Salivary Film Expresses a Complex, Macromolecular Binding Site for Streptococcus sanguis*

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Teeth in the oral cavity are coated with a salivary film or pellicle, which lacks apparent intermolecular organization. This heterogeneous film facilitates binding of early commensal colonizing bacteria, including Streptococcus sanguis. To test the hypothesis that sufficient intermolecular organization exists in salivary films to form binding sites for S. sanguis, an in vitro model of salivary-coated teeth was probed with murine anti-idiotypic monoclonal antibodies (mAb2, anti-ids). The anti-ids were harvested from hybridomas that were developed in response to first generation murine hybridomas that produced anti-S. sanguis adhesion monoclonal antibodies (mAb1). The anti-ids (i) reacted with experimental salivary films and inhibited S. sanguis adhesion in a dose-dependent fashion. In Western blots, the anti-ids (ii) recognized a high molecular weight salivary antigen and (iii) secretory IgA (sIgA) light chain and α-amylase. After isolation by gel filtration from whole saliva or mixed secretory IgA and α-amylase, the high molecular weight component, containing amylase activity and sIgA, bound to hydroxyapatite to promote adhesion of S. sanguis. Therefore, a complex enriched in secretory immunoglobulin A and α-amylase forms a S. sanguis-binding site.

Naturally occurring biofilms generally develop when a conditioning film adsorbs to a surface, promoting the selective adherence of microbes from the surrounding environment (1). Yet the mechanisms by which heterogeneous conditioning films promote selective microbial adhesion are ill defined (2). As the biofilm matures, certain of the attached microbes will colonize the surface. Depending in part on the specificity for adherence to the conditioning film, microbial biofilms may be beneficial (e.g. the gastrointestinal commensal flora) or harmful (e.g. infected catheters and medical implants and dental plaque).

Dental plaque is an example of a complex microbial biofilm; Streptococcus sanguis are among the first or "pioneer" bacteria to adhere selectively and colonize the salivary-coated teeth (3). Representing a small proportion (~1%) of more than 300 species of bacteria in the oral cavity (4), S. sanguis first adheres to a conditioning film of heterogeneous salivary proteins and glycoproteins. The film includes α-amylase and secretory IgA (sIgA) (5, 6) and forms largely by adsorption from the surrounding salivary milieu in which it remains. Like most naturally occurring conditioning films, the salivary film on enamel (~98% hydroxyapatite, HA) has ill defined macromolecular organization and surface boundaries. Salivary macromolecules also change conformation upon adsorption to HA in in vitro models; interactions of the bacteria with salivary components in solution are not representative of adhesion to a salivary film (7–9). Actinomyces viscosus and certain other prominent dental plaque bacteria adhere to conformationally sensitive domains on purified salivary proline-rich proteins adsorbed to HA but not in solution (9), and Staphylococcus aureus and Pseudomonas aeruginosa bind to a heterotypic complex of low molecular weight mucin and sIgA in solution but not in solid phase (10). In solution, sIgA may also complex with other salivary macromolecules to form binding receptors of different specificity for bacterial adhesins (11). When adsorbed on hydroxyapatite (saliva-coated hydroxyapatite (sHA)), kinetic data (7, 8, 12, 13) suggest that the salivary film promotes adhesion of S. sanguis by specific (14–16) and nonspecific (17) mechanisms. Because the salivary film lacks apparent intermolecular organization, we tested the hypothesis that conditioning film macromolecules form structures that serve as binding sites for S. sanguis.

To overcome the paucity of structural information about the salivary film, we developed a strategy to predict the molecular determinants that serve as binding sites for streptococci on sHA. First, murine anti-S. sanguis 133–79 adhesion monoclonal antibodies (mAb1s) were developed and characterized in a sHA adhesion assay (18, 19). S. sanguis 133–79 was selected to model adhesion, because it binds 3.6 times more effectively to sHA than HA. Anti-S. sanguis 133–79 mAb1.1 and mAb1.2 Fab fragments (or intact mAbs) each showed dose-dependent partial inhibition of adhesion. Together, these two mAbs inhibited adhesion of S. sanguis 133–79 to sHA by a maximum of 63%. Although additional adhesins are expressed, the mAb1s recognized 87- and 150-kDa antigens (19). The 150-kDa adhesin contained two different adhesive epitopes, each reacting with either mAb1.1 or -1.2.

To immunochemically simulate the specificity of the two respective adhesion epitopes, murine monoclonal anti-idiotypic antibodies (mAb2s; anti-ids) were developed using the mAb1.1 and mAb1.2 as antigens (18). The antigen-combining site of anti-idiotypic antibodies can express a surface substructure or internal image that is a molecular mimic of the original immunizing antigen (20, 21). Reacting with the probes, a complex containing secretory immunoglobulin A and α-amylase was identified as a S. sanguis-binding site.

EXPERIMENTAL PROCEDURES

S. sanguis Strain and S. sanguis-sHA Adhesion Assay—Strain 133–79 was stored and grown as described previously (19, 24). The in vitro adherence assay is a modification of that used by Liljemark et al. (22) and Tellefson and Germaine (23). Briefly, the assay was performed in 1.5-ml polypropylene microcentrifuge tubes with 1 ml KH2PO4, K2HPO4 buffer, pH 6.8, with 50 mM KCl, 1 mM CaCl2, 0.1 mM MgCl2 (Gibbons' buffer) at 20 °C (ambient temperature). Human whole saliva

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‡ The abbreviations used are: sIgA, secretory IgA; HA, hydroxyapatite; sHA, saliva-coated hydroxyapatite; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.
was collected from five adult volunteer donors (procedure reviewed and approved by the Committee on the Use of Human Subjects in Research of the University of Minnesota) into a chilled tube on ice, pooled, and clarified by centrifugation (1,972 × g for 20 min at 4 °C). Whole saliva supernatant (1 ml) was incubated for 60 min to coat 10 mg of HA that had been immobilized onto 2 bed volumes of 0.5 M methyl-2-H-thymidine to a specific activity of 1.6 × 10^7 cpm/280 bacteria/ml (mean of five experiments, three determinations of each, ± S.E.). Radiolabeled cells (10^8) were added and mixed with sHA for 60 min by continuous inversion on a roto-torque at ambient temperature. Unattached bacteria were removed, and cells loosely associated with the sHA were removed by washing. The radioactivity associated with sHA was monitored by liquid scintillation counting.

Preparation of mAbs—mAbs against S. sanguis adhesins (mAb1.1 and mAb1.2) were prepared as previously reported (18, 19). In brief, BALB/c mice were immunized with live cells of the adhesion positive strain 133–79. Hybridomas were screened for reaction with an adhesion positive strain (133–79) but not with an adhesion negative strain (ATCC 10556) and for the ability to react with strain 133–79 to inhibit adhesion to sHA. These selected mAb1 hybridomas were then injected intraperitoneally into BALB/c mice to produce anti-idiotypic mAb2s. The enlarged spleens were harvested, and mAb2 hybridomas were prepared and screened in indirect enzyme-linked immunosorbent assay for reaction with rabbit polyclonal IgG antibodies against the 87- and 150-kDa adhesin antigens. mAb2s from positive clones were then incubated with sHA and tested for inhibition of adhesion of strain 133–79.

Identification of Salivary Film Antigens That Bind S. sanguis—To learn if mAb2s bind to specific adhesion receptors on salivary film, sHA was blocked with 5% bovine serum albumin, pretreated with increasing amounts of mAb2a, and then incubated for 1 h with S. sanguis in the adhesion assay (19, 24). To identify components in the salivary film that may have formed immunologically unique adhesion receptors for cells of S. sanguis, freshly collected and clarified whole saliva was separated by SDS-PAGE in reduced or nonreduced conditions and reacted with the mAb2s in Western immunoblots (25, 26). In each experiment, nonspecific mouse IgG was used as the negative control. Based on their estimated sizes, isozymes of α-amylase (reduced 56-kDa and unreduced 56- and 58-kDa bands) and immunoglobulin light chain (reduced 25-kDa antigens) were suggested to react with mAb2a. To verify this, the whole saliva was then allowed to react with rabbit anti-α-amylase, anti-IgA (α-chain), or anti-γ light chain in identical conditions or nonspecific rabbit IgG as a negative control. Purified sIgA (colostrum) and purified α-amylase were also allowed to react with mAb2a.

To test the possibility that sIgA (or its light chain) and α-amylase form a complex with unusual electrophoretic mobility, human colostral sIgA and α-amylase were mixed (1:16 ratio) for 30 min at 37 °C. The mixture was then separated by SDS-PAGE and allowed to react with mAb2a.

Collection and Fractionation of Saliva—Whole human saliva from at least five healthy donors was collected into chilled tubes for each experiment. EDTA was added to a final concentration of 0.01%. Pooled human whole saliva was clarified by centrifugation and then dialyzed overnight at 4 °C against 0.1 M NH₄HCO₃ buffer, pH 8.0, with 0.05% 2-ME. To inhibit adhesion of S. sanguis 133–79, 10 pmol each of mAb2.1 and -2.2 were mixed and then incubated with the experimental films for 1 h, and then cells of S. sanguis were added. Unrelated murine IgG1 mAbs and products of the IgG1 myeloma clones MOPC-21 and S1–68.1 (unknown specificities) were used as negative controls for the IgG1 mAb2s.

RESULTS

The anti-idiotypic antibody mAb2.1 (66.7 pmol) bound to sHA, inhibited adhesion of S. sanguis by a maximum of 69%, and showed an ID₅₀ (inhibitory dose, 50%) of 5 pmol/ml (Fig. 1). When its idiotype counterpart mAb1.1 was preincubated with sHA in the same conditions, adhesion of strain 133–79 was not significantly affected. In identical conditions, mAb2.2 inhibited adhesion maximally by 35%. Together, these two anti-idiotypic antibodies (20 pmol each) inhibited adhesion by 86%.

As mimics of the adhesin macromolecules, mAb2.1 and mAb2.2 recognized salivary antigens of 300 and 365 kDa (estimated Mr; unreduced) in Western immunoblot (Fig. 2, lanes 2 and 3, respectively). Nonspecific mouse IgG reacted only with macromolecules of 180 and 120 kDa as indicated by the asterisks. mAb2.2 also reacted weakly with bands of 56 and 58 kDa (lane 3). After reduction of disulfide bonds with 2-mercaptoethanol (2-ME + ), the mAb2a reacted only with salivary antigens of 25 and 58 kDa, and nonspecific mouse IgG did not appear to react. The unreduced 300-kDa band also reacted with rabbit anti-immunoglobulin k-chain (Fig. 2B, lane 1; 25 kDa after reduction), anti-immunoglobulin light chain (for ≈ 60 kDa after reduction), and anti-α-amylase (lane 2; 58 kDa after reduction). Nonspecific rabbit IgG reacted only with an unreduced macromolecule of 180 kDa (asterisk). The specific salivary antigens probably complex with other proteins because unreduced antigens of other sizes were also detected. mAb2.2 also reacted with purified sIgA (colostrum) and light chain before and after reduction, respectively, and (in identical conditions) purified α-amylase.
α-amylase (Fig. 2C). mAb2.1 reacted more weakly with amylase and light chain (data not shown). To test the possibility that the 300-kDa salivary macromolecule may be a complex with unusual electrophoretic mobility, human colostral sIgA and α-amylase were mixed (1:16 ratio) for 30 min at 37 °C. Both mAb2s reacted with the mixture of α-amylase and sIgA, including a fraction that migrated at 300 kDa, and reduced light chain and α-amylase (Fig. 2D). Nonspecific mouse IgG reacted weakly only with an unreduced macromolecule of 180 kDa (asterisk).

The mAb2-reactive component was then isolated from whole human saliva by gel filtration chromatography. A representative chromatogram from fractionation of a 12-ml sample of whole saliva shows the four peaks that typically resolved (Fig. 3). Serial dilutions of whole saliva or the four pooled fractions (P1–P4) were used to coat HA beads. A film formed by a 40% dilution of whole saliva, or pooled fraction 2 (P2) or 4 (P4), supported adhesion of S. sanguis maximally. All dilutions of P1 and P3 weakly promoted adhesion of S. sanguis (data not shown). Among 40% dilutions of the pooled fractions, P2 formed films with the greatest adhesion promotion activity per μg of soluble protein (Fig. 3). Fresh saliva and fractions were necessary for adhesion. After denaturation or dialysis and lyophilization, salivary fractions P1–P4 promoted similar low levels of adhesion, and no dilution effect was seen (data not shown).

When visualized by SDS-PAGE, fraction P1 contained trace amounts of the putative 300-kDa component, P2 contained the 300-kDa component, and P3 contained trace amounts of a 56-kDa protein, whereas P4 was enriched in a 56-kDa protein (Fig. 4). The 300-kDa component in P2 reacted in Western blots with mAb2s, rabbit anti-human κ-chain, Igα-chain, and α-amylase (data not shown). Most amylase activity (2.75 units) eluted in P4 as expected (Table I). By comparison, P2 contained 0.045 unit of amylase activity, whereas P1 contained 0.001 unit and P3 contained no detectable activity.

After colostral sIgA and α-amylase were mixed and chromatographed, four fractions resolved (chromatogram not shown), each with an Rf similar to eluted whole saliva. As noted in the fraction of whole saliva, P2 isolated from the
mixture of sIgA and α-amylase also contained amylase activity (Table II). Separate experimental HA films formed from this P1 (sIgA) and P4 (amylase) promoted adhesion by only 7 and 5%, respectively. In contrast, when coated on HA, P2 from the sIgA and amylase mixture promoted S. sanguis adhesion by about 16%. Furthermore, adhesion of strain 133–79 to the HA film formed from P2 isolated from the mixture was inhibited in a dose-response manner by mAb2.1 (Fig. 5). The mAb2s also showed dose-dependent inhibition of binding of strain 133–79 to P2 from saliva (data not shown). In contrast, adhesion of S. sanguis to buffer-treated or P3-coated HA was unaffected by mAb2.1 (Table II).

**DISCUSSION**

The data from this study and previous reports suggest strongly that specific interactions between cells of S. sanguis and sIgA predominate in this model of adhesion (24). As mimics of S. sanguis adhesins, the mAb2s react with specific epitope(s) to define an apparent binding receptor for S. sanguis formed on experimental salivary films. The mAb2.1 and mAb2.2 express...
similar but non-identical specificities (18). They appear to act independently to bind different epitopes on sHA for S. sanguis adhesins, inhibit adhesion additively, and differ in their ID₅₀ values for adhesion of cells of S. sanguis, but they showed similar patterns of reaction on Western blots with salivary macromolecules.

Identified by mAb2s, the sHA binding receptor for S. sanguis is a heterotypic complex of the light chain of sIgA and α-amylase. Macromenzyme complexes of α-amylase and IgA or IgG form in serum and can be elevated in autoimmune and liver diseases (29). We now show that complexes of sIgA-light chain and amylase form in saliva or are reconstituted from purified individual proteins. Whether formed by purified macromolecules or isolated from saliva, the sIgA-amylase complexes are surrogates for a binding receptor expressed in the film formed on HA by whole saliva. The mAb2s do not bind the complex in saliva. A mAb2.2 affinity column failed to bind antigens from whole saliva (data not shown). The conformational dependence of the binding complex may enable S. sanguis to bind to salivary films formed on high energy surfaces such as HA or nitrocellulose but avoid anti-adhesion interactions with these proteins in the salivary fluid.

It is now clear that there is ample immunochemically defined structure in experimentally produced salivary films to serve as specific adhesion receptors. In the heterogeneous salivary biofilm, it would also be expected that interactions be promoted between multiple salivary proteins. The complexes that form may provide binding receptors of different specificities for the pioneer streptococci and other early colonizers. The structural characteristics of these binding receptors need to be further studied. To define space-fitting models of the receptors, we speculate that predictions could be made from structural characterization of the idiotopes of the mAb1s and new mAb3s, now in development. If proteinaceous complexes serve as a general adhesion mechanism for adhesion to conditioning films, binding sites and biofilm development may be modified for prevention and therapy by the development of novel antibiotic and biomimetic compounds.

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