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Streptococcus sanguis Expresses a 150-Kilodalton Two-Domain Adhesin: Characterization of Several Independent Adhesin Epitopes

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Streptococcus sanguis **binds to saliva-coated hydroxylapatite (sHA), an in vitro model of the enamel pellicle. To learn if more than one adhesin functions during adhesion, 12 reactive monoclonal antibodies (MAbs) were isolated by screening against both adhesive and nonadhesive strains. Two of these MAbs, 1.1 and 1.2, inhibited adhesion in a dose-dependent fashion, although maximum inhibition with either was only 37%. When these two MAbs plus a polyclonal antibody to P1-like adhesin were combined, the inhibition was additive to about 82%. These data indicated that there were at least three distinct, functional adhesion epitopes on the surface of *S. sanguis*. Western blot analyses of *S. sanguis* surface macromolecules showed antigens at 36 and 56 (with MAb 1.2), 87 and 150 (with both MAb 1.1 and MAb 1.2), and 100, 130, and 170 kDa (with anti-P1 antibody). The antigens were eluted from gels. Isolated antigens and corresponding antibodies inhibited adhesion similarly. Additivity experiments suggested the distinct epitopes were in three groups: (i) 36/56 kDa, (ii) 87/150 kDa, and (iii) 100/130/170 kDa. The 150-kDa antigen reacting with both MAbs was isolated from gels and digested with trypsin. The digestion revealed a series of tryptic bands. A band at 38 kDa reacted with MAb 1.1 whereas a band at 54 kDa reacted with MAb 1.2 in Western blot analysis, indicating two distinct adhesive epitopes on the 150-kDa antigen. These data strongly suggest that *S. sanguis* adhesion to sHA is maximized when several adhesin epitopes are coexpressed on surface antigens of different sizes.**

Streptococcus sanguis is usually among the earliest colonizers of the tooth surface (3). Adhesion of *S. sanguis* to the salivary film or pellicle that coats enamel surfaces precedes successful colonization and thereby initiates dental plaque formation (13, 14). When analyzed in vitro by using saliva-coated hydroxylapatite (sHA), adhesion of *S. sanguis* occurs via a complex set of potential mechanisms. Strains of *S. sanguis* may adhere through lectin-like (33), hydrophobic (34), or specific protein (adhesin) interactions (7, 26, 37). Some strains have been suggested to express combinations of the three (14, 20). Yet, it is unclear if several adhesins function together to promote adhesion. While such adhesins may be expressed simultaneously on more than one macromolecule, a singular adhesin may contain more than one adhesive domain or epitope.

For example, *S. sanguis* 12 binds to pH- and neuraminidase-sensitive binding sites on sHA, suggesting expression of at least two adhesins (11, 31, 32). The strongly hydrophobic, variant strain 12na, which has reduced adherence characteristics, binds only to the pH-sensitive site. Similarly, *S. gordonii* may express more than one functional adhesin, consistent with the presence of more than one potential binding site on sHA. One site may be comprised of members of the acidic proline-rich protein family (15), salivary agglutinin may constitute a second site (23), and salivary amylase may constitute a third (39, 40). A candidate adhesin for the proline-rich proteins and salivary agglutinin on *S. gordonii* M5 has been suggested to be a 162-kDa sialic acid-binding lectin (23). Hence, an adhesin such as SspB appears to contain adhesive domains with specificity for more than one salivary protein. In contrast, *S. gordonii* adhesion to amylase involves cell surface proteins of 20 to 36 and 82 to 87 kDa, varying among different strains (17, 39). *S. gordonii*, therefore, appears to express more than one adhesin protein, each specific for one or more salivary macromolecular binding sites.

An adhesin domain may appear to be expressed on proteins of different sizes. For example, polypeptide fragments of a SspB homolog may be expressed on the cell surface after posttranslational proteolytic processing (24). Products of alternative genes, including SspA and SspB (5) and CshA and CshB (29, 30), are also expressed as similar but nonidentical cell surface adhesins. Use of deletion mutants of SspA and SspB, for example, indicates that one gene product serves as an adhesin in the absence of its alternative (5). Indeed, the products of more than one adhesin gene family are suggested to each contribute partially to adhesion when another is deleted or blocked with specific antiserum (22). Therefore, adhesion of streptococci to a substrate such as sHA may be mediated by adhesins that are (i) expressed as identical specificities on posttranslationally modified fragments of one protein, or similar products of alternatively expressed genes, and (ii) complementary and nonredundant products of different genes, which independently and noncompetitively bind to different sites to increase adhesion.

To probe adhesive specificities on *S. sanguis* 133-79 and distinguish between these hypothesized mechanisms, murine monoclonal antibodies (MAbs) 1.1 and 1.2 raised against *S. sanguis* 133-79 (16), MAb D10 to *S. parasanguis* FW213 type 1 fimbrial adhesin (7), and rabbit anti-*S. mutans* P1 adhesin antiserum (10) were used. Potential adhesin antigens were isolated from the cell surface as described previously (21), using mutanolysin digestion in osmotically stabilized conditions, and used to inhibit *S. sanguis*-sHA adhesion. The data provided by this study suggest strongly that there are multiple adhesins for sHA expressed on the surface of *S. sanguis* 133-79. Some adhesins have redundant specificities found on several

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antigens. Adhesins with nonidentical, complementary specificities contributed independently to increase adhesion. One adhesin, a 150-kDa antigen, has two different adhesin epitopes. Adhesion appears to be maximal when several different adhesin specificities operate to promote adhesion to sHA.

MATERIALS AND METHODS

***S. sanguis* strains and growth.** *S. sanguis* 133-79 and ATCC 10556 (16) were stored in skim milk at −80°C. The bacterial cells were transferred from frozen stocks onto plates of mitis-salivarius agar and incubated for 48 h at 37°C in 5% CO_2. A single colony was picked, inoculated into 50 ml of Todd-Hewitt broth, and allowed to grow overnight at 37°C in 5% CO_2. The cells were washed three times in 0.01 M sodium phosphate buffer (pH 7.4) with 0.9% sodium chloride (phosphate-buffered saline [PBS]) and resuspended to an optical density at 620 nm (OD_{620}) of 0.5 (10^9 cells/ml).

***S. sanguis*-sHA adhesion assay.** This in vitro adherence assay is a modification of that used by Liljemark et al. (25, 26) and Tellefson and Germaine (43). Briefly, the assay was performed in 1.5-ml polypropylene microcentrifuge tubes with 0.01 M phosphate buffer (pH 6.8) (PB), at room temperature (RT) in all experiments. Cells of *S. sanguis* 133-79 or 10556 were grown overnight in Todd-Hewitt broth with 10 μCi of [*methyl*-^{3}H]thymidine (Research Products International Corp., Mount Prospect, Ill.) per ml. Radiolabeled cells were washed three times with PB, sonicated three times for 3 s each to break the bacterial chains, and resuspended in the same buffer at 10^9 cells/ml. The average specific activity for labeled *S. sanguis* was $1.49 \times 10^3 \pm 55$ bacteria per cpm (mean of 20 experiments, five determinations each, ±SEM [standard error of the mean]).

Whole saliva was collected in ice-cooled containers by expectoration without stimulation from a pool of five consenting adult volunteer donors according to procedures approved by the Committee on the Use of Human Subjects of the University of Minnesota. After centrifugation at 10,000 × *g* for 20 min at 4°C, fresh, clarified whole salivary supernatant (1 ml) was incubated for 60 min to coat 10 mg of HA (Gallard-Schlessinger, Carle Place, N.Y.) that had been equilibrated for 60 min with PB. After coating with saliva, sHA was washed two times and then transferred into a new microcentrifuge tube. Radiolabeled cells (10^9) were added and mixed with sHA for 60 min by continuous inversion on a Roto-Torque heavy-duty rotator (Cole-Palmer, Chicago, Ill.). Unattached bacteria were aspirated, and cells loosely associated with the sHA were removed by washing. The radioactivity associated with sHA was monitored by liquid scintillation counting.

The data are presented as percentage of total input (10^9) bacterial cells adhering to 10 mg of sHA and calculated as (radioactivity associated with sHA × specific activity/10^9) × 100. From preliminary experiments, *S. sanguis* 133-79 binds to sHA 3.6 times more effectively than to naked HA. Within 60 min of incubation, 31.5% ± 4.9% of cells adhered (mean of five experiments, three determinations each, ±SEM). ATCC 10556 binds more poorly to both sHA (3.7%) and HA (3.9%) and was therefore selected as a negative adhesion control.

Preparation of MAbs. MAbs were raised by immunizing 2-month-old BALB/c mice intraperitoneally with 2×10^7 live cells of *S. sanguis* 133-79. Spleen cells were harvested and fused with FOX-NY myeloma cells (16, 42), and hybridomas were screened for anti-*S. sanguis* immunoglobulin G (IgG) production by reaction with immobilized cells of strains 133-79 and 10556 in an enzyme-linked immunosorbent assay (ELISA) (6, 16). Clones reactive with strain 133-79, but not 10556, were selected.

To select MAbs specific for putative adhesins, selected culture supernatants were tested for the ability to react with strain 133-79 to inhibit adhesion to sHA. Suspensions of strain 133-79 (5×10^8 radiolabeled cells) were preincubated with culture supernatants for 2 h at RT. After preincubation, *S. sanguis* cells were washed three times with 1 ml of PB and tested for adhesion to sHA or HA. Controls included *S. sanguis* cells preincubated with (i) PB or (ii) 1 μg of mouse IgG/ml of FOX-NY myeloma cell culture supernatant. Percent inhibition of *S. sanguis* 133-79-sHA adhesion was calculated as [(adhesion PB − adhesion MAb)/adhesion PB] × 100, where adhesion PB is adhesion occurring in the presence of buffer and adhesion MAb is adhesion occurring in the presence of added antibody.

Clones inhibiting adhesion of *S. sanguis* 133-79 to sHA were selected as presumptive anti-adhesin MAbs. MAbs from presumptive antiadhesin clones were purified, and Fab fragments were prepared as described previously (16, 28). Purified MAbs, Fab fragments, or nonspecific mouse IgG (control) were tested as inhibitors of *S. sanguis*-sHA adhesion.

Determination of antibody concentration in ascites and serum. Murine anti-*S. parasanguis* FW213 type 1 fimbrial adhesin MAb D10 ascites was gift of Paula Fives-Taylor, University of Vermont. Rabbit sera developed against *S. mutans* P1 adhesin were gifts of Howard F. Jenkinson, University of Otago, Dunedin, New Zealand, and Neil Hunter, Institute of Dental Research, Sydney, New South Wales, Australia. Gamma globulin was isolated from ascites or antisera with E-Z-SEP (Middlesex Sciences, Inc., Foxborough, Mass.) as instructed by the manufacturer. Briefly, 30 μl of ascites, antisera, or mouse IgG (control; 2 mg/ml; Sigma, St. Louis, Mo.) was mixed with 90 μl of E-Z-SEP-A for 30 min at RT and then centrifuged at 4°C for 30 min at 5,000 rpm. The supernatant was decanted, and the precipitate was completely resuspended in 30 μl of distilled, deionized water. The suspension was allowed to stand for 10 min at RT and was then mixed with 30 μl of E-Z-SEP-B for 30 min and centrifuged. The final precipitate was resuspended in 210 μl of 0.1 M Tris (pH 9). Dilutions of the samples were made, and IgG concentrations were determined from the OD_{280}, using a standard curve of serial dilutions of 2 mg of mouse IgG per ml. All samples were tested in triplicate. The recovery of mouse IgG ranged from 90 to 96% with this method.

Analysis of adhesins with antiadhesin antibodies. To learn if more than one adhesin specificity is expressed on cells of *S. sanguis* 133-79, the murine MAbs 1.1, 1.2, and D10 and rabbit anti-P1 antibody were preincubated alone or in combinations with cells of *S. sanguis* in the sHA adhesion assay. Concentrations of MAbs 1.1, 1.2, and D10 and rabbit anti-P1 antibody required for half-maximal and maximal inhibition of adhesion were determined.

To identify specific putative adhesins, cell surface macromolecules of *S. sanguis* 133-79 were released by digestion with mutanolysin (Sigma) in osmotically stabilized conditions as described previously (21) and reacted with specific antibodies in Western immunoblot assays. The digests (10 μg/well) were resolved by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) on 5 to 10% gradient polyacrylamide gels at 30 mA and transferred electrophoretically to nitrocellulose (Bio-Rad, Richmond, Calif.) at 200 mA for 75 min (12, 45). The nitrocellulose was blocked overnight with 3% bovine serum albumin (BSA) in PBS and then incubated overnight with 35 ml of culture supernatant of MAb 1.1 or 1.2 (diluted 1:2), MAb D10 ascites (diluted 1:100), or mouse IgG in FOX-NY culture supernatant at 1 μg/ml (control) or incubated for 1.5 h with rabbit anti-P1 serum (diluted 1:800). The nitrocellulose sheet was washed three times, incubated with alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit IgG (Bio-Rad) for 3 h, and developed with 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium color development solution (Bio-Rad).

To ensure relative purity, the mutanolysin was assayed for contaminating proteolytic activity as described by Siegel et al. (41). Azocoll (Calbiochem, La Jolla, Calif.) was suspended in PB at 10 mg/2.5 ml and mixed with 200 U of mutanolysin. After incubation at 37°C for 1 or 2 h, the mixture was centrifuged at 2,500 rpm for 20 min. The supernatant was then read at OD_{520} against Azocoll blanks that did not contain mutanolysin. All samples were tested in triplicate, and no proteolytic activity was detected.

Isolation of adhesins. Antigens recognized on Western blots by antiadhesin antibodies were subsequently isolated from SDS–5 to 10% acrylamide gradient gels and tested for adhesin-related activity. In brief, mutanolysin digests (3.75 mg per gel) were loaded onto 1.5- by 27-mm preparative polyacrylamide gels, resolved at 30 mA, and stained by Coomassie blue. The respective bands were excised and crushed. Antigens were eluted by diffusion into a buffer containing 0.1% SDS, and SDS was then removed by acetone precipitation as described by Hager and Burgess (18). The precipitated protein was dissolved in 6 M guanidine-HCl and diluted 50-fold in PBS. The quantity of isolated, eluted, and reconstituted protein was determined by the Lowry protein assay (27). The eight selected antigens were then assayed for unique adhesin determinants. (A ninth putative adhesin could not be resolubilized and was not studied further.) First, sHA was blocked with 1% BSA for 30 min and then pretreated with isolated antigen (40 pmol), alone or in combinations. For two antigens, amounts less than and greater than 40 pmol were also tested to estimate maximal inhibitory concentrations. As a negative control, sHA was pretreated with BSA in PBS with 0.12 M guanidine-HCl. Next, pretreated sHA was washed and then incubated with radiolabeled *S. sanguis* in the adhesion assay. Percent inhibition of *S. sanguis* adhesion to sHA was calculated. The data from individual antigens were compared by Duncan's test with each other (valid $n = 24$) and combinations (valid $n = 75$).

Oligospecificity of an adhesin. To learn if it contains more than one adhesive specificity (oligospecificity), the 150-kDa antigen (15 μg in 60 μl of PBS) was incubated with *N*-*p*-tosyl-L-lysine chloromethyl ketone–trypsin (0.3 μg) at 37°C for 1.5 h. To control for the fidelity of digestion, antigen (5 μg/20 μl) was also incubated in separate tubes with PBS only or mutanolysin (2 U). Immediately after incubation, the digests were boiled for 5 min in SDS sample buffer and loaded on SDS–10% polyacrylamide gels at 5 μg/well. Subsequently the gels were transferred onto nitrocellulose and allowed to react with MAbs 1.1 and 1.2 in Western blots. To confirm relative purity, the 150-kDa antigen was isolated, SDS was removed, and the antigens were subjected to electrophoresis on nondenaturing polyacrylamide gels as described previously (8).

RESULTS

Twelve anti-*S. sanguis* MAb clones reacted in ELISA with strain 133-79 but not strain 10556. Of these, representative clones MAb 1.1 and 1.2 were selected for further study. MAb culture supernatants were preincubated with cells of strain 133-79, which adhered significantly better to sHA than HA in buffer ($P < 0.00003$). *S. sanguis* 133-79 adhesion to sHA was inhibited significantly by MAb 1.1 (reduced 21.4%; $P < 0.0001$) and MAb 1.2 (reduced 12.3%; $P < 0.0059$) (Table 1). These MAbs did not affect adhesion to HA. Adhesion of *S.*

TABLE 1. Specific inhibition of *S. sanguis* 133-79 adhesion to sHA by MAbs 1.1 and 1.2 culture supernatant[a]

S. sanguis preincubated with:	% Inhibition of adhesion	
	S. sanguis to sHA	*S. sanguis* to HA
Mouse IgG	−2.3 ± 0.5	−2.6 ± 0.4
MAb 1.1	21.5 ± 2.2	−5.1 ± 1.6
MAb 1.2	12.3 ± 0.7	−0.2 ± 3.0

[a] *S. sanguis* cells (5×10^8) were preincubated for 2 h with culture supernatants from MAb 1.1, MAb 1.2, or mouse IgG (1 μg/ml) in FOX-NY culture medium or phosphate buffer (PB) at 20°C. The concentration of each MAb in culture supernatant was approximately 1 μg/ml. After incubation, the bacterial cells were washed three times with 1 ml of PB and tested for adhesion to sHA. The inhibition of adhesion to sHA or HA relative to that of *S. sanguis* cells preincubated with PB was calculated. Results are means of one experiment done in triplicate ± SEM.

sanguis to sHA and HA was also unaffected by nonspecific mouse IgG in FOX-NY culture medium.

Purified MAbs 1.1 and 1.2, D10 ascites, and anti-P1 antibody each showed dose-dependent partial inhibition of adhesion of strain 133-79 to sHA (Fig. 1). MAb 1.1 inhibited by a maximum of 37% at 130 pmol of IgG/10^9 cells/ml; half-maximal inhibition (16.5%) occurred at 33.5 pmol. MAb D10 inhibited by a maximum of 30% at 130 pmol (half-maximal inhibition at 54 pmol), MAb1.2 inhibited similarly at 100 pmol (half-maximal inhibition at 31 pmol), and anti-P1 inhibited by 28% at 130 pmol (half-maximal inhibition at 45 pmol). Fab fragments prepared from MAbs 1.1 and 1.2 were preincubated with *S. sanguis* 133-79 in separate experiments. MAb 1.1 Fab fragments reacted with strain 133-79 to inhibit adhesion by 42.9% at 260 pmol/10^9 cells/ml; MAb 1.2 Fab fragments inhibited by 32.1% at 200 pmol/10^9 cells/ml. Commercial mouse IgG did not affect adhesion. Since Fc-mediated or nonspecific steric interactions were not obvious in these conditions, intact antibodies were used in all other experiments.

To learn if different adhesin epitopes were functional, two or three antibodies were mixed at the maximal inhibitory concentration of each. The combinations of different antibodies were

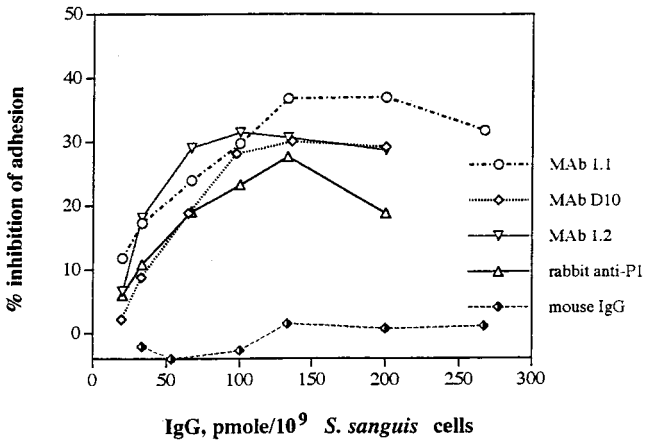

FIG. 1. Inhibition of *S. sanguis* adhesion to sHA by antiadhesin antibodies. In separate experiments, MAbs 1.1, 1.2, and D10, rabbit anti-P1 antibody, or mouse IgG (control) was incubated with tritium-labeled *S. sanguis* 133-79 cells (10^9/ml) for 2 h at RT. Pretreated bacteria were then mixed with 10 mg of sHA for 1 h. Nonadherent bacterial cells were removed by washing, and the number of bacteria adherent to the sHA was determined by scintillation counting. Percent inhibition of bacterium-sHA adhesion was determined by comparing with *S. sanguis* 133-79 cells preincubated with PB. Data shown are the means of three experiments, each done in triplicate.

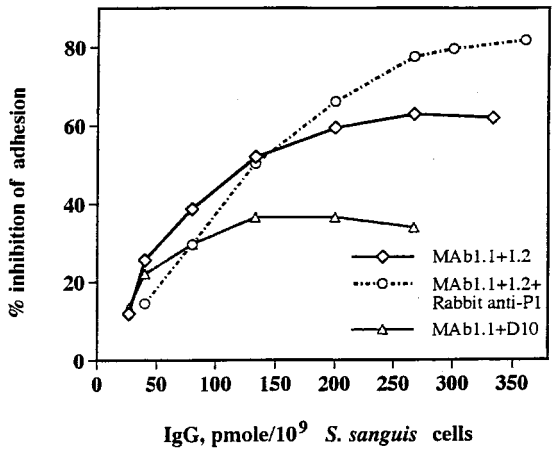

FIG. 2. Incremental inhibition of *S. sanguis*-sHA adhesion by combinations of antiadhesin antibodies. *S. sanguis* 133-79 cells (10^9/ml) were incubated for 2 h at RT with the indicated total amount of equimolar mixtures of either MAb 1.1 plus D10, MAb 1.1 plus 1.2, or MAb 1.1 plus 1.2 plus rabbit anti-P1 antibody. Pretreated bacterial cells were assayed for adherence to sHA. Data shown are the means of three experiments, each done in triplicate.

then preincubated with strain 133-79, which was then tested for adhesion to sHA. When mixed, MAbs 1.1 and D10 did not increase inhibition of adhesion from a maximum of 37% (Fig. 2). In contrast, the combination of MAbs 1.1 and 1.2 inhibited additively to a maximum of about 63%. Similarly, the combination of MAb 1.1, MAb 1.2, and anti-P1 inhibited adhesion by 82%.

Western blots were performed to identify the putative adhesin antigens reacting with each antibody. As expected, MAbs 1.1 and D10 reacted with the same group of cell surface antigens of 40, 87, 150, and 205 kDa (Fig. 3). MAb 1.2 recognized antigens of 40, 87, 150, and 205 kDa and additional bands at 36 and 56 kDa. Anti-P1 reacted with antigens of 100, 130, 170, and 205 kDa.

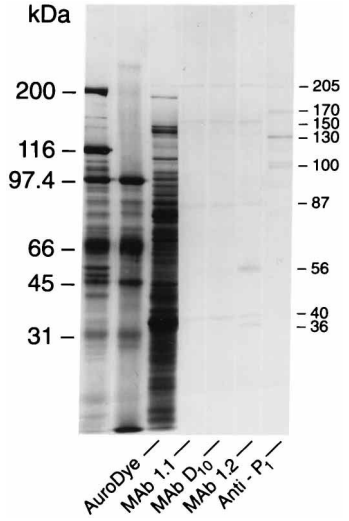

FIG. 3. Potential adhesins of *S. sanguis* 133-79 recognized by antiadhesin antibodies in Western blot analyses. To identify the putative adhesins reacted with the selected MAbs, Western blot analyses were performed. Mutanolysin digests of *S. sanguis* 133-79 were resolved by SDS-PAGE (5 to 10% gel) and electrotransferred to nitrocellulose. The proteins were either stained with AuroDye or allowed to react with antiadhesin antibodies as described in Materials and Methods.

TABLE 2. Inhibition of *S. sanguis*-sHA adhesion by isolated adhesins, individually or in combination

Molecular mass (kDa) of antigen added in combination	% Inhibition upon preincubation of sHA with isolated antigen having molecular mass (kDa) of:							
	36	40	56	87	100	130	150	170
36	25.3 ± 5.8[a]	25.7 ± 2.3[b]	26.2 ± 1.7[b]	53.6 ± 13[b]	52.5 ± 4.6[b]			
40		7.7 ± 4.4[a]	22.6 ± 4.5[b]	34.6 ± 1.1[b]			41.0 ± 9.0[b]	
56			21.5 ± 6.9[a]	53.9 ± 13[b]		50.7 ± 2.8[b]	41.9 ± 5.8[b]	
87				33.8 ± 3.5[a]		52.6 ± 3.4[b]	38.5 ± 4.8[b]	
100					28.2 ± 0.9[a]	26.2 ± 2.5[b]		25.1 ± 5.8[b]
130						26.7 ± 2.8[a]	74.6 ± 3.3[b]	24.9 ± 3.4[b]
150							41.8 ± 11[a]	
170								24.2 ± 11[a]

[a] sHA was blocked with 1% BSA for 30 min, treated with an isolated antigen (40 pmol/ml) for 1 h, and then mixed with *S. sanguis* 133-79 in the adhesion assay. The value given is the means of three experiments, each done in triplicate, ± SEM.

[b] One antigen was combined with another isolated antigen (40 pmol/ml) and incubated with sHA. Pretreated sHA was then tested in the adhesion assay. The value given is the mean of three experiments, each in triplicate, ± SEM.

To test for adhesin-like function, each of the nine surface antigens identified by Western blotting was isolated by preparative SDS-PAGE. The 205-kDa antigen was excluded from further study, since it could not be reconstituted in buffer after isolation. Next, each isolated antigen alone or in combinations (40 pmol of each) was preincubated with sHA, which was then tested for adhesion with *S. sanguis* (Table 2). In data not shown, 40 pmol of either the 36- or 150-kDa antigen was shown to inhibit binding maximally. It was assumed that this amount of all other antigens would approximate maximal inhibition also. Compared with all others, the 150-kDa (reduced by 41.8% ± 11%; $P < 0.03$) and 87-kDa (reduced by 33.8% ± 3.5%) antigens inhibited adhesion best and were similar to each other ($P = 0.157$) (Duncan's test, $n = 3$; for all groups, $n = 24$).

Isolated antigens of 40, 87, and 150 kDa, recognized by MAbs 1.1, 1.2, and D10, respectively, in Western blots (Fig. 3), were mixed in pairs and preincubated with sHA (Table 2). When combined, these antigens inhibited adhesion of strain 133-79 no more than the 87- or 150-kDa protein alone (Duncan's test, $n = 3$; for all groups, $n = 75$). The 40-kDa antigen contributed little to inhibition alone or in combination with any other antigens (8% reduction). Combining the 36- and 56-kDa antigens (which react only with MAb 1.2) did not increase inhibition; likewise, inhibition was unaffected when the 56- and 150-kDa antigens were combined. In contrast, when the 87-kDa antigen was mixed with the 36- or 56-kDa antigen, inhibition increased significantly, from about 26 to 53% ($P < 0.001$). It appears that the 87-kDa antigen is the minimal functional adhesin identified by MAbs 1.1, 1.2, and D10, while the 36- and 56-kDa antigens contain another specificity reacting with MAb1.2.

Similarly, mixing P1-cross-reactive adhesins of 100, 130, or 170 kDa with each other did not increase inhibition (Table 2). When the 130-kDa antigen was mixed with the 56- and 87-kDa antigens, each apparently representing a different functional adhesin epitope, inhibition was increased to 76.7% ± 0.9%. Alternatively, when antigens of 130 and 150 kDa were combined, inhibition was also increased significantly, to 74.6% ± 3.3% ($P < 0.00002$).

Adhesion of strain 133-79 to sHA was inhibited independently by MAbs 1.1 and 1.2. Reacting separately with MAb 1.1 or 1.2, the isolated 150-kDa antigen also inhibited adhesion. Hence, the 150-kDa antigen appears to express two different adhesin epitopes. To test this hypothesis, the 150-kDa antigen was isolated by preparative SDS-PAGE and retested for reactions with MAbs 1.1 and 1.2 by Western blotting. The antibodies reacted only with the 150-kDa band (data not shown) and not the higher-molecular-weight contaminant seen in Fig. 4A. The isolated 150-kDa antigen was then resolved on a native polyacrylamide gel, migrating as a single band (data not shown). This relatively pure 150-kDa antigen was digested with trypsin, and the products were analyzed by SDS-PAGE and Western blotting. The digest contained several components, including protein fragments of 38 and 54 kDa (Fig. 4A). The 38-kDa protein fragment reacted only with MAb 1.1, while the 54-kDa antigen reacted with MAb 1.2 in Western blots (Fig. 4B). The other fragments reacted with neither MAb. Mutanolysin did not digest the isolated 150-kDa antigen (Fig. 4A).

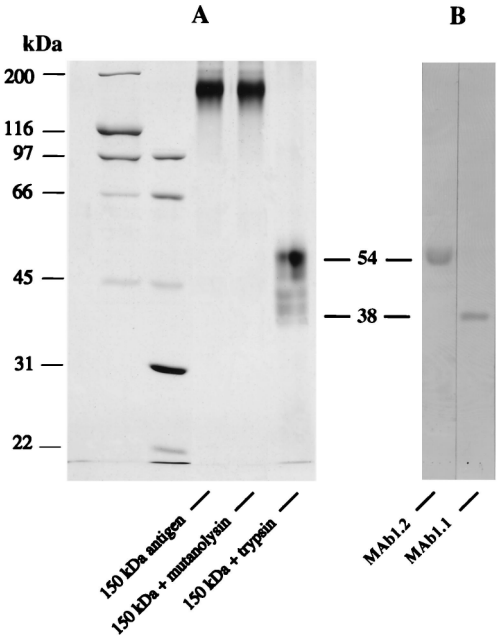

FIG. 4. Two adhesin domains in the 150-kDa protein. The 150-kDa protein was isolated on an SDS–5 to 10% gradient polyacrylamide gel and eluted by diffusion into a buffer containing 0.1% SDS. SDS was removed by acetone precipitation, and the precipitated protein was dissolved in PBS. The protein (5 μg in 20 μl of PBS) was incubated either with trypsin (0.1 μg) or with mutanolysin (2 U) at 37°C for 1.5 h. The antigen incubated with PBS only was used as a control. After incubation, the digests were immediately boiled for 5 min with SDS sample buffer and loaded on an SDS–10% polyacrylamide gel at 5 μg/well (A). (B) The trypsin digest of 150 kDa was transferred onto nitrocellulose and allowed to react with MAbs 1.1 and 1.2 in a Western blot assay.

DISCUSSION

These data suggest strongly that *S. sanguis* 133-79 expresses adhesins with several different specificities, which interact cooperatively to maximize adhesion to sHA. The three distinct adhesin epitopes are characterized as 36/56 kDa (defined by MAb 1.2), 40/87/150 kDa (MAbs 1.1 and D10), and 100/130/170 kDa (anti-P1). These adhesins were first defined by reactions with rabbit anti-P1 IgG and murine MAbs 1.1, 1.2, and D10. These antibodies inhibited adhesion in a dose-response manner, each inhibiting only partially at maximal concentrations. At these concentrations, several antibodies could be mixed together to inhibit adhesion additively. Hence, MAbs 1.1 and 1.2 and anti-P1 defined unique adhesin epitopes. In contrast, inhibition was unaffected when maximal concentrations of MAbs 1.1 and D10 were mixed, indicating that these two MAbs reacted with related adhesin epitopes. The adhesin epitopes were shown to be expressed on an array of surface macromolecules and fragments that were resolved by Western blotting. The isolated adhesins and the antibodies that reacted with them showed a generally consistent pattern of inhibition of adhesion in experiments with sHA. Likewise, combined fractions of isolated adhesins or the corresponding antibodies inhibited adhesion additively or appeared to compete, confirming the expression of both unique and related adhesin domains among the surface macromolecules of *S. sanguis* 133-79.

Indeed, adhesins isolated by mutanolysin digestion of *S. sanguis* 133-79 can be grouped based on their apparent specificities. While it is not clear that these fractions are pure, the data indicate that the adhesins in fractions of 36 and 56 kDa (36/56-kDa adhesion fractions) express similar specificities. The 36/56-kDa adhesin fractions differ from the 87- and 100/130/170-kDa fractions.

The 100/130/170-kDa antigens reacted with anti-*S. mutans* P1 antiserum, suggesting that *S. sanguis* 133-79 expresses homologs of P1. P1 is a major high-molecular-weight adhesin protein of *S. mutans* (2, 10, 19, 38). P1 is immunologically and biochemically identical to *S. mutans* antigens Pac and I/II (35, 38). The molecular masses of *S. mutans* P1 antigens vary among different strains (1) from 185 to 200 kDa (2). *S. sobrinus* also expresses P1-like protein, SpaA. The molecular mass of SpaA is 170 kDa (44). *S. sanguis* P1-like antigens identified in this study include smaller forms of 100 and 130 kDa in addition to a 170-kDa antigen.

The 100/130/170-kDa antigens appear to be unrelated to the 36/56- and 87-kDa antigens since (i) the polyclonal anti-P1 fails to cross-react with these other antigens, (ii) combining the P1-like antigen group with other adhesin groups increased the inhibition of adhesion, and (iii) the 87- and 150-kDa antigens also reacted with MAb D10. MAb D10, like MAb F51, reacts with a 150-kDa fimbria-associated adhesin of *S. parasanguis* FW213, which is apparently unrelated to P1 (7). The fact that both rabbit anti-P1 antibody and isolated 100/130/170-kDa antigens inhibited adhesion to sHA indicated that *S. sanguis* 133-79 use P1-like antigens as adhesins.

MAbs 1.1 and 1.2 were developed against *S. sanguis* 133-79. They appeared to react with adhesins of different specificities on the bacteria to inhibit adhesion to sHA (i) in a dose-response manner, (ii) independently and additively, and (iii) similarly to their respective MAb Fab fragments. In contrast, MAbs 1.1 and D10 recognized the same or proximal epitopes on *S. sanguis* 133-79, since each (i) at maximal concentrations inhibited *S. sanguis*-sHA adhesion only by about 30% and showed no additive effect on inhibition when mixed together and (ii) recognized the same antigens of the 40/87/150-kDa group.

Only MAb 1.2 reacts with the 36/56-kDa antigen in Western immunoblots. Antigens of 36 and 56 kDa contain similar adhesin epitopes. In the adhesion assay, these two antigens (i) independently inhibited *S. sanguis*-sHA adhesion to about 25%, (ii) showed no additive effect on adhesion when mixed together, and (iii) when mixed with the 87-kDa antigen separately, increased inhibition of adhesion to about 60%. That the 87-kDa antigen combined with the 36/56-kDa antigen showed additive inhibition suggested that each functioned through independent adhesin domains. When the 150-kDa antigen was mixed with the 36/56-kDa antigen, no additive effect was noted.

The findings with the soluble 150- and 87-kDa fractions when mixed with 36/56-kDa fraction are somewhat inconsistent with the specificity of the antibodies that reacted with each. As an inhibitor of adhesion, the 87-kDa antigen functioned as if it was defined solely by MAb 1.1 (or MA6 D10). The 87-kDa adhesin appeared to differ from the MAb1.2-reactive 36/56-kDa adhesins and the P1-like 100/130/170-kDa adhesins. The reason why the 87-kDa adhesin reacted with MAb 1.2 is not clear. The 87-kDa antigen may contain a 36/56-kDa cross-reactive epitope as a contiguous or internal segment. This segment may be cryptic in the isolated, soluble adhesin. Note that the 87-kDa adhesin may be related to the type I fimbrial adhesin of *S. parasanguis* FW213, which reacts specifically with MAb D10 (7). As a soluble molecule in solution, the 150-kDa antigen behaved similarly to the 87-kDa antigen but showed no incremental inhibition of adhesion in the presence of the 36/56-kDa antigens. The solution conformation of the 150-kDa antigen may make the adhesin specificity ambiguous.

The 150-kDa antigen does, however, contain two adhesin epitopes. Relatively pure 150-kDa antigen was sensitive to trypsin, and tryptic fragments were resolved. While the 150-kDa adhesin reacted with MAbs 1.1 and 1.2, the 38-kDa tryptic fragment reacted with MAb 1.1 and the 54-kDa fragment reacted with MAb 1.2. The different antigenic fragments represent two unique adhesin epitopes or domains of the 150-kDa adhesin, which appear to be expressed on the cell surface. A similar two-domain structure also has been shown in protein F of *S. pyogenes* (36) and CshA and CshB of *S. gordonii* (30). These adhesins have been described as multifunctional (30) but should also be considered oligospecific. Using the 150-kDa adhesin antigen as an example, oligospecific adhesins express more than one functional domain, each with a unique structural domain or specificity.

The fact that MAbs 1.1, 1.2, and D10 and anti-P1 antibody reacted with the 205-kDa antigen strongly suggested that this antigen is also oligospecific, containing three different adhesin domains or epitopes. It has been reported (4) that *S. sanguis* M5 has an anti-P1 reactive 205-kDa receptor, SSP-5, for mucin-like salivary glycoproteins. The deduced amino acid sequences of *S. sanguis* SSP-5 and *S. mutans* P1 (PAc) show 59% identity. Similar levels of homology are also observed with the *S. sobrinus* P1-like protein, SpaA (4). The 205-kDa antigen on *S. sanguis* 133-79, therefore, may be related to SSP-5.

The chemical composition of the antigens in the mutanolysin digest from strain 133-79 is largely undefined. That similar adhesin specificities were found on macromolecules of differing sizes might suggest fragmentation of a parent macromolecule by artifactual proteolysis. The mutanolysin used in this study did not digest Azocoll or isolated 150-kDa adhesin. While protein fragmentation by unexpected autolysin activity could not be ruled out, the tryptic fragments of 38 and 54 kDa, comprising the two domains of the trypsin-sensitive 150-kDa

adhesin, were not recovered from or identified in the mutanolysin digest. Hence, endogenous trypsin-like enzymes are unlikely to have produced other smaller sized fragments isolated from the mutanolysin digest.

The adhesins within each group may represent the products of alternatively expressed genes. For example, *S. gordonii* DL1 expresses multifunctional adhesins CshA and/or CshB (30). These proteins have no significant sequence similarities with I/II or P1 family and can mediate the bacterial binding to other oral bacteria and immobilized human fibronectin. Lactose-insensitive interactions of *S. gordonii* DL1 with *Actinomyces naeslundii* T14V and WVU627, *S. oralis* C104 and 34, and with immobilized fibronectin were dependent on expression of both CshA and CshB. The smaller variants may also represent incomplete transcripts or posttranslational modifications that are expressed on the cell wall. If such adhesin size variants exist, they may reside on different cells in the culture. For example, cells within a static broth culture vary in growth phase and in proximity to neighboring cells and the wall of the vessel. Each condition may regulate the expression of adhesins. Since the adhesion assay is a bulk measure, it is unclear that each cell in a culture expresses the several adhesin specificities or all or some of the proposed size variants of a particular specificity.

It is interesting that MAbs 1.1 and 1.2 mark independent surface antigens on *S. sanguis* 133-79 that are also required for interactions with human platelets (16). Strain 133-79 is prototypic of many isolates obtained from positive blood cultures of patients with infective endocarditis. It adheres selectively through the MAb 1.1-reactive adhesin and induces human platelets to aggregate in vitro with a MAb 1.2-reactive antigen (platelet aggregation-associated protein). Since receptors for oral streptococci found in saliva and on epithelial cells are antigenically related (46), the corresponding binding sites or receptors on platelets and sHA for *S. sanguis* 133-79 are also likely to share essential structural features.

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