Separate Oligosaccharide Determinants Mediate Interactions of the Low-Molecular-Weight Salivary Mucin with Neutrophils and Bacteria†

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ABSTRACT: The low-molecular-weight human salivary mucin (MG2) coats oral surfaces, where it is in a prime location for governing cell adhesion. Since oligosaccharides form many of the interactive facets on mucin molecules, we examined MG2 glycosylation as it relates to the molecule's adhesive functions. Our previous study of MG2 oligosaccharide structures showed that the termini predominantly carry T, sialyl-T, Lewis^x (Le^x), sialyl Le^x (sLe^x), lactosamine, and sialyl lactosamine determinants [Prakobphol, A., et al. (1998) *Biochemistry 37*, 4916−4927]. In addition, we showed that sLe^x determinants confer L-selectin ligand activity to this molecule. Here we studied adhesive interactions between MG2 and cells that traffic in the oral cavity: neutrophils and bacteria. Under flow conditions, neutrophils tethered to MG2-coated surfaces at forces between 1.25 and 2 dyn/cm^2, i.e., comparable to the shear stress generated at the tooth surface by salivary flow (∼0.8 dyn/cm^2). MG2 was also found in association with neutrophils isolated from the oral cavity, evidence that the cells interact with this mucin in vivo. Since MG2 serves as an adhesion receptor for bacteria, the MG2 saccharides that serve this function were also identified. Seven of 18 oral bacteria strains that were tested adhered to MG2. Importantly, six of these seven strains adhered via T antigen, sialyl-T antigen, and/or lactosamine sequences. No adherence to Le^x and sLe^x epitopes was detected in all the strains that were tested. Together, these results suggest that distinct subsets of MG2 saccharides function as ligands for neutrophil L-selectin and receptors for bacterial adhesion, a finding with interesting implications for both oral health and mucin function.

Human saliva has several critical functions, including lubrication (*1*−*3*), digestion (*4*), formation of a bioactive semipermeable barrier (i.e., pellicle; *5*−*7*) that coats oral surfaces, and regulation of the oral flora. Saliva fulfills the latter function by virtue of its antimicrobial activity (*8*, *9*) and by promoting selective microbial clearance or adherence (*10*−*13*). The diverse functions attributed to saliva are allocated among its many components, which include amylases, cystatins, proline-rich proteins, proline-rich glycoproteins (PRGs), carbonic anhydrases, peroxidases, statherins, histatins, lactoferrin, lysozyme, sIgA, and mucins.

To date, at least two human salivary mucin populations have been identified: the high-molecular-weight mucin encoded by *MUC5B* (MG1,[1] M_r > 1 000 000) (*14*−*16*) and the low-molecular-weight mucin encoded by *MUC7* (MG2, M_r = 120 000) (*17*). Studies of the purified mucins indicate that they are structurally distinct and heavily glycosylated. MG1, which consists of large, disulfide-linked peptide subunits, contains 15% protein and 78% carbohydrate (*18*, *19*), whereas MG2, which exists as a much smaller, single-peptide chain, contains 30% protein and 68% carbohydrate (*18*−*20*). Because of their high level of glycosylation, it is likely that the peptide cores of both molecules are encased in a shell of saccharide residues.

The importance of carbohydrate-mediated interactions to cell adhesion is recognized in many normal and pathological processes (*21*−*23*). With regard to the latter, a variety of organisms have lectins that interact with carbohydrate receptors presented by host glycoproteins and glycolipids. For example, P-fimbriated *Escherichia coli*, which is implicated in human urinary tract infections, adheres via Galα1→4Gal sequences carried by glycolipids in vitro (*24*). In vivo, the wild-type strain, but not a strain that lacks the

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[1] Abbreviations: MG1, high-molecular weight salivary mucin; MG2, low-molecular weight salivary mucin; PRG, proline-rich glycoprotein; SMSL, submandibular-sublingual; Le^x, Lewis^x; sLe^x, sialyl Lewis^x; ECL, enhanced chemiluminescence; HBSS, Hank's Balanced Salt Solution; HBSS/BSA, Hank's Balanced Salt Solution with 0.2% bovine serum albumin; SD, standard deviation.

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relevant adhesin (PapG), causes pyelonephritis in cynomolgus monkeys (*25*). Very recently, interactions between *E. coli* and host cell carbohydrate receptors were shown to transmit signals into both host and bacterial cells, a result with interesting implications for pathogenesis (*26*). *Helicobacter pylori*, a bacterium associated with gastritis, ulcers, and stomach cancer, possesses a specific adhesin, BabA, which recognizes the Lewis^b (Le^b) antigen carbohydrate sequence (*27*). This carbohydrate structure is expressed by only a subset of humans and other primates who carry the appropriate fucosyltransferase gene, suggesting an explanation for why only certain individuals are affected. Oral bacteria can also adhere via carbohydrate receptors presented by salivary glycoproteins. For example, we showed that *Fusobacterium nucleatum*, a bacterium associated with periodontal disease, adheres to the highly glycosylated PRG via its N-linked saccharides that present lactosamine sequences in the context of the Le^y determinant (*10*). In contrast, *Streptococcus sanguis* strains adhere to the low-molecular weight mucin via a sialic acid-containing receptor (*11*).

Another interesting function discovered for carbohydrates is their role in governing lymphocyte trafficking, the process whereby vascular endothelia, extracellular matrixes, and resident tissue present "traffic signals" that regulate lymphocyte movement (reviewed in refs *28*−*32*). These adhesive interactions occur in a stepwise manner and involve the sequential actions of several molecular families. Initially, lymphocytes extracted from the circulation begin rolling on endothelium, a Ca^{2+}-dependent process that is mediated by molecules with C-type lectin domains (e.g., selectins). To date, three selectins are known. E-Selectin is expressed on endothelial cell surfaces, whereas P-selectin is expressed on the surfaces of activated platelets and endothelial cells. Both E- and P-selectins mediate adhesion of neutrophils, monocytes, and certain subsets of lymphocytes. L-Selectin is expressed on leukocyte cell surfaces and mediates the adherence of these cells to various postcapillary venules, including high endothelial venules in secondary lymphoid organs. The biological importance of the selectin−ligand interactions with regard to systemic infections is highlighted by studies of individuals with genetic defects that preclude appropriate glycosylation of selectin ligands. For example, patients with leukocyte adhesion deficiency syndrome II (LAD II) have greatly reduced levels of sialyl Lewis^x (sLe^x) expression and recurrent bacterial infections (*33*). In accord with these data, genetic ablation of α1→3 fucosyltransferase expression in mice eliminates E- and P-selectin activity and significantly impairs L-selectin-mediated processes (*34*).

Dental plaque formation is a unique infection process that can lead to diseases of the teeth (dental caries) and the tissues that support them (gingivitis and subsequently periodontitis). Although these pathological processes are the end result of a complex interplay between microbial and host factors, the critical first step is adhesion of oral bacteria to the pellicle of salivary components that is adsorbed to the tooth surface. These components include MG1 and MG2, which are the primary sources of host-derived oligosaccharides that function as adhesion receptors (*6*, *35*). Once a tooth surface is colonized, bacteria adhere to one another by another carbohydrate-dependent process, termed co-adherence/aggregation (*36*−*38*).

Previously, we characterized the oligosaccharides carried by MG2 obtained from a single donor. The nuclear magnetic resonance and mass spectrometric data showed that, of the 41 different structures we detected, the major species were (i) the T antigen (Galβ1→3GalNAc), (ii) the sialyl-T antigen (NeuAcα2→3Galβ1→3GalNAc), (iii) a type 2 core pentasaccharide that also carried this motif, (iv) a type 2 core hexasaccharide with a Le^x determinant [Galβ1→4(Fucα1→3)-GlcNAc], and (v) a type 2 core hexasaccharide with a sLe^x determinant (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc) (*39*). Di-, tri-, and pentasaccharides with one sulfate group were also detected. Further investigation showed that the oligosaccharide termini carried by MG2 can vary among individuals. For example, we used an antibody that specifically reacted with sLe^x to show that MG2 from five of our 67 saliva donors failed to express this determinant (ref *39* and unpublished data).

Here we studied the role of MG2 and its oligosaccharides in cell adhesion. Since sLe^x and related sulfated species are a feature of several selectin ligands, we hypothesized that neutrophils that emerge into the oral cavity could interact with MG2, either immobilized to a substrate or in solution. Since MG2 also serves as a receptor for bacterial adhesion (*11*), we wanted to know whether the same or a different subset of the MG2 saccharides serves this function. We were also interested in the force of these adhesive interactions, which is one way to estimate their ability to resist distractive forces due to salivary flow, and thus to judge their biological importance. Together, our data indicate that MG2 adhesive functions are apportioned among its oligosaccharides. As a result, this molecule potentially serves a dual role by coordinating both neutrophil and bacterial adhesion.

EXPERIMENTAL PROCEDURES

Materials. All chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose membranes (0.45 μm) were obtained from Schleicher & Schuell (Keene, NH). [^{35}S]Methionine (1159 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Enhanced chemiluminescence (ECL) detection reagents, Hyperfilm ECL and Hyperfilm βmax, were products of Amersham (Arlington Heights, IL). Vectastain ABC reagents and biotinylated peanut agglutinin were from Vector Laboratories (Burlingame, CA). Mouse anti-sLe^x epitope monoclonal antibody was obtained from Chemicon International Inc. (Temecula, CA). Goat anti-mouse IgM (heavy and light chains) was from Jackson Immuno Research Laboratories Inc. (West Grove, PA). Falcon flexible flat-bottomed polyvinyl chloride 96-well microtiter plates were from Becton Dickinson (Oxnard, CA). Pressure-sensitive tape was obtained from Scotch 3M (St. Paul, MN). Trypticase soy broth was purchased from BBL Microbiology Systems, Becton Dickinson Co. (Cockeysville, MD), and yeast extract from DIFCO Laboratories (Detroit, MI). Synthetic oligosaccharide polymers (M_r = 30 000), in the form of multivalent receptors in which every fifth amide of a polyacrylamide matrix carries an oligosaccharide unit on a spacer arm, were purchased from GlycoTech (Rockville, MD). The polymers represented (i) the T antigen (Galβ1→3GalNAc), (ii) the sialyl-T antigen (NeuAcα2→3Galβ1→3GalNAc), (iii) the Le^x determinant [Galβ1→4(Fucα1→3)GlcNAc], (iv) the sLe^x determinant [NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc], (v)

a lactosamine unit (Galβ1→4GlcNAc), and (vi) HSO_3-Le^x [3′HSO_3-Galβ1→4(Fucα1→3)GlcNAc]. Polymers i−v presented the nonreducing termini of major MG2 oligosaccharides. Polymer v also mimicked the internal sequence of some MG2 saccharides. Polymer vi corresponded to a high-affinity selectin-binding motif (*39*).

Preparation of Peripheral Blood Neutrophils. Peripheral blood neutrophils were isolated as follows. Blood was obtained by venipuncture from healthy donors. The informed consent protocol for collecting blood samples was reviewed and approved by the Committee on Human Research, University of California, San Francisco. To prevent clotting and lysis, EDTA was added to a final concentration of 5 mM; then the samples were diluted (1:2, v/v) with Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS) and layered over Histopaque 1119 and 1077. After centrifugation at room temperature (RT) (500*g* for 20 min), the neutrophil fraction was diluted in Ca^{2+}- and Mg^{2+}-free HBSS supplemented with 0.2% bovine serum albumin (HBSS/BSA) and centrifuged again. Isolated cells were stored at RT in HBSS/BSA (10^7 cells/mL) for a maximum of 2 h. Just prior to the start of each experiment, the neutrophils were diluted 1:10 (v/v) with HBSS/BSA containing 1 mM Ca^{2+} and 1 mM Mg^{2+}.

Laminar Flow Assays. The laminar flow assay was adapted from a previously described method (*40*). Briefly, purified MG2 was diluted in Tris-buffered saline (pH 8.5) to a final concentration of 5 μg/mL. This solution was used to coat (4 °C, overnight) a small area of a bacteriological Petri dish. The dish was then washed with phosphate-buffered saline (PBS), and sites of nonspecific adherence were blocked by incubation in 2% BSA/PBS for 2 h at RT. After another washing, the MG2-coated area was incorporated as the lower wall in a parallel plate flow chamber and mounted on the stage of an inverted phase-contrast microscope. The system consisted of the following components: (i) an inverted phase-contrast microscope (Diaphot TMD; Nikon Inc., Garden City, NY), (ii) a programmable syringe pump (Harvard Apparatus, Natrick, MA), (iii) a black-and-white video camera (Cohu, Inc., San Diego, CA), (iv) a super VHS video recorder (Panasonic AG-1980P, Cypress, CA), and (v) a power Macintosh 8500/150 computer with a frame-grabber card (Scion Corp., Frederick, MD). Macros for the NIH image cell counting program, as well as for quantitating cell velocities, were created in collaboration with S. Vössner (Department of Engineering Economic System and Operations Research, Stanford University, Stanford, CA). All the assays were performed at RT. The wall shear stress was calculated by assuming that the buffer had a viscosity equal to that of water at RT.

To assess tethering, neutrophils (10^6 cells/mL in HBSS/BSA containing Ca^{2+} and Mg^{2+}) were infused into the chamber at shear stresses equal to 1, 1.25, 1.5, 2, or 3 dyn/cm^2. After equilibration (3 min), the number of cells that were either proximal to, or rolling on, the substrate was determined. The percentage of tethered cells was calculated as follows: (number of rolling cells)/(number of rolling cells + number of proximal cells) × 100. Rolling velocities were measured after first allowing cells to accumulate in the field of view for 3 min at 1.5 dyn/cm^2. Then the shear stress was raised every 10 s, in 2-fold increments, until a final value of 25 dyn/cm^2 was achieved. Displacement (micrometers per second) was followed for 1−3 s at each level. The fraction of cells that rolled at successively greater shear stresses was calculated as a percentage of the number that rolled initially.

Incubation of Peripheral Blood Neutrophils with Human Saliva. Peripheral blood neutrophils (5×10^5 cells/100 μL) were incubated for 1 h at RT with 100 μL of submandibular-sublingual (SMSL) saliva collected as the ductal secretion. The cells were washed three times in PBS and lysed in 40 μL of SDS−PAGE loading buffer. Control cells were incubated in PBS and lysed in loading buffer.

Preparation of Oral Neutrophils. Oral neutrophils were obtained from the same donor as the SMSL saliva sample by using a modification of the method described by Ashkenazi and Dennison (*41*). Briefly, the donor swirled 10−15 mL of HBSS in the mouth for 1 min and then expectorated into a beaker. This procedure was repeated for 20 min (∼20 cycles). The collected solution was passed three times through a 100 mm nylon mesh filter and three times through a 20 mm nylon mesh filter. The effluent containing neutrophils was centrifuged (250*g* for 15 min) to obtain the cell pellet. The resulting cells were washed twice in HBSS containing 20 mM HEPES and resuspended in the same buffer. The identity of the isolated cells was investigated by fluorescence-activated cell sorting to assess the expression of CD15, a neutrophil marker (*42*), CD14, a monocyte marker (*43*), and CD9, an eosinophil marker (*44*). Of the $1-2 \times 10^6$ cells that were routinely obtained from each collection, 90−95% were neutrophils. The isolated cells (5×10^5 cells) were then lysed in 40 μL of SDS−PAGE loading buffer.

Western Blot Analysis. Cell lysate samples of oral and peripheral blood neutrophils were subjected to SDS−PAGE (10% gel), and the separated proteins were transferred to nitrocellulose membranes. Immunoblot analyses with anti-sLe^x were performed as described previously (*39*).

Preparation of Radiolabeled Bacteria. Sources of *F. nucleatum* FN2, *S. sanguis* 72-40 and 804, and *Actinomyces viscosus* T14-V and T14-AV were described previously (*10*, *11*). All other bacterial strains (see Table 1) were from the American Type Culture Collection. Methods by which they were maintained, cultured, and metabolically labeled with [^{35}S]methionine were also described previously (*11*). The labeled cells were harvested by centrifugation, washed three times with PBS, and resuspended at a concentration of $1-10 \times 10^8$ cells/mL in 5% BSA/PBS or $1-2.5 \times 10^8$ cells/mL in 1% BSA/PBS.

Bacterial Adhesion Assays. Bacterial adhesion to saccharide receptors was assessed by two different techniques. First, we used an overlay assay to examine the adherence of bacterial strains to whole saliva, purified MG2, and synthetic saccharide polymers dot-blotted onto nitrocellulose membranes. The informed consent protocol for collecting the saliva sample was reviewed and approved by the Committee on Human Research, University of California, San Francisco. Briefly, 2 μL of undiluted whole saliva, MG2 purified as previously described (*39*) and diluted (4 μg/μL) in PBS, or a saccharide-conjugated polyacrylamide solution (1 μg/μL PBS) was spotted onto a nitrocellulose membrane and allowed to dry at RT for 1 h. Sites of nonspecific adhesion were blocked by incubating the membrane in 10% BSA/PBS for 1 h at RT. The blot was then overlaid with [^{35}S]-methionine-labeled bacteria ($1-10 \times 10^8$ cells/mL in 5%

Table 1: Bacterial Adhesion to Whole Saliva (WS), MG2, and Carbohydrate Polymers[a]

bacterial strain	WS	MG2	T Ag[b]	s-T Ag	Le^x	sLe^x	$3'\text{-}HSO_3\text{-}Le^x$	lactosamine
Streptococcus sanguis 804	64	62	0	72	0	0	0	8
S. sanguis 72-40	69	63	0	79	0	0	0	54
S. sanguis 10556	30	30	0	0	0	0	0	0
Streptococcus anginosis 27335	0	0	0	0	0	0	0	0
Streptococcus salivarius 13419	0	0	0	0	0	0	0	0
Streptococcus oralis 10557	100	83	0	51	0	0	0	0
Streptococcus gordonii 10558	20	26	0	0	0	0	0	36
Streptococcus mutans 25175	100	9	0	0	0	0	0	0
Actinomyces viscosus 19246	20	7	0	0	0	0	0	0
A. viscosus T-14V	70	8	0	0	0	0	0	0
A. viscosus T-14AV	40	0	0	0	0	0	0	0
A. naeslundii 12104	72	40	100	0	0	0	0	46
Fusobacterium nucleatum FN2	53	43	46	0	33	0	0	44
Fusobacterium periodonticum 33693	15	0	0	0	0	0	0	0
Capnocytophaga sputigena 33612	0	0	0	0	0	0	0	0
Capnocytophaga gingivalis 33624	88	0	0	0	0	0	0	0
Prevotella intermedia 25261	0	0	0	0	0	0	0	0
Porphyromonas gingivalis 33277	0	0	0	0	0	0	0	0

[a] Binding of radiolabeled bacteria to immobilized protein fractions or glycoconjugates in a dot blot assay. Adherence was quantified as described in Experimental Procedures. Scores from 0 to 100 were assigned. The background (e.g., no binding) was scored as 0, and maximal binding was scored as 100. [b] T Ag, T antigen; s-T Ag, sialyl-T antigen.

BSA/PBS) for 2 h at RT. After three washes, spots to which the bacteria bound were visualized by autoradiography. The autoradiogram was scanned by using a transmission scanner (Sharp, model JX-330) equipped with Image Master software (Amersham Pharmacia Biotech, Inc., Piscataway, NJ). Since different bacterial strains exhibited different specific activities, the values were relative approximations. In some cases, we confirmed bacterial adhesion to specific salivary components (intact or desialylated) by performing whole-cell ligand blotting on nitrocellulose replicas of electrophoretically separated salivary proteins as previously described (*11*, *45*). Binding properties of each bacterium were assessed in at least two separate experiments.

Second, we used a centrifugal force assay to determine the binding strength of adhesive interactions between bacteria and synthetic carbohydrate receptors. In this case, polyvinyl chloride microtiter wells were coated by adding 20 μL of the saccharide-conjugated polyacrylamide solution (0.1 μg/μL PBS) to the wells, which were allowed to dry at RT overnight. As a control, some wells were incubated with PBS alone. The rest of the assay was performed essentially as described previously (*46*, *47*). Briefly, the coated wells were washed with PBS, and sites for nonspecific adherence were blocked by incubating with 10% BSA/PBS for 1 h at RT. After another washing with PBS, 125 μL of 1% BSA/PBS was added to the wells, followed by 50 μL of radiolabeled bacteria ($1-2.5 \times 10^8$ cells/mL in 1% BSA/PBS) and another 125 μL of 1% BSA/PBS. The plate was sealed with pressure-sensitive tape. To bring the bacteria into contact with the substrates, the plates were centrifuged at RT for 10 min (1100*g*, spin on). After incubation at RT for 1 h, the plates were inverted and centrifuged again for 10 min at RT (spin off); replica plates were subjected to different centrifugal forces, ranging from 100*g* to 800*g*. The plates were then removed from the centrifuge in the inverted position and submerged in ethanol/dry ice to freeze the contents. The bottom 3 mm of each well was clipped off and transferred to a scintillation vial. The number of cells that remained bound was determined by quantification of the radioactivity in each well. The percentage of cells bound equals the number of cells bound after the spin-off step divided by the number of cells brought into contact by the spin-on step times 100.

RESULTS

Tethering and Rolling of Neutrophils on Immobilized MG2. Previously, we showed that MG2 could serve as an L-selectin ligand and that the sLe^x determinant was critical for the ligand activity (*39*). This finding led us to hypothesize that neutrophils that emerged in the oral cavity could interact with MG2 presented in both immobilized and solubilized forms.

Adhesive interactions with immobilized molecules in the mouth are opposed by shear stresses on oral surfaces created by salivary flow. In an earlier study, we used published values of salivary fluid velocity, depth, and viscosity to calculate a value for the shear stress at the tooth surface of $\sim$0.8 dyn/cm^2 (*46*). To simulate the in vivo situation, we assayed the ability of MG2 immobilized on the wall of a flow chamber to support neutrophil tethering and rolling as a function of shear stress. With regard to tethering, 10−20% of the neutrophils in proximity of the MG2 substrate attached at shear stresses between 1.25 and 2 dyn/cm^2 (Figure 1A). No interactions were detected in the absence of Ca^{2+} (data not shown), a characteristic of selectin-mediated processes. As is typical of selectin-mediated adhesive interactions under flow conditions (*48*, *49*), there was a threshold shear stress of 1 dyn/cm^2, below which cells did not attach to MG2. Also, no cell attachment was observed at shear stresses of $\geq$3 dyn/cm^2. This unusual characteristic has been attributed to the short lifetime of selectin−ligand bonds, which permits tethering initially (*48*), followed by rolling above a threshold of shear stress (*50*).

With regard to rolling, the calculated velocities ranged from 120 to 210 μm/s at shear stresses between 1.25 and 6 dyn/cm^2. Between 1.25 and 2 dyn/cm^2, the velocities (120−160 μm/s; Figure 1B, white bars) were approximately 5−7-fold slower than those of noninteracting cells in solution (Figure 1B, striped bars). We also examined the strength of the rolling interaction. Approximately 40% of the cells that tethered at a shear stress of 1.25 dyn/cm^2 remained rolling

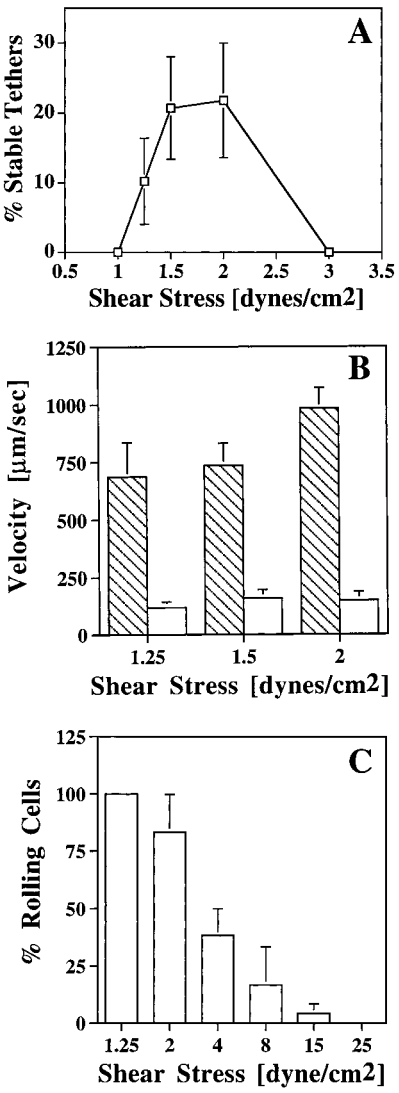

FIGURE 1: Immobilized MG2-mediated neutrophil tethering, followed by rolling under shear flow conditions. (A) The percentage of neutrophils in the proximity of the MG2 substrate that attached at shear stresses between 1 and 3 dyn/cm^2. Data points and error bars represent the mean and SD of stable tethers in a 4× microscope field in three separate experiments. (B) The velocities of neutrophils rolling on MG2 substrates (white bars) compared to the velocities of noninteracting cells in solution (striped bars) at shear stresses between 1.25 and 2 dyn/cm^2. Values represent the mean and SD of 30 rolling cells. The experiment was repeated twice with similar results. (C) The percentage of rolling cells that remained attached as the shear stress was increased from 1.25 to 15 dyn/cm^2. The percentage of rolling cells at 1.25 dyn/cm^2 was assumed to be 100. Values represent the mean and SD of three separate experiments.

at 4 dyn/cm^2; 20% remained rolling at 8 dyn/cm^2. All of the cells detached above 15 dyn/cm^2 (Figure 1C). Together, the results of these experiments suggest that MG2 can support neutrophil tethering and/or rolling over a range of shear stresses likely to be encountered at oral surfaces.

Interaction of Oral Neutrophils with MG2 in Saliva. Next, we investigated whether MG2 was found in association with neutrophils that were isolated from the oral cavity. The low-molecular-weight mucin was detected by immunoblotting with an antibody that recognizes one of its most abundant carbohydrate epitopes (anti-sLe^x). In accord with our previous results (*39*), we found that a band with the same molecular weight as MG2 (120 000) was the major immunoreactive

FIGURE 2: Oral neutrophils interact with MG2 in saliva. The presence or absence of the low-molecular-weight mucin was demonstrated by immunoblotting with an antibody that recognizes one of its most abundant carbohydrate epitopes (anti-sLe^x). A band with the same molecular weight as MG2 (120 000) and a mucin dimer were the major immunoreactive components in human SMSL saliva (lane 1). No immunoreactivity was detected in association with peripheral blood neutrophils (lane 2), but lysates prepared from peripheral blood neutrophils that had been incubated with SMSL saliva and from oral leukocytes contained immunoreactive bands with the same estimated molecular weight as those found in the SMSL saliva sample (lanes 3 and 4, respectively). This experiment was performed three different times with essentially the same results.

component in human SMSL saliva (Figure 2, lane 1). The presence of a higher-molecular-weight band, of variable intensity, that also reacted with the antibody is likely due to the recently described ability of this mucin to form dimers, as well as high-order multimers (*51*). As expected, no immunoreactivity was detected in association with peripheral blood neutrophils (Figure 2, lane 2), but the lysates prepared from oral leukocytes contained immunoreactive bands with the same estimated molecular weight as those found in the SMSL saliva sample (Figure 2, lane 4). Finally, a lysate prepared from peripheral blood leukocytes that were incubated for 1 h in SMSL saliva also contained the same two immunoreactive bands (Figure 2, lane 3). Together, these results suggest that neutrophils also interact with soluble MG2 and, importantly, that these interactions occur in vivo.

Bacterial Adhesion to MG2 Oligosaccharides. We used a dot blot overlay assay to screen 18 bacterial strains that colonize the oral cavity for their ability to bind to whole saliva, purified MG2, or a set of synthetic oligosaccharide structures mimicking those presented on MG2. The results are summarized in Table 1. Adherence was quantified as described in Experimental Procedures. Scores from 0 to 100 were assigned. The background (e.g., no binding) was scored as 0, and maximal binding was scored as 100. Thirteen of the 18 strains we tested interacted with whole saliva; of this subset, seven exhibited substantial adherence to purified MG2. Six of the seven strains that adhered to MG2 also adhered to T antigen, sialyl-T antigen, and/or lactosamine sequences. Except for *F. nucleatum* FN2, which exhibited a relatively weak interaction with the Le^x sequence, none of the strains that adhered to MG2 bound to Le^x, sialyl Le^x, or 3′-HSO$_3$-Le^x saccharide sequences. These results suggested that certain bacteria adhere to the MG2 molecule via a subset

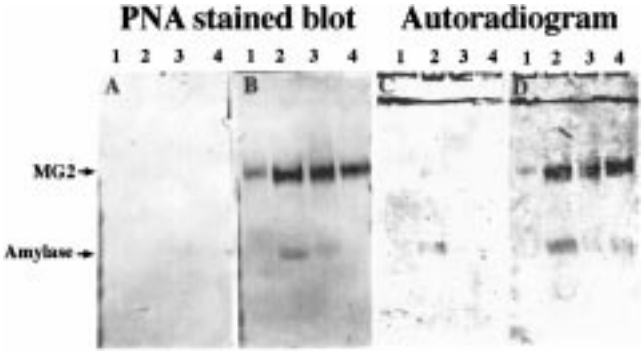

FIGURE 3: Adherence of *A. naeslundii* 12104 to intact vs desialylated MG2. Components from four different submandibular and/or sublingual saliva samples were electrophoretically separated on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane. The replicas were either stained with peanut agglutinin (A and B; PNA), a lectin that recognizes (asialo) Galβ1→3GalNAc sequences, or overlaid with metabolically ^{35}S-labeled *A. naeslundii*, in which case binding was detected by autoradiography (C and D; autoradiogram). Very low or no binding of peanut agglutinin (A) or *A. naeslundii* (C) to intact MG2 was observed. After removal of sialic acid by treatment of a duplicate replica with 0.1 N HCl for 1 h at 70 °C, both the lectin (B) and the bacterium (D) bound strongly to the desialylated MG2. In some cases, bacterial binding to a band with the same M_r as α-amylase was also detected.

of terminal epitopes (e.g., T antigen, sialyl-T antigen, or lactosamine) that does not include the known selectin ligands (e.g., sLe^x and 3′-HSO$_3$-Le^x). We further explored adherence mechanisms used by three strains that adhered to carbohydrate receptors that mimicked the nonreducing termini of MG2 oligosaccharides. With regard to *Actinomyces naeslundii*, the dot blot assay suggested that this organism does not adhere to the sialyl-T antigen, but that removal of sialic acid from this oligosaccharide permits strong binding. We found the same receptor preference when the sialyl-T and T antigens were presented in the context of the MG2 peptide backbone. In these experiments, we first assessed T antigen levels in SMSL saliva by overlaying a replica of four electrophoretically separated samples with peanut agglutinin, a lectin that recognizes (asialo) Galβ→3GalNAc sequences (*52*). No binding was detected (Figure 3A). In contrast, after mild acid treatment to remove sialic acid (*11*), lectin binding to all four samples was readily detected (Figure 3B). Then we tested binding of *A. naeslundii* by whole-cell ligand blotting to electrophoretically separated salivary proteins. Binding of *A. naeslundii* to MG2 mimicked that of peanut agglutinin, in that either no or very low levels of adherence to intact MG2 were observed (Figure 3C), but after desialylation, adherence was once again readily detected (Figure 3D). In some cases, *A. naeslundii* binding to a band with the same molecular weight as α-amylase was also enhanced after mild acid treatment of the samples (compare panels C and D of Figure 3).

Finally, we used the centrifugal force assay to estimate the strength of adhesive interactions between this organism and synthetic polymers that presented T antigen, sialyl-T antigen, and lactosamine sequences. In accord with data from the other adherence assays, we found that *A. naeslundii* bound with greater force to Galβ1→3GalNAc than to NeuAcα2→3Galβ1→3GalNAc (Figure 4A). For example, at 800*g* (1.9×10^{-7} dyn/cell), the highest centrifugal force it was technically possible to generate, 20% of the organisms

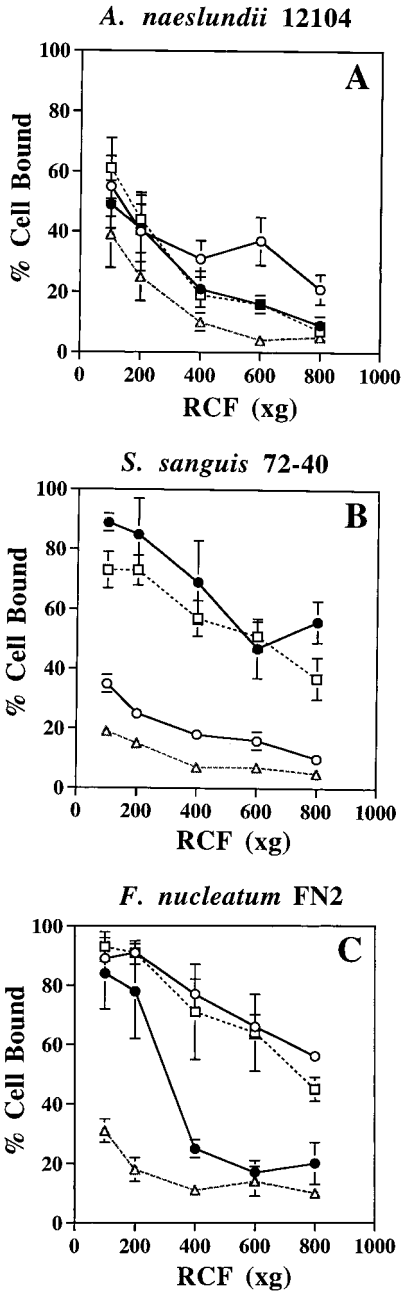

FIGURE 4: Adhesive strength of interactions between bacteria and synthetic polymers presenting oligosaccharides that mimic MG2 carbohydrate structures. The assay is based on the principle that the centrifugal force required to remove cells from a substrate is a direct measure of the adhesive strength of the interaction(s) being disrupted. This method was used to estimate the strength of adhesive interactions between *A. naeslundii* 12104 (A), *S. sanguis* 72-40 (B), or *F. nucleatum* FN2 (C) and synthetic polymers that presented either T antigen (○), sialyl-T antigen (●), or lactosamine (□) sequences. Control wells were coated with PBS (△). RCF is the relative centrifugal force; error bars represent the SD. Each experiment was carried out at least twice, with five to eight replicates per experiment.

remained attached to the T antigen substrate; less than half that number of cells adhered to sialyl-T antigen-coated wells. Lactosamine binding was similar to that observed with the sialyl-T antigen substrate.

With regard to *S. sanguis* 72-40, we previously showed that this organism adheres to intact MG2, whereas desialylation abolishes binding (*11*). Here we replicated this result

in the centrifugal force adhesion assay, using carbohydrate polymers as substrates (Figure 4B). At 100*g* (3.6×10^{-9} dyn/cell), ~90% of the cells bound to sialyl-T antigen-coated wells whereas only 35% of the cells bound to T antigen-coated wells. At 600*g* (2.2×10^{-8} dyn/cell), more than 50% of the organisms remained attached to sialyl-T antigen whereas less than 20% of the cells remained attached to T antigen. In addition, *S. sanguis* 72-40 appeared to bind strongly to lactosamine-coated wells.

We also used the centrifugal force adhesion assay to study *F. nucleatum* adherence. Data from our previous studies showed that this organism adheres to the PRG, a component of parotid saliva; no interactions with SMSL salivary components were detected (*10*). More recently, we observed, by whole-cell ligand blotting, that this organism sometimes adheres to MG2 in saliva samples after long-term storage (data not shown) or purification (see Table 1). This finding suggested that *F. nucleatum* might adhere via neo-Galβ1→3GalNAc and/or neo-lactosamine sequences. Here we show that this is the case (Figure 4C). Strong binding to both sialyl-T and T antigen substrates was observed at relatively low centrifugal forces (≤200*g*). Above 400*g*, adherence only to the latter polymer was detected. *F. nucleatum* bound to both the T antigen and lactosamine substrates with approximately equal forces.

DISCUSSION

The results of these experiments show that MG2, a major source of oligosaccharides that coat oral surfaces, can serve as a receptor for neutrophil and bacterial adhesion. With regard to neutrophils, immobilized MG2 can mediate both tethering and rolling of these cells in shear flow. The interactions were Ca^{2+}-dependent, and there appeared to be a threshold shear stress below which cells did not attach to MG2; both of these are hallmarks of selectin-mediated processes. We were particularly interested in the dynamic range (1−3 dyn/cm^2) that allows initiation of the interaction, noting that these experimental values fit remarkably well with our theoretical calculation of shear force at the tooth surface due to salivary flow (~0.8 dyn/cm^2) (*46*). Although shear stress is not homogeneously distributed in the oral cavity, our findings suggest that this force could be one factor that influenced the evolution of MG2 glycosylation, and consequently the density of L-selectin binding sites on the molecule (*53*). This would also account for the fact that neutrophil interactions with MG2 were weaker than those with CD34. For example, neutrophils rolled at higher velocities on MG2 at maximal coating densities (120−160 μm/s) than on CD34 coated at low site densities (30−100 μm/s) (*54*). The rolling interactions supported by the low-molecular weight salivary mucin were also less stable. Most neutrophils detached from MG2 substrates at shear stresses above 8 dyn/cm^2, whereas complete detachment from CD34 occurred at shear stresses above 25 dyn/cm^2 (*54*). Although the neutrophil−MG2 interactions are weaker, it should be noted that neutrophils encounter much lower shear stresses near oral surfaces than at the walls of postcapillary venules (5.6−8.3 dyn/cm^2) (*55*).

These data, together with our discovery that MG2 is found in association with neutrophils isolated from the oral cavity, allow us to suggest a stepwise process by which neutrophils marginate in the oral cavity. In the first step, common to many sites throughout the body, circulating leukocytes use selectin−ligand interactions to tether and roll on inflamed endothelium, a transient interaction that is rapidly followed by integrin-mediated firm arrest and extravasation. In the second step, specialized to the oral environment, neutrophils emerging at the gingival crevice (*41*, *56*) use L-selectin to attach to MG2-coated surfaces, thereby resisting the shear stress they once again encounter in the mouth. Data showing that L-selectin expression is comparable on neutrophils collected from peripheral blood and from healthy gingiva at sites of extravasation support the two-step process we propose (*57*). Furthermore, our observation that salivary neutrophils associate with MG2 supports the in vivo relevance of the interactions we propose. It is important to note that freshly isolated salivary neutrophils are viable. For example, these cells actively phagocytose streptococcal strains in vitro (A. Prakobphol and S. J. Fisher, unpublished data). Finally, we also found that neutrophils can interact with soluble MG2. We do not, as yet, understand the biological significance of the latter interactions, but we are testing several possibilities. For example, soluble mucin could act as a competitor inhibitor facilitating the release of adherent neutrophils. We are also investigating the possibility that MG2 multimers could facilitate aggregation and clearance of salivary neutrophils.

We also found that MG2 oligosaccharides support adhesion of certain strains of bacteria. The results of these experiments offer interesting insights, at a molecular level, into dental plaque formation. For example, we identified several bacterial strains that used lectin-like adherence mechanisms to interact with MG2 carbohydrate sequences, primarily T and sialyl-T antigens, as well as lactosamine units. Given that the low-molecular-weight mucin is a major glycoprotein in pellicle (*58*), this observation could help explain why some of these organisms are better adapted than others for early colonization. Additionally, we showed that a subset of strains expressed multiple lectins. We speculate that the relative importance of individual receptors to the organism's overall adhesion strategy could change over time. For example, *S. sanguis* 72-40 can bind to sialyl-T sequences that cap the nonreducing termini of newly synthesized MG2 that is adsorbed to oral surfaces. As the pellicle matures, this epitope could be lost due to the potent sialidase activity of certain oral bacteria (*59*, *60*). The newly generated T antigen epitope could then serve as a receptor for *A. naeslundii* and *F. nucleatum*. In fact, we found that MG2 in samples of whole saliva occasionally did support the binding of *A. naeslundii* and *F. nucleatum*. This was particularly evident when the samples were stored (frozen at −20 °C) for a long period of time before they were analyzed (unpublished data).

Our study also showed that *A. naeslundii*, *S. sanguis*, *Streptococcus gordonii*, and *F. nucleatum* all have a lectin activity that may permit relatively strong adherence to lactosamine units on some MG2 saccharides (see Figure 4A−C). This would suggest another strategy these organisms may use to adhere via desialylated MG2. It is interesting that *F. nucleatum* can also bind to the PRG, another highly glycosylated salivary component, via a lactosamine sequence on the unique N-linked saccharide structures presented by this molecule (*10*). Most *S. sanguis* strains (*11*) and *A.*

naeslundii (unpublished data), however, do not adhere to the PRG. This indicates that among these bacterial strains, the adhesins that recognize the lactosamine sequence, although functionally related, are not structurally identical.

Together, the results of our studies suggest that MG2 presents oligosaccharide sequences that mediate at least two types of adhesive interactions. In the first, neutrophils tether and roll on immobilized MG2. In the second, bacteria adhere via T antigen, sialyl-T antigen, and lactosamine sequences. Since distinct subsets of oligosaccharides mediate adherence of neutrophils and bacteria, the possibility exists that in coordinating both adhesive interactions MG2 facilitates opsonization. Given the potential biological importance of these interactions, it is likely that individual variations in glycosylation of MG2 among the population may indicate a risk factor for certain oral diseases in which bacterial infection and/or immune dysfunction are the presumed etiologic agents (e.g., periodontal diseases; *61*). For example, we hypothesize that failure to express sLe^x would result in a functional imbalance; MG2 could promote bacterial adhesion, but its interactions with neutrophils would be compromised. In this regard, it is interesting that MG2 samples from some individuals lack sLe^x epitopes but express T and sialyl-T antigen sequences (*39*), suggesting that such a situation can occur in vivo. Currently, we are investigating whether expression of the latter MG2 glycoforms is associated with particular oral diseases.

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