salivary agglutinin-coated hydroxyapatite beads. Purified IgG was used in these assays. An irrelevant anti-*Actinobacillus actinomycetemcomitans* MAb IgG and anti-P1 preimmune rabbit IgG were included as negative controls. These results are summarized in Table 3. Both polyclonal anti-P1 and anti-SpaA were able to inhibit the binding of NG8 to agglutinin-coated beads. Anti-P1 appeared to be a better inhibitor of NG8 adherence than was anti-SpaA at all IgG concentrations used. We have shown previously that although both of these antisera cross-react with the heterologous proteins, their specificities are not identical. Anti-P1 and anti-SpaA antisera can be rendered monospecific for P1 and SpaA by cross-adsorption with SpaA- and P1-expressing strains, respectively (4a). The anti-P1 preimmune negative control IgG did not inhibit adherence.

The anti-P1 MAbs demonstrated a range of inhibitory activity. MAb 2-8G_{1d} was unable to prevent adherence of NG8 even at the highest concentration tested (1.5 mg/ml), and MAb 3-10E_{4d} demonstrated only minimal inhibition (≤15%) at the highest concentrations. MAb 6-11A_{3a} was also only a weak inhibitor, and MAbs 3-8D_{2a} and 3-3B_{5e} were moderately inhibitory. MAbs 1-6F_{6b} and 6-8C_{1a} appeared to be the strongest inhibitors of aggregation, although 4-9D_{4c},

TABLE 2. Aggregation of mutans streptococci in the presence of fluid-phase agglutinin

Species (serotype)	Isolate	% Decrease in OD_{700}	
		Without agglutinin	With agglutinin
S. cricetus (a)	AHT	62.28 ± 4.89	65.53 ± 3.49
	E49	23.18 ± 6.29	56.70 ± 1.08
S. rattus (b)	BHT	2.85 ± 1.23	32.34 ± 10.23
	FA-1	4.34 ± 8.47	37.99 ± 8.82
S. mutans (c)	Ingbritt 175	12.26 ± 7.91	47.73 ± 4.97
	NG7	21.38 ± 4.04	61.14 ± 4.38
	NG8	8.79 ± 2.88	63.48 ± 3.89
	DP8	7.61 ± 2.07	54.72 ± 4.71
	DP7	17.69 ± 7.06	59.91 ± 7.62
P1 retainers	DP6	13.57 ± 12.94	67.83 ± 3.68
	DP5	5.10 ± 11.04	39.53 ± 0.65
	MT8148-SM	38.14 ± 3.21	55.47 ± 14.32
	MT8148-SH	9.43 ± 21.57	50.36 ± 11.93
	Ingbritt 162	3.17 ± 3.47	4.71 ± 2.72
P1 nonretainers	NG5	1.92 ± 6.62	0.16 ± 12.29
	GS5	3.17 ± 18.84	7.71 ± 1.26
P1-deficient mutant	834	7.63 ± 1.52	22.03 ± 3.13
S. mutans (e)	V100	7.16 ± 5.18	52.93 ± 5.82
S. mutans (f)	OMZ175	2.73 ± 7.78	59.46 ± 3.58
S. sobrinus (d)	SL-1	7.47 ± 5.98	55.78 ± 5.02
S. sobrinus (g)	6715 (ATCC 27352)	37.86 ± 2.01	44.26 ± 2.87
	6715-13-WT	7.47 ± 3.13	12.00 ± 9.64
	6715-RH	15.13 ± 3.34	55.97 ± 10.95
	K1-R	9.26 ± 6.05	13.67 ± 3.44
S. downeii (h)	ATCC 33748	50.42 ± 3.04	56.26 ± 0.67
S. pyogenes	647	56.19 ± 7.87	58.01 ± 6.12
	Type 5	13.52 ± 7.96	53.31 ± 5.45

TABLE 3. Percent inhibition of adherence of *S. mutans* NG8 to agglutinin-coated hydroxyapatite beads by polyclonal and monoclonal antibodies

Antibody	% Inhibition at IgG concn (mg/ml) of:					
	1.500	1.125	0.750	0.300	0.150	0.030
Polyclonal anti-P1	74.4	64.5	61.4	45.0	43.9	49.9
Polyclonal anti-SpaA	39.2	24.2	19.3	5.0	0.0	0.0
Anti-P1 pre-immune	18.1	ND[a]	0.0	3.9	0.4	0.0
$1\text{-}6F_{6b}$	80.2	69.6	74.3	49.7	38.8	16.6
$2\text{-}8G_{1d}$	0.0	0.0	0.0	0.0	0.0	1.5
$3\text{-}3B_{5e}$	38.0	40.3	29.5	20.5	5.6	0.0
$3\text{-}8D_{2a}$	35.5	22.9	12.9	11.4	8.6	3.7
$3\text{-}10E_{4d}$	12.0	15.2	14.1	4.2	3.3	2.1
$4\text{-}9D_{4c}$	55.3	48.6	36.0	25.9	18.7	2.3
$4\text{-}10A_{8c}$	ND	ND	43.3	34.0	33.2	45.5
$5\text{-}3E_{5e}$	ND	ND	48.6	1.0	1.3	0.0
$5\text{-}5D_{6a}$	ND	ND	61.3	25.6	0.0	0.0
$6\text{-}8C_{1a}$	60.8	56.0	46.2	45.2	23.6	8.3
$6\text{-}11A_{3a}$	28.4	22.6	19.6	ND	0.0	0.0
$1\text{-}5F_{2a}$[b]	ND	ND	0.0	ND	0.0	0.0

[a] ND, not determined.
[b] Anti-*A. actinomycetemcomitans*.

$4\text{-}10A_{8c}$, and $5\text{-}5D_{6a}$ appeared to be quite effective inhibitors as well. Sufficient quantities of IgG were unavailable to test each of the MAbs at the highest concentrations. MAb $5\text{-}3E_{5e}$ was consistently noninhibitory at lower concentrations but demonstrated a pronounced increase in inhibitory activity at an IgG concentration of 0.75 mg/ml. The negative control MAb, anti-*A. actinomycetemcomitans*, failed to inhibit adherence of NG8 at all IgG concentrations tested.

Inhibition of fluid-phase salivary agglutinin-mediated aggregation by anti-P1 MAbs. The 11 anti-P1 MAbs which were tested for the ability to inhibit the adherence of NG8 to agglutinin-coated hydroxyapatite beads were also tested for the ability to inhibit aggregation of NG8 in the presence of fluid-phase agglutinin. Whole murine ascites fluids were used in these experiments. Again, anti-*A. actinomycetemcomitans* MAb $1\text{-}5F_{2a}$ was used as a negative control. Aggregation inhibition assays using the negative control MAb and an inhibitory anti-P1 MAb, $2\text{-}8G_{1d}$, are shown as examples in Fig. 3. When NG8 cells were incubated in buffer alone with no salivary agglutinin, no decrease in OD_{700} was detected after 1 h. However, when salivary agglutinin was added to the bacterial suspension, the cells aggregated and an approximately 50% decrease in OD_{700} was observed during the 1-h incubation period (Fig. 3). When murine ascites fluid containing the negative control MAb was added to the NG8 bacterial suspension containing salivary agglutinin, no difference was observed between aggregation in the presence or absence of antibody (Fig. 3A). In contrast, MAb $2\text{-}8G_{1d}$ demonstrated significant inhibition of aggregation (Fig. 3B). The amount of inhibition of aggregation decreased from 100% in the presence of 150 μl of ascites fluid to 53% in the presence of 25 μl of ascites fluid. A summary of the percent inhibition of agglutinin-mediated aggregation observed in the presence of 150, 100, 50, and 25 μl of each anti-P1 murine ascites fluid is shown in Table 4. As was seen with the

TABLE 4. Percent inhibition of agglutinin-mediated aggregation of *S. mutans* NG8 by anti-P1 MAbs

Antibody	% Inhibition at murine ascites fluid vol (μl) of:			
	150 μl	100 μl	50 μl	25 μl
$1\text{-}6F_{6b}$	16	45	16	0
$2\text{-}8G_{1d}$	100	100	77	53
$3\text{-}3B_{5e}$	22	22	0	9
$3\text{-}8D_{2a}$	83	80	75	40
$3\text{-}10E_{4d}$	77	43	50	6
$4\text{-}9D_{4c}$	83	80	75	40
$4\text{-}10A_{8c}$	89	89	61	54
$5\text{-}3E_{5e}$	19	30	7	0
$5\text{-}5D_{6a}$	67	48	12	0
$6\text{-}8C_{1a}$	96	100	96	90
$6\text{-}11A_{3a}$	0	0	31	0
$1\text{-}5F_{2a}$[a]	0	0	0	0

[a] Anti-*A. actinomycetemcomitans*.

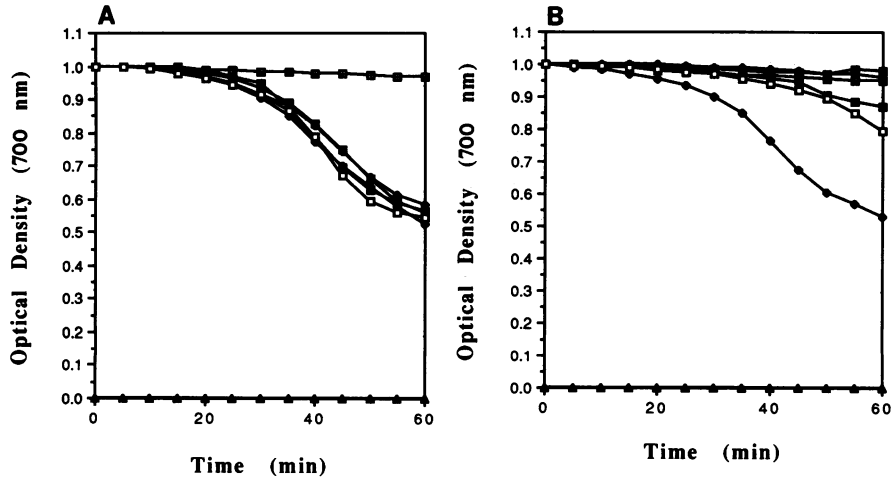

FIG. 3. Inhibition of salivary agglutinin-mediated aggregation of *S. mutans* serotype c strain NG8 by an irrelevant anti-*A. actinomycetemcomitans* MAb (A) and by anti-P1 MAb $2\text{-}8G_{1d}$ (B). Symbols: ⊡, no agglutinin or MAb added to the bacteria; ◆, agglutinin but no MAb added to the bacteria; ⊡, ◇, ■, and □, bacteria preincubated with 150, 100, 50, and 25 μl, respectively, of murine ascites fluid before addition of agglutinin.

adherence inhibition assays, a range of inhibitory activity was demonstrated by the different MAbs. MAbs 6-8C_{1a} and 4-9D_{4c} exhibited the greatest degree of aggregation inhibition. MAbs 4-10A_{8c}, 2-8G_{1d}, 3-8D_{2a}, 3-10E_{4d}, and 5-5D_{6a} were also able to inhibit aggregation and demonstrated different degrees of inhibition with decreasing concentrations of antibody. MAbs 3-3B_{5e} and 5-3E_{5e} were weakly inhibitory after 1 h. Calculation of percent inhibition of aggregation after 30 min (data not shown) compared with percent inhibition of aggregation after 60 min indicated that these two MAbs delayed but could not prevent aggregation. Two other MAbs, 6-11A_{3a} and 1-6F_{6b}, were interesting in that they consistently demonstrated a moderate degree of inhibition at an intermediate, but not at the highest or lowest, concentration of antibody.

DISCUSSION

The nature of the interaction of the cariogenic oral microorganism *S. mutans* with a high-molecular-weight salivary glycoprotein, salivary agglutinin, may determine the fate of the invading bacterium in the oral cavity. Mutans and viridans streptococci which express the cell surface protein molecule P1, or immunologically related antigens, have been shown to react with salivary agglutinin (13–16, 21, 39, 40). Presumably, aggregation of organisms in the presence of fluid-phase agglutinin represents a mechanism of nonimmune clearance of invading bacteria, while binding of organisms to immobilized agglutinin on tooth surfaces represents a mechanism of bacterial adherence. We have tested a number of strains of mutans streptococci, including those both surface positive and negative for P1 or related molecules, and have used our panel of anti-P1 MAbs to demonstrate that although both agglutinin-mediated aggregation of *S. mutans* and adherence of *S. mutans* to agglutinin-coated hydroxyapatite involve an interaction of P1 with salivary agglutinin, the interactions are not identical in nature.

Strains of *S. mutans* serotype c which express P1 on their cell surfaces were able to adhere well (≥35%) to salivary agglutinin-coated hydroxyapatite beads, while P1 nonretainer strains were not (≤10%) (Table 1). Mutant strain 834, derived from parent strain NG8 (40), which expresses a truncated P1 polypeptide corresponding to the amino-terminal 612 to 1,561 amino acids of the complete protein (6), was also nonadherent (5%). *S. mutans* serotype e and f strains, which express P1 molecules that are immunologically indistinguishable from that of serotype c strains (6), were also adherent (≥40%). A P1 cross-reactive antigen has been reported to be expressed by serotype a strains (59), and the *S. cricetus* isolates tested in this study were found to be adherent. Strains of *S. rattus* serotype b have not been found to react with anti-P1 MAbs, and only 1 of 15 of these antibodies reacted with a serotype h strain (3). Hybridization was not detected with DNA from either of these serotypes with use of a cloned *spaP* DNA probe (39). More recently, Ma et al. (46) have shown that one of five polymerase chain reaction-generated DNA probes spanning the *spaP* gene hybridized not only to DNA from serotype b and h strains but also to non-mutans alpha-hemolytic streptococci, indicating that limited regions of the *spaP* gene are highly conserved. There is as yet no immunological evidence to suggest that P1-related polypeptides are expressed by *S. rattus* isolates. The *S. rattus* strains tested in this study were nonadherent (≤7%), whereas the *S. downeii* strain tested was able to adhere (30%). This finding suggests that an adhesin, possibly related to P1, is expressed by the *S. downeii* serotype h strain. Strains of *S. sobrinus* serotypes d and g, which express the P1 cross-reactive antigen SpaA on their surfaces, were also adherent with the exception of 6715-WT-13 and K1-R. The reason for the inability of these two strains to adhere is not understood, since they were as reactive with polyclonal anti-SpaA rabbit antiserum as were the other *S. sobrinus* strains tested (data not shown). An observed difference in isolates with the same strain designation, e.g., 6715, is troublesome in that it indicates that long-term laboratory storage and passage may affect the behavior of these related isolates in in vitro model systems designed to understand biologically relevant properties. The differences in adherence among the *S. sobrinus* isolates suggests that if the P1 cross-reactive antigen, SpaA, acts as the major agglutinin-binding adhesin, the molecule may be altered in some way in strains 6715-WT-13 and K1-R. Alternatively, a molecule other than SpaA may function as the adhesin in *S. sobrinus* strains. Gibbons et al. (24) have reported that the adherence of *S. mutans* serotype c and *S. sobrinus* serotype g strains to pellicles of glucosyltransferase or saliva-glucosyltransferase mixtures on hydroxyapatite beads was strikingly different, suggesting that these two species attach to different receptors on experimental pellicles. Their *S. sobrinus* 6715 isolate adhered in high numbers to dextran-treated hydroxyapatite, while their *S. mutans* serotype c strains did not. Comparison of the results of their study with our results suggest that at least for some strains of *S. sobrinus*, multiple mechanisms of adherence may be employed. Finally, both strains of *S. pyogenes* tested were found to adhere to the agglutinin-coated beads. This result is consistent with the finding that this species is able to interact with a salivary molecule which can be removed by adsorption with *S. sanguis* (9). The P1 cross-reactive molecule SSP-5, which interacts with the same salivary agglutinin as does P1, is expressed by *S. sanguis* (13–16).

The ability of mutans streptococci to aggregate in the presence of fluid-phase agglutinin was also found to correlate with surface expression of P1 and related molecules, but not as tightly as the correlation of adherence with expression of P1. All strains of all serotypes tested aggregated in the presence of salivary agglutinin except for *S. mutans* serotype c P1 nonretainer strains. In contrast to results of the adherence assay, the P1-deficient mutant 834 demonstrated some degree of agglutinin-mediated aggregation (22%). A substantial degree of self-aggregation in the absence of agglutinin (>20%) was observed for several strains and species tested. Serotype c strain MT8148-SM self-aggregated (38%), whereas MT8148-SH did not (9%). Again, this is an example of discrepant behavior by related isolates with the same strain designation. The two *S. rattus* strains tested both aggregated in the presence of agglutinin but did not self-aggregate, suggesting that they express a surface molecule unrelated to P1 which can interact with fluid-phase agglutinin. In contrast, the *S. downeii* serotype h strain showed virtually the same degree of aggregation in the absence of fluid-phase agglutinin (50%) as with agglutinin (56%). The same two *S. sobrinus* isolates 6715-13-WT and K1-R, which were nonadherent to agglutinin-coated hydroxyapatite, also were not aggregated by agglutinin. This result suggests that adherence and aggregation of *S. sobrinus* are related by whatever modification differentiates these two isolates form the other *S. sobrinus* isolates tested. Again, both strains of *S. pyogenes* tested aggregated in the presence of agglutinin; however, 647 (type M49) self-aggregated (56%) whereas the M type 5 strain did not (13%).

A subset of anti-P1 MAbs were effective inhibitors of

adherence of *S. mutans* serotype c strain NG8 to salivary agglutinin immobilized on hydroxyapatite beads. A review of Table 3 shows that MAbs 1-6F_{6b}, 4-9D_{4c}, 4-10A_{8c}, 5-5D_{6a}, and 6-8C_{1a} were the most inhibitory of the antibodies tested, while 3-3B_{5e}, 3-8D_{2a}, and 5-3E_{5e} were moderately inhibitory and 2-8G_{2a}, 3-10E_{4d}, and 6-11A_{3a} were only weakly or not at all inhibitory. An overlapping, but not identical, subset of anti-P1 MAbs were able to inhibit aggregation of NG8 in the presence of fluid-phase agglutinin. In aggregation inhibition assays, MAbs 2-8G_{1d}, 3-8D_{2a}, 3-10E_{4d}, 4-9D_{4c}, 4-10A_{8c}, and 6-8C_{1a} demonstrated marked inhibition, while 5-5D_{6a} was somewhat less inhibitory and 1-6F_{6b}, 3-3B_{2a}, 5-3E_{5e}, and 6-11A_{3a} were only weakly or not at all inhibitory (Table 4). The negative control anti-*A. actinomycetemcomitans* MAb had no effect on either adherence or aggregation. The fact that some but not all of the anti-P1 MAbs were able to interfere with adherence and/or aggregation indicates that there is a degree of specificity to these P1-agglutinin interactions, i.e., that specific functional domains of the P1 molecule are involved. The fact that certain MAbs were effective inhibitors of aggregation but not of adherence, and vice versa, indicates that the interactions of cell surface P1 with salivary agglutinin differ depending on whether the agglutinin is free in solution or is immobilized on a solid surface. This suggests that different regions of the agglutinin molecule may be exposed in these two situations. The phenomenon of recognition by bacterial adhesins of cryptic segments or cryptitopes exposed on adsorbed or protease-treated molecules has been observed for several other oral organisms and their attachment to host tissues (for a review, see reference 23).

Anti-P1 MAbs which inhibit agglutinin-mediated adherence or aggregation or both do not map to a discrete segment within the P1 molecule (see Fig. 1). It is unclear whether multiple functional domains of P1 are involved in these two phenomena or whether MAbs which map to different regions of P1 have different effects which impact on a single functional domain. Little is known regarding the secondary or tertiary structure of P1 and related molecules. It is possible that antibodies which appear to bind to distant sites on the primary sequence may actually bind quite close to one another when the molecule is in its native configuration on the intact cell. Therefore, MAbs may inhibit the P1-agglutinin interaction directly by binding to a functionally important site or indirectly by binding to a spatially proximal but nonfunctional site. Alternatively, other MAbs may exert conformational constraints on the molecule which may affect a functional domain some distance away. These possibilities can be resolved in future studies in which antibody Fab fragments are used for inhibition experiments and by using a molecular approach in which P1 deletion mutants and truncated P1 polypeptides are used to characterize further both MAb-binding domains and domains which can bind directly to agglutinin.

The most inhibitory antibody preparation in the adherence inhibition assays was anti-P1 polyclonal rabbit IgG. Polyclonal anti-SpaA rabbit IgG was not nearly as effective an inhibitor of *S. mutans* serotype c adherence as was the anti-P1 preparation. This finding, coupled with the results that cross-reactive polyclonal anti-P1 and anti-SpaA antisera can be rendered monospecific by cross-adsorption with the heterologous antigens (4a), that 5 of 11 anti-P1 MAbs (including 4-9D_{4c} and 1-6F_{6b}, which are highly inhibitory in the adherence inhibition assays described above) do not demonstrate any cross-reactivity with SpaA (6), and that variability is observed in the salivary agglutinin-mediated adherence and aggregation properties among *S. sobrinus* isolates which show no difference in their abilities to react with polyclonal anti-SpaA antiserum, suggests that at least subtle functional differences exist between the immunologically related P1 and SpaA molecules.

The high degree of inhibitory activity of anti-P1 polyclonal IgG suggests that a combination of antibodies of different specificities is a more effective inhibitor of adherence than any single antibody recognizing one discrete epitope. Attempts to perform adherence inhibition assays using combinations of anti-P1 MAbs were confounded by the fact that most of these MAbs interfere with the binding of the other MAbs, as evidenced by negative cooperativity in enzyme-linked immunosorbent assays (ELISA) (data not shown). Negative cooperativity by different MAbs in ELISA was seen despite evidence that all of the 11 MAbs tested mapped to different epitopes by Western blot, cross-reactivity, and radioimmunoassay experiments (6). Although steric inhibition of one MAb's ability to bind to its cognate epitope by another MAb is certainly one explanation for these observations, it appears that at least some of the anti-P1 MAbs are able to modify the conformation of the P1 molecule in some way.

Some clues have been uncovered at the molecular level to begin to understand the interaction of P1 with salivary agglutinin and the resultant biological effects. Mutant 834, which expresses a truncated polypeptide corresponding to amino acid residues 1 to 612 of P1, showed limited aggregation but no adherence ability (Tables 1 and 2). Consistent with this finding, MAb 3-8D_{2a}, the only one of the 11 anti-P1 MAbs which mapped to the amino terminus of P1 (Fig. 1), was a much better inhibitor of aggregation than of adherence (compare Tables 3 and 4). Taken together, these results suggest that the amino-terminal region of P1, which includes the alanine-rich tandem repeats, may be involved in aggregation mediated by fluid-phase agglutinin but is not significantly involved in adherence to immobilized agglutinin. We have also demonstrated that a truncated recombinant-specified P1 polypeptide (M_r = 150,000) which lacks the carboxy-terminal 254 amino acids is an excellent inhibitor of adherence (Fig. 2), suggesting that the carboxy terminus of P1 does not significantly affect its ability to interact with immobilized agglutinin. In seeming contradiction of this result, MAb 6-8C_{1a}, which appears to map to the region of P1 missing from the truncated recombinant M_r-150,000 molecule (Fig. 1), is an extremely efficient inhibitor of both adherence and aggregation. We therefore cannot rule out the possibility that the MAbs which map to the carboxy terminus of P1 may not react directly within that region but may be dependent on its presence for formation of their cognate epitopes. Another interesting finding is that two MAbs, 1-6F_{6b} (a strong inhibitor of adherence) and 6-11A_{3a} (a weak inhibitor of adherence), consistently demonstrate moderate inhibition of aggregation at intermediate, but not high or low, concentrations of antibody. Both of these MAbs map to the central region of P1 (Fig. 1), which includes the proline-rich tandem repeats. It is tempting to speculate that these antibodies may bind to repeated epitopes within that domain, which could explain their concentration-dependent antiaggregation effects. Finally, MAb2-8G_{1d}, which also maps to the carboxy terminus of P1, is one of the most effective inhibitors of aggregation yet has virtually no effect on adherence. This result strongly reinforces the idea that aggregation and adherence of *S. mutans* are both mediated by the interaction of the P1 and agglutinin molecules but that the interaction is in some way different.

Immunization of laboratory animals with P1 has resulted in protection against dental caries (32, 41, 43). Use of anti-P1 antibodies to protect against *S. mutans* colonization in passive immunization studies has also shown some promise (42, 45, 47, 54). It has been suggested, however, that caution be exercised in this approach of immune protection against dental caries since extensive sequence homology exists between P1 and the surface protein SSP-5 expressed by *S. sanguis*, a common oral organism not associated with oral disease (15). The results of this study indicate that we have begun to dissect the interaction of P1 and agglutinin at the molecular level and are beginning to understand that aggregation and adherence represent different manifestations of the interaction of the same two molecules. This suggests the potential for the development of yet another therapeutic modality in which adherence of pathogenic bacteria to agglutinin-coated surfaces might be prevented, possibly by using a small synthetic peptide as a competitive inhibitor, without interfering with the nonimmune clearance mechanism afforded by agglutinin-mediated aggregation.

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