Identification of Monoclonal Antibody-Binding Domains within Antigen P1 of *Streptococcus mutans* and Cross-Reactivity with Related Surface Antigens of Oral Streptococci

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Eleven monoclonal antibodies (MAbs) specific for P1, the major protein surface antigen of *Streptococcus mutans* serotype c, were characterized by Western blot (immunoblot) analysis and by radiolmmunooassay using whole bacterial cells. The approximate binding domains of the MAbs were determined by using full-length and truncated P1 polypeptides. The accessibility of these binding sites on the surfaces of intact bacteria was determined by radiolmmunooassay. The ability of each MAb to cross-react with related proteins from strains of *S. mutans* serotypes e and f, *S. sanguis*, and *S. sobrinus* serotype g is also reported.

Mutans streptococci are present in large numbers in dental plaque. There is a strong correlation between the presence of these organisms in plaque and the occurrence of dental caries in humans (22, 38). A number of species make up the group of mutans streptococci, including *Streptococcus mutans* (serotypes c, e, and f), *S. sobrinus* (serotypes d and g), *S. cricetus* (serotype a), *S. rattus* (serotype b), *S. macacae* (serotype h), and *S. downei* (serotype h) (4, 8, 9, 52, 61). These species are distinguished by differences in the guanine and cytosine content of their DNA, sugar fermentation, bacitracin sensitivity, colony morphology on sucrose-containing media, and cell protein profiles obtained by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. All species of mutans streptococci except *S. rattus* express a high-molecular-weight surface protein which is believed to function as an adhesin enabling the organisms to bind to the salivary pellicle or other plaque microorganisms on tooth surfaces (16, 19, 29, 30, 34, 41, 55). This 185,000- *M* subunit protein was first identified on *S. mutans* serotype c strains and has been referred to by various investigators as P1 (3, 18, 28, 33, 34), antigen B (50, 51), P1 II (49, 62), IF (24), PAc (45, 46, 57), and MSA-1 (13). We will use the designation P1 to this article. Immunologically cross-reactive proteins have been designated antigen SR (2) (*M* = 74,000) for *S. mutans* serotype f strains, SpaA (1, 10, 20, 23) and PAg (44, 57) (*M* = 210,000) for *S. sobrinus* serotype d and g strains, and SSP-5 (11, 12) (*M* = 205,000) for *S. sanguis* strains.

A number of genes encoding the related oral streptococcal surface adhesin proteins have been cloned and sequenced. The genes include *spaP* (26, 33), *pac* (45, 46), and *MSL-1* (13) from *S. mutans* serotype c; *sr* (43, 54) from *S. mutans* serotype f; *spaA* (23, 32, 56) and *pag* (57) from *S. sobrinus* serotype g; and *SSP-5* (11, 12) from *S. sanguis*. Primary amino acid sequences predicted on the basis of nucleic acid sequences demonstrate high but variable degrees of homology among these antigenically related proteins (32). In addition to the level of homology with one another at the primary sequence level, all of the proteins demonstrate highly conserved common structural features. All possess an amino-terminal leader sequence which is removed by post-translational processing. The amino-terminal third of each of the molecules contains a series of alanine-rich tandem repeat sequences predicted to result in an alpha-helical structure typical of coiled-coil proteins. All of these proteins also have a series of proline-rich tandem repeat sequences in the central region of the molecules predicted to result in a structure exhibiting many beta turns. In addition, the proteins all possess a second proline-rich sequence at their carboxy termini which is similar to that described for other streptococcal surface proteins and is believed to span the cell wall (17). All but SSP-5 demonstrate a conserved carboxy-terminal consensus sequence, L-P-X-T-G, which was first identified for M protein and is common to a number of streptococcal surface antigens. This sequence is believed to serve as a signal for the posttranslational modification of proteins whereby they are cleaved by a thiol-dependent membrane anchor-cleaving enzyme and subsequently attached to a putative cell membrane anchor molecule (48).

There has been considerable interest in using P1 as an immunogen to protect against dental caries (25, 35, 37). In addition, several researchers have reported promising results using anti-P1 antibodies to protect against *S. mutans* colonization in passive immunization studies (36, 39, 40, 47). Characterization of the interaction of P1 with anti-P1 antibodies will be useful in determining the immunogenic regions of the protein as well as understanding the mechanism of protection observed with certain anti-P1 antibodies. The goal of this study was to characterize our panel of monoclonal antibodies (MAbs) prepared against antigen P1 (3). The approximate locations of reactivity of 11 MAbs were determined by Western blot (immunoblot) analysis using full-length and truncated P1 polypeptides. A radioimmunoassay (RIA) was used to determine the accessibility of epitopes to each of the MAbs on the surface of intact *S. mutans* serum type c bacterial cells. In addition, the ability of the MAbs to cross-react with the P1 molecule from a serotype d isolate and with antigens SR, SpaA, and SSP-5 was determined by Western blot analysis and by whole-cell RIA. The results of this study have allowed these MAbs to be used as tools for understanding the interaction between P1 and salivary components at the molecular level (7a). These MAbs will also prove useful for the analysis of functional similarities and differences among the immunologically related adhesin molecules. In addition, their cross-reactivity profile will be valuable in epidemiological studies, as it will allow certain

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anti-P1 MAb to be added to those MAb already available to differentiate between different species of mutants streptococci (14, 15).

MATERIALS AND METHODS

Bacterial strains and growth media. The streptococcal strains used in this study were S. mutans serotype e, C. britt 175, NG5, and NG8 (from K. Knox, Institute of Dental Research, Sydney, Australia), PS2K2 (from D. Malamud, University of Pennsylvania, Philadelphia), MT8148 (from S. Michalek, University of Alabama, Birmingham), and 834, a P1- isogenic mutant derived by insertional inactivation of the spaP gene in NG8 (34); S. mutans serotype e V100 (from R. McKinney, Centers for Disease Control, Atlanta, Ga.); S. mutans serotype f OMZ175; S. sanguis M5 from D. Malamud; and S. sobrinus serotype G 6715 (from R. Holt, Meharry Medical College, Nashville, Tenn.). All streptococcal isolates were grown aerobically for 16 h at 37°C in the chemically defined medium (TDM) described by Terleckyj et al. (59). Escherichia coli JM109 (International Biotechnological Institute, Netar, Conn.) and JM109 containing recombinant plasmids pSM2949 (33) and p26R3 (5) were prepared by harvesting cells from 200-ml LB medium cultures by centrifugation at 10,000 × g for 30 min at 4°C. The cells were washed once with 25 ml of 50 mM Tris buffer, pH 7.5, and resuspended in 2 ml of the same buffer. The cells were broken by sonication (Microson Ultrasonic Processor; Heat Systems-Ultrasonics, Farmingdale, N.Y.) with four 20-s blasts at 20% output power with cooling on ice in between blasts. Cellular debris was removed by centrifugation at 10,000 × g for 30 min at 4°C.

Recombinant truncated 150,000-M1, P1 was purified by resuspending E. coli harboring plasmid pSM2949 to 1/50 of the original culture volume in 10 ml Tris, pH 8.0, containing 25% sucrose. EDTA and phenylmethylsulfonyl fluoride (Sigma Chemical Co.) were each added up 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 of the original culture volume with ice-cold water containing 1 mM each EDTA and phenylmethylsulfonyl fluoride and incubated on ice for 15 min. Periplasmic contents were present in the supernatant after centrifugation at 7,000 × g for 15 min. Tris (pH 8.0) was added up to a final concentration of 10 mM, and the periplasmic preparation was filtered through a 0.2-μm-pore-size Acrodisc. The 150,000-M1 recombinant P1 was separated from other periplasmic proteins by passage over a gel filtration column of Sepharose CL-4B (Pharmacia) equilibrated with 10 mM Tris, pH 8.0. Fractions containing P1 were assayed for purity by SDS-polyacrylamide gel electrophoresis on a 7.5% acrylamide gel. Fractions containing a single 150,000-M1 band were pooled and stored at −20°C.

SDS-polyacrylamide gel electrophoresis and Western blotting. Proteins (approximately 1 to 2 mg) were separated by SDS-polyacrylamide gel electrophoresis on 7.5% acrylamide preparative gels (12 cm by 14 cm by 1.5 mm) by the method of Laemmli (31). Proteins were electroblotted onto nitrocellulose membranes for 3 h at 70 V by the method of Towbin et al. (60). To reduce nonspecific antibody binding, membranes were washed with PBS containing 0.25% gelatin and 0.25% Tween 20 (PBS-Gel-Tw), with four changes of medium, for 15 min per wash. The blots were cut into 0.5-cm strips and reacted in the individual troughs of an Incubray (Schleicher & Schuell, Keene, N.H.) on a rocking platform, overnight at ambient temperature, with 3 ml of 1:500 dilutions of mouse hybridoma ascitic fluids in PBS-Gel-Tw. The blot strips were washed four times (15 min each) with 5 ml of PBS-Gel-Tw to remove unbound antibodies and probed with 3 ml of a 1:1,000 dilution of peroxidase-conjugated goat

Precipitation of bacterial proteins from culture supernatant. Cells were removed from a 1-liter overnight TDM culture of strain NG5 by centrifugation, and the supernatant was filtered through a 0.2-μm-pore-size Nalgene Disposable Filterware unit (Nalgene Co., Rochester, N.Y.). Proteins were precipitated by the addition of ammonium sulfate (Fisher Scientific, Fair Lawn, N.J.) to 65% saturation for 48 h at 4°C. Precipitated proteins were removed by centrifugation at 15,000 × g for 5 h at 4°C, and the pellet was resuspended in 2 ml of PBS. The resuspended pellet was dialyzed for 48 h at 4°C against 4 liters of PBS with two changes of medium.

Phosphate buffer extraction of mutants streptococci. Bacterial cells from a 1-liter overnight TDM cultures of strains NG8, V100, OMZ175, 834, and 6715 were harvested by centrifugation at 10,000 × g for 30 min at 4°C, washed once with 100 ml of PBS, and resuspended in 3 ml of 0.5 M phosphate buffer, pH 6.0, as described previously (53). The cell suspensions were subjected to vigorous shaking for 1 h at 4°C by tapping the test tubes to the platform of a Vortex Genie 2 mixer (Allied Fisher Scientific, Orlando, Fla.). Cells were removed by centrifugation at 30,000 × g for 30 min at 4°C, and the supernatants were filtered through 0.2-μm-pore-size Acr disc (Gelman Sciences, Ann Arbor, Mich.). Supernatants containing phosphate-extracted proteins were exchanged into PBS with G25 gel filtration columns (PD 10; Pharmacia, Piscataway, N.J.).

Source of SSP-5 protein from S. sanguis M5. SSP-5 protein was kindly provided by D. Demuth and D. Malamud, University of Pennsylvania.

Preparation of recombinant truncated P1 polypeptides. Cellular proteins from E. coli JM109 containing pUC18 or recombinant plasmid pSM2949 (33) or p26R3 (5) were prepared by harvesting cells from 200-ml LB medium cultures by centrifugation at 10,000 × g for 30 min at 4°C. The cells were washed once with 25 ml of 50 mM Tris buffer, pH 7.5, and resuspended in 2 ml of the same buffer. The cells were broken by sonication (Microson Ultrasonic Processor; Heat Systems-Ultrasonics, Farmingdale, N.Y.) with four 20-s blasts at 20% output power with cooling on ice in between blasts. Cellular debris was removed by centrifugation at 10,000 × g for 30 min at 4°C.
anti-mouse immunoglobulin G (IgG) (Cappel, Organon Teknika Corp., West Chester, Pa.) diluted in PBS-Gel-Tw. Next, the blot strips were washed twice (15 min each time) with 5 ml of PBS-Gel-Tw and twice (15 min each time) with PBS and then developed at ambient temperature for 30 min with 2 ml of 4-chloro-1-naphthol solution (7 ml of PBS, 1 ml of 4-chloro-1-naphthol [Sigma Chemical Co.; 3 mg/ml in ice-cold methanol], 8 μl of 30% hydrogen peroxide [Fisher Scientific]). The blot strips were allowed to develop further by storage overnight at 4°C under water in the dark.

Two-stage RIA of intact bacterial cells. A two-stage RIA (7) was used to determine the reactivity of anti-P1 MAbs with P1 or P1-like molecules on the surface of intact bacterial cells. Bacteria were harvested from 25-ml TDM overnight cultures by centrifugation at 2,000 × g for 10 min, and the cells were resuspended in 5 ml of PBS (pH 7.2). These suspensions were found routinely to correspond to readings of 400 Klett units (Klett-Sumner photoelectric colorimeter; Klett Manufacturing Co., Inc., Long Island City, N.Y.). Tubes containing 100 μl of bacterial suspension were incubated for 1 h at 37°C with 100 μl of 1:500 dilutions of each anti-P1 mouse hybridoma ascitic fluid in PBS-Gel-Tw to reduce nonspecific binding. Serial titration of all ascitic fluids was performed first to ensure that the dilution used contained antibody in excess and that observed differences in ability to bind bacterial cells were not merely a reflection of differences in titer. Anti-Actinobacillus actinomycetemcomitans mouse hybridoma ascitic fluid was included in each assay as a negative control to indicate nonspecific binding of immunoglobulins. After incubation, the cells were washed once with 2 ml of PBS to remove unbound antibodies and pelleted by centrifugation at 2,000 × g for 10 min, and the supernatants were decanted. The cell pellets were resuspended in residual buffer (~100 μl) by vortexing. Bacterially bound antibody was quantitated by incubating the cell suspension for 1 h at 37°C with 100 μl of 125I-labeled sheep anti-mouse IgG (Amersham Corp., Arlington Heights, Ill.) containing approximately 30,000 cpm in PBS-Gel-Tw.

After incubation, the cells were washed twice with 2 ml of PBS-Gel-Tw containing 0.01 M EDTA to remove any label not associated with a bacterial antigen-antibody complex. The bacterium-associated radioactivity was measured in a Beckman 5500 auto-gamma counter (Beckman Instruments, Palo Alto, Calif.). The background level of radioactivity was determined with control tubes containing bacteria and 125I-sheep anti-mouse IgG only. All assays were performed in triplicate.

RESULTS

Reactivity of anti-P1 MABs with S. mutans serotype c, e, and f P1 proteins. Eleven anti-P1 MABs were tested for their reactivity by Western blot analysis against P1 and P1-like proteins isolated by phosphate buffer extraction from S. mutans NG8, V100, and OMZ175 (Fig. 1). These strains represent serotype c, e, and f isolates, respectively (Fig. 1A, B, and C, respectively). All anti-P1 MABs (lanes 1 to 11) demonstrated reactivity, to greater or lesser extents, with a major high-molecular-weight protein band with an M, of 185,000 from all three serotypes. In addition to demonstrating weak reactivity with the full-length protein from all three serotypes, anti-P1 MAB 3-8D2a (lane 1) also reacted with a number of lower-molecular-weight polypeptide bands. These are thought to represent breakdown products of the parent molecules, since P1 preparations have been reported to undergo proteolytic degradation (18, 28). Anti-P1 MAB 1-6F6e (lane 11) reacted very weakly with the 185,000-M, protein isolated from all three serotypes by this procedure. An anti-A. actinomycetemcomitans MAb used as a negative control (lane 12) was not reactive with proteins from the serotype c and e strains (Fig. 1A and B) but did cross-react with the protein isolated from the serotype f strain (Fig. 1C). The results of the Western blot reactivities of the anti-P1 MABs with P1 from serotype c, e, and f isolates of S. mutans are summarized in Table 1.

All 11 anti-P1 MABs tested retained reactivity with P1 on
Western blots which had been subjected to mild periodate oxidation, although the reactivity of a control MAb directed against a carbohydrate epitope of the high-molecular-weight salivary agglutinin glycoprotein was destroyed by this procedure (data not shown). This suggests that, unlike several periodate-sensitive MABs raised against antigen I/II from S. mutans serotype c (42), the anti-P1 MAbs in our panel recognize protein rather than carbohydrate epitopes.

**Reactivity of anti-P1 MABs with full-length and truncated P1 polypeptides.** Soluble bacterial proteins were precipitated from the culture supernatant of *S. mutans* serotype c strain NG5. This strain is a nonretainer in that P1 is primarily released from the cell surface rather than being retained as part of the outer "fuzzy coat" (3, 28). The mechanism by which retention as opposed to nonretention occurs is unclear. In addition to full-length 185,000-Mr, P1 released by NG5, a major 140,000-Mr truncated P1 polypeptide is found (5a). Knox et al. (28) have shown not only that different strains of *S. mutans* serotypes demonstrate different quantities of extracellular P1 but that at least three patterns of expression of P1 polypeptides with *M*~s~ <185,000 can be observed. Again, these are presumed to represent breakdown products of the parent P1 molecule (18, 28). The 11 anti-P1 MABs were tested for their reactivity with 185,000- and 140,000-Mr MABs and lower-molecular-weight P1 polypeptides present in NG5 culture supernatants by Western blot analysis (Fig. 2A). In general, much stronger reactivities were observed with ammonium sulfate-precipitated antigen from NG5 than with the antigen present in the phosphate buffer extract of NG8 (compare Fig. 1A and 2A). Several patterns of reactivity with the NG5 polypeptides were observed. For example, MABs 6-11A_{3a} (Fig. 2A, lane 5) and 1-6F_{2b} (lane 11) were not reactive with P1 polypeptides with *M*~s~ <140,000 in comparison with the remainder of the antibodies, and MAB 3-8D_{2a} (lane 1) demonstrated a broad reactivity profile with lower-molecular-weight polypeptides that differed from those of the other MABs. Certain MABs (lanes 7 to 10) reacted with NG5 polypeptides with *M*~s~ of 140,000-Mr and generally did not react with the 140,000-Mr polypeptide. MAB 2-8G_{1d} (lane 9) was not consistently reactive with the 140,000-Mr polypeptide. The Western blot reactivity of the anti-P1 MAbs with NG5 P1 polypeptides are summarized in Table 1. No reactivity was observed with the anti-*A. actinomycetemcomitans* negative control MAB (lane 12).

The 11 anti-P1 MABs were also tested by Western blotting for their reactivity with a truncated recombinant P1 polypeptide (Fig. 2B). A 150,000-Mr polypeptide is encoded by recombinant plasmid pSM2949 (33), which contains 4,019 of the 4,782 bp that compose the complete *spaP* gene of NG5 (26). This recombinant plasmid lacks the 3′-terminal 763 bp of the gene, and the encoded polypeptide therefore lacks the carboxy-terminal 254 amino acids of P1. A comparison of *N*-chlorosuccinimide and hydroxylamine peptide maps indicates that, like the 150,000-Mr recombinant P1, the NG5 140,000-Mr truncated P1 polypeptide lacks the carboxy terminus of the protein (5a). Except for MAB 5-3E_{5e} (Fig. 2B, lane 7), the reactivities of MABs with recombinant 150,000-Mr P1 paralleled their reactivities with NG5 140,000-Mr P1.

Three of the eleven anti-P1 MABs reacted with polypeptides isolated from a P1-deficient mutant designated 834 (Fig. 2C). This strain was derived by allelic exchange of the *spaP* gene in serotype c strain NG8 with an insertionally inactivated recombinant *spaP* gene fragment (34). Sequence analysis of the entire gene (26) subsequent to the construction of 834 indicated that the insertion interrupted the gene between bp 1937 and 3345 of *spaP*. Strain 834 would therefore be capable of expressing the amino-terminal 612 amino acids of P1 immediately prior to the insertional inactivation site. MAB 3-8D_{2a} (Fig. 2C, lane 1) reacted very strongly with polypeptides extracted from this mutant, while two other MABs, 4-10A_{5c} and 4-9D_{4c} (lanes 2 and 4, respectively), reacted weakly.

Only one of the eleven MABs, 3-8D_{2a}, reacted with the polypeptide encoded by recombinant plasmid p26R3 (Figure 3, lane 1, and Table 1). This plasmid contains *spaP* DNA encoding the amino-terminal 480 amino acids of P1 including the alanine-rich repeat region and is predicted to express a polypeptide product with an *M*~s~ of 47,600. The *spaP* DNA is ligated in frame with the β-galactosidase gene in p26R3, resulting in the expression of a fusion protein. MAB 3-8D_{2a} was also reactive with the product of recombinant plasmid pSM2949, which encodes amino acids 1 to 1307 of P1 (lane 12).
2), but not with any other proteins present in sonicates of the host *E. coli* strain harboring the pUC18 plasmid vector alone (lane 3).

**Cross-reactivity of anti-P1 MAbs with SSP-5 and SpaA proteins.** The ability of the 11 anti-P1 MAbs to cross-react with the surface adhesin molecules from *S. sanguis* M5 and *S. sobrinus* serotype g 6715 also was determined by Western blot analysis (Fig. 4). MAb 3-8D_{2a} reacted with both SSP-5 and SpaA proteins (lane 1). As had been observed with P1 and SR (Fig. 1, lanes 1), this MAb reacted more strongly with lower-molecular-weight polypeptides than with the full-length proteins. MAbs 4-10A_{ac}, 5-5D_{sa}, and 5-3E_{se} were also cross-reactive with both SSP-5 and SpaA proteins (Fig. 4, lanes 2, 3, and 7), although differences in the relative intensities of their reactivity with SSP-5 versus SpaA were seen. MAbs 3-10E_{id} and 3-3B_{sa} were cross-reactive with SSP-5 but not with SpaA (lanes 6 and 8), while MAb 6-8C_{la} was cross-reactive with SpaA but not with SSP-5 (lane 10). MAbs 4-9D_{ac}, 6-11A_{la}, 2-8G_{ld}, 1-6F_{eb}, and the anti-*A. actinomyctemcomitans* negative control did not bind to either SSP-5 or SpaA (lanes 4, 5, 9, 11, and 12). These results are summarized in Table 1.

**Reactivity of anti-P1 MAbs with *S. mutans* serotype c intact bacterial cells.** In order to determine whether the epitopes recognized by the anti-P1 MAbs are exposed on the surface of intact bacteria, a two-stage RIA was performed. The reactivities of the 11 anti-P1 MAbs were tested by RIA against four strains of *S. mutans* serotype c, NG8, Ingbritt 175, KP SK2, and MT8148 (Fig. 5). The amount of ^{125}I-radiolabeled anti-mouse IgG tracer associated with the bacterial pellets reflects the degree of binding of each MAb to the bacteria in the first stage of the assay. As with Western blot analysis, different degrees of reactivity were evident for each MAb. For example, MAb 1-6F_{eb}, which was only marginally reactive on most Western blots (Fig. 1 and 2 and Table 1), was the most reactive antibody with whole cells. The patterns of reactivity of the 11 anti-P1 MAbs were
cross-reactive antibodies and by adsorbing polyclonal rabbit anti-SpaA antiserum with S. mutans serotype c NG8 to remove anti-P1 cross-reactive antibodies (5a). The resulting monospecific anti-P1 and anti-SpaA antisera demonstrated significant residual reactivity against NG8 and 6715, respectively, by whole-cell RIA. The ability to differentiate between P1 and SpaA, as well as other members of this family of closely related adhesin molecules, with both monoclonal antibodies and polyclonal reagents will be quite useful in studies directed at analyzing common functional properties as well as differences.

**DISCUSSION**

A composite of the results of Western blotting experiments using full-length and truncated P1 polypeptides has allowed the locations of the binding sites of the anti-P1 MAbs to be estimated. A schematic diagram of the approximate locations of reactivity of the MAbs in the context of the molecular architecture of P1 is shown in Fig. 7. Only one MAb, 3-8D2a, reacted with P1 polypeptides encoded by both recombinant p26R3 (amino acids 1 to 480) and mutant strain 834 (amino acids 1 to 612) in which only the amino-terminal portion of the molecule is expressed. This suggests that the recognized epitope for 3-8D2a lies within the first 480 amino acids of the protein (following cleavage of the 38-residue leader sequence). This region includes the series of three tandem 82-residue alanine-rich tandem repeats (A region) predicted on the basis of the nucleotide sequences of spaP and pac. The A-region repeats exhibit a seven-residue periodicity (27), also demonstrated by other streptococcal surface proteins such as group A streptococcal M protein (17) and pneumococcal PspA (58), which is predicted to result in an alpha-helical coiled-coil structure. Analysis of the predicted secondary structure of P1 with the IBI MacVector software package (data not shown) indicates that the A region does not have a high probability of being exposed on the surface of the molecule, nor does it have a high antigenic index. Goldschmidt and Curtiss (20) have reported that antigenic determinants of the recombinant SpaA protein are distributed along the carboxy-terminal two-thirds of the molecule and are not encoded by the region of the spaP gene specifying the first 56 kDa of the protein. Consistent with these predictions, only one of the eleven MAbs tested, 3-8D2a, was reactive with the amino terminus of P1. This antibody demonstrated unique reactivity on Western blots in that it was at least as reactive, if not more so, with lower-molecular-weight P1 polypeptides as with full-length proteins (Fig. 1, 2, and 4, lanes 1 and 1) and its RIA reactivity with intact bacterial cells also was only moderate (Fig. 5 and 6, bars 1). Taken together, these results suggest that 3-8D2a recognizes an epitope which is partially masked on full-length native molecules. All P1-like molecules tested, including P1 from S. mutans serotypes e and f, SSP-5, and SpaA, were reactive with 3-8D2a indicating that a conserved epitope lies within the amino-terminal third of this family of proteins.

Five MAbs, 4-10A8c, 5-5D6a, 4-9D4a, 6-11A3a, and 3-10E6a, mapped to the central region of P1 (Fig. 7). Two of these MAbs, 4-10A8c and 4-9D4a, reacted with the truncated product of insertionally inactivated mutant 834 but not with that of recombinant p26R3, suggesting that they are at least partially reactive within the region containing amino acids 480 to 612. These five MAbs were all reactive with truncated P1 polypeptides lacking an intact carboxy terminus, including the 150,000-Mr, product of pSM2949 (amino acids 1 to

**FIG. 4.** Western immunoblots to test the reactivity of anti-P1 MAbs with SSP-5 and SpaA proteins. SSP-5 protein from S. sanguis M5 (A) or a phosphate buffer extract of S. sobrinus serotype g 6715 (B) were electrophoresed in parallel on SDS-7.5% polyacrylamide gels and electroblotted onto nitrocellulose membranes. The blot strips were reacted with anti-P1 MAbs 3-8D2a, 4-10A8c, 5-5D6a, 4-9D4a, 6-11A3a, 3-10E6a, 5-5D6a, 3-8D2a, and 1-6F6a (lanes 1 to 11, respectively). The negative control anti-A. actinomy cetecomitans MAb is shown in lane 12. Positions of molecular mass standards, in kilodaltons, are indicated.

Remarkably similar for the four S. mutans serotype c strains tested. No binding of the negative control anti-A. actinomy cetecomitans MAb to intact cells was detected (data not shown). All of the anti-P1 MAbs are in the IgG1 subclass, except 3-3B5e, which is in the IgG2a subclass. Radiolabeling of sheep anti-mouse IgG with 125I did not interfere with its ability to bind to any of the MAbs used in this study when tested by a dot blot assay (data not shown).

**Reactivity of anti-P1 MAbs with S. mutans serotypes e and f, S. sanguis, and S. sobrinus serotype g intact bacterial cells.** In addition to determination of the cross-reactivities of the anti-P1 MAbs by Western blot analysis, intact cells of strains V100 (S. mutans serotype e), OMZ175 (S. mutans serotype f), M5 (S. sanguis), and 6715 (S. sobrinus serotype g) were tested by whole-cell RIA (Fig. 6). The patterns of reactivity with the S. mutans serotype e (Fig. 6A) and serotype f (Fig. 6B) strains were nearly identical to that seen with the serotype c strains (compare Fig. 6 with Fig. 5). The reactivity profiles of anti-P1 MAbs with whole cells of S. sanguis M5 (Fig. 6C) and S. sobrinus 6715 (Fig. 6D) were similar, but not identical to, their reactivity profiles with SSP-5 and SpaA proteins on Western blots (compare Fig. 6 with Fig. 4 and Table 1). In general, as was seen on Western blots, the level of cross-reactivity of anti-P1 MAbs appeared to be lower with SpaA from S. sobrinus 6715 than with SSP-5 from S. sanguis M5. Differences in Western blot and RIA cross-reactivity profiles of the anti-P1 MAbs against SSP-5 and SpaA most likely reflect differences between the accessibility of epitopes on denatured proteins and their accessibility on native proteins on whole cells.

Further evidence for immunological variation between P1 and SpaA was obtained by adsorbing polyclonal rabbit anti-P1 antiserum with S. sobrinus 6715 to remove anti-SpaA...
1307) and the 140,000-M₉ breakdown product present in the culture supernatant of nonretainer strain NG5. The approximate binding sites for these MAb, except 4-10A₈c and 4-9D₄c, can therefore be localized between amino acid 612 and that residue calculated to occur approximately 10 kDa amino terminal to residue 1307, i.e., amino acid 1218. MAb 6-11A₃a was the only one of these five antibodies which was not very reactive with NG5 P1 polypeptides with M₉ of <140,000 by Western blot analysis, suggesting that it recognizes a more conformationally dependent epitope. Among these five MAbs, 4-10A₈c and 5-5D₆a were reactive with all P1-like molecules tested, suggesting that two more conserved epitopes lie within amino acids 480 to 1218 of P1. These boundaries span the series of 39-residue proline-rich tandem repeats (amino acids 840 to 963 of P1) identified for all surface adhesion molecules of mutants streptococci analyzed to date (12, 13, 26, 32, 43, 46, 57) as well as the variable region (amino acids 679 to 823 of P1) identified by restriction fragment length polymorphism analysis of S. mutans serotype c isolates (6). Secondary structure analysis indicates that, in general, the central region exhibits a higher antigenic index than does the region consisting of first 480 residues of P1 and that the P region in particular has a high surface probability score. Indeed, all of these five MAbs demonstrated significant reactivity with intact S. mutans cells when tested by RIA, indicating that their binding sites are well exposed when P1 is in its native configuration on the cell surface (Fig. 5 and 6, bars 2 to 6). The ability of certain MAbs to react to a greater extent than others with P1 in its native configuration on the surface of intact cells may reflect the accessibility of epitopes as a result of the conformation of the P1 protein itself. Alternatively, some epitopes may be buried or masked by adjacent surface structures other than P1.

Four MAbs, 5-3E₅c, 3-3B₅c, 2-8G₁₄d, and 6-8C₁₄a mapped to the carboxy terminus of P1 (Fig. 7). These four MAbs demonstrated the common property of decreased whole-cell reactivity when tested by RIA (Fig. 5, bars 7 to 10). This result is not surprising. Like the carboxy termini of a number of streptococcal surface proteins, the carboxy terminus of P1 is believed to be involved in anchoring the protein to the cell surface; therefore, it would be expected to be less accessible to the external environment than other regions of the molecule. Some subtle variation of reactivity of three of these antibodies with the four S. mutans serotype c isolates tested by RIA was observed (Fig. 5, bars 7, 8, and 10). The reactivities of these MAbs, especially 3-3B₅c, demonstrated some day-to-day variability. The reason for this variability is
unclear. Differences in binding of 3-3B_{5a}, 5-3E_{5a}, and 6-8C_{1a} do not correlate with restriction fragment length polymorphisms observed among \textit{S. mutans} serotype \textit{c} strains (6). That is, the reactivity of any given MAb is not favored by \textit{spaP}-like strains (Ingbrit 175 and KPSK2) or \textit{pac}-like strains (NG8 and MT8148). MAb 5-3E_{5a} consistently reacted with the 150,000-\textit{M}, recombinant product of pSM2949 but not with the 140,000-\textit{M}, NG5 P1 breakdown product, sug-

FIG. 6. Two-stage RIA to test the reactivity of anti-P1 MAbs with \textit{S. mutans} serotypes \textit{e} and \textit{f}, \textit{S. sanguis}, and \textit{S. sobrinus} serotype \textit{g} intact cells. Bacterial suspensions of \textit{S. mutans} serotype \textit{e} V100 (A), \textit{S. mutans} serotype f OMZ175 (B), \textit{S. sanguis} M5 (C), and \textit{S. sobrinus} serotype \textit{g} 6715 (D) were reacted with anti-P1 MAbs 3-8D_{2a}, 4-10A_{6c}, 5-5D_{6a}, 4-9D_{6c}, 6-11A_{3a}, 3-10E_{6a}, 5-3E_{5a}, 3-3B_{5a}, 2-8G_{1a}, 6-8C_{1a}, and 1-6F_{5b} (bars 1 to 11, respectively) and probed with ^{125}\text{I}-radiolabeled sheep anti-mouse IgG. All assays were performed in triplicate.

FIG. 7. Schematic representation of the P1 molecule from \textit{S. mutans} serotype \textit{c} strain NG5. Numbers refer to the amino acid positions deduced from the nucleotide sequence of the cloned \textit{spaP} gene (26). 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 \textit{spaP} (6). The bars below the map indicate the approximate binding domains of the panel of 11 anti-P1 monoclonal antibodies, deduced from Western blot experiments. The order of antibodies within each segment is arbitrary and does not reflect their location of binding on the P1 molecule.
gesting that its binding site lies within the region comprising amino acid residues 1218 to 1307. This MAb also was cross-reactive with proteins from serotype e and f strains as well as with SSP-5 and SpaA. Thus, there is yet another conserved epitope shared among these molecules. MAbs 5-3Ese, 3-3Bse, 2-8G1d, and 6-8lc1a may react directly with the carboxy terminus of P1 or may be dependent on its presence for formation of their binding sites. These two possibilities can be tested directly once the construction of spaP subclones which express carboxy-terminal polypeptides has been completed. Reactivity of these MAbs with common ~40,000- and ~50,000-Mr NG5 P1 polypeptides, presumably including the carboxy terminus, is a preliminary argument against the second possibility. Some degree of binding of MAb 2-8G1d with 140,000-Mr P1 on the Western blot shown in Fig. 2A (lane 9) was observed, although this was not a consistent result. This sporadic reactivity may reflect cross-reactivity within other regions of the intact molecule or may be an indication of different levels of renaturation of carboxy-terminally truncated P1 on Western blots performed on different days.

Lastly, 1-6F6b is different from the other 10 anti-P1 MAbs tested in that it is more reactive with S. mutans intact cells by RIA than would be predicted on the basis of its weaker Western blot reactivity. This MAb reacts with truncated polypeptides lacking a carboxy terminus and was nonreactive with amino-terminally truncated molecules, suggesting that it also binds within the region comprising amino acids 612 to 1218 of P1 (Fig. 7). Like MAb 6-11A3a, 1-6F6b is not reactive with NG5 P1 polypeptides with Ms<140,000 (Fig. 2A, lanes 5 and 11). It may be more dependent on the native conformation of P1 than are other MAbs, either because its epitope is discontinuous rather than sequential or as a result of differential exposure of the epitope on native and denatured molecules. A major immunodeterminant of SpaA from S. sobrinus serotype g recently has been shown to be encoded by discontinuous segments of DNA (20, 21). Alternatively, MAb 1-6F6b may recognize an epitope comprising P1 in conjunction with another surface molecule. 1-6F6b was the only one of the eleven anti-P1 MAbs generated by using intact bacterial cells as the immunogen. All others were generated by using mutanolysin-digested cell walls as the immunogen (3).

All 11 anti-P1 MAbs tested demonstrated Western blot and RIA reactivity profiles that were virtually indistinguishable among S. mutans serotype c, e, and f strains. Only the anti-A. actinomycetemcomitans negative control MAb differentiated between these proteins in that it cross-reacted with the product of OMZ175 (serotype f). The predicted amino acid sequences of the P1 and PAc (serotype c) and SR (serotype f) proteins are 88% homologous (43). Although the gene encoding the P1 molecule from a serotype e strain has not been cloned and sequenced, one would predict a high degree of amino acid homology with both the serotype c and f proteins on the basis of MAb reactivity. The reason why we found the product of the serotype f strain OMZ175 to have an Mr of 185,000 rather than 74,000 as described by other researchers (2) is unknown. Perhaps a polymorphism between the strains used in the two laboratories exists. The 74,000-Mr SR molecule is believed to result from posttranslational processing of a larger molecule, as the recombinant product of the cloned sr gene is reported to have an Mr of 195,000 (43, 54).

Significant homology at the amino acid level between P1 (MSL-1 and PAc), SSP-5, and SpaA (PAg) also has been reported (12, 13, 32, 56). Because SSP-5 and SpaA show some differences in their anti-P1 cross-reactivities, careful scrutiny of alignments of predicted amino acid sequences of these proteins with anti-P1 MAbs can be accomplished. This will be feasible once the approximate binding sites of the MAbs are determined. Construction of subclones of spaP which express defined truncated polypeptides and deletion mutagenesis approaches are under way in our laboratory and will allow those types of experiments to be done.

In addition to providing useful tools for analysis of immunological similarities and differences among the surface adhesin molecules of mutans streptococci, characterization of these MAbs is very important from the standpoint of being able to use them to answer questions regarding the structure and function of P1. To this end, experiments in our laboratory have been performed to analyze the ability of these MAbs to inhibit adherence of intact bacteria to salivary agglutinin-coated hydroxyapatite beads and to inhibit agglutinin-mediated aggregation of bacteria (7a). Comparison of the results of all experiments performed to date with this panel of anti-P1 MAbs indicates that they can all be differentiated from one another. Therefore, 11 MAbs demonstrating unique specificities are available to further our understanding of P1 and related molecules at the molecular level.

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