Highly Glycosylated Human Salivary Molecules Present Oligosaccharides That Mediate Adhesion of Leukocytes and *Helicobacter pylori*†

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ABSTRACT: Glycoproteins display carbohydrate facets that serve as adhesion receptors for cells including leukocytes and bacterial cells. Our aim was to understand the role of the specialized carbohydrate motifs carried by highly glycosylated human salivary proteins in regulating the oral ecology. To date, our structural studies suggest that these molecules display a wide array of oligosaccharide structures, including many species with highly charged and/or fucosylated termini. Here, we used an immunoblot approach to gain additional information about the nature of these oligosaccharides. The results showed that MG1 and the salivary agglutinin express the MECA-79 epitope, an unusual sulfated carbohydrate structure that belongs to an important class of high-affinity (endothelial) L-selectin ligands. Unexpectedly, we discovered that in many women the expression of this epitope is hormonally regulated. Additional experiments revealed that MG1, MG2, and the salivary agglutinin also present Lewis blood group antigens, the exact repertoire varying on an individual basis. In parallel, we explored the functions of these carbohydrate motifs. Using an assay that detects L-selectin ligands, we found that the subset of MECA-79-reactive oligosaccharides displayed on salivary molecules specifically bind an L-selectin/Fc chimera. In contrast, the Lewis blood group structures are receptors for many strains of *Helicobacter pylori*, an organism that is implicated in the development of gastric ulcers and cancer. Together, these results suggest that MG1, MG2, and the salivary agglutinin play important roles in governing leukocyte and bacterial adhesion. Our findings suggest novel strategies, based on the relevant carbohydrate structures, for promoting or inhibiting these processes.

Human saliva has several critical functions including lubrication (*1*, *20*, *26*), digestion (*41*), formation of a bioactive semipermeable barrier that coats oral surfaces (i.e., pellicle [*2*, *5*, *17*, *41*]), and regulation of the composition of the oral flora. Saliva fulfills the latter function by virtue of its antimicrobial activity (*42*, *47*) and by promoting selective microbial clearance or adherence (*22*, *24*, *45*, *60*). The diverse functions of saliva are allocated among its many soluble components which are secreted from parotid, submandibular/sublingual, and minor salivary glands. These components include amylase, cystatins, proline-rich proteins, proline-rich glycoproteins, carbonic anhydrases, peroxidases, statherins, histatins, lactoferrin, lysozyme, secretory immunoglobulin (Ig)[1] A, salivary agglutinin (gp-340), and mucins.

To date, at least two human salivary mucin populations have been identified: the high-molecular-weight mucin encoded by *MUC5B* (MG1, M_r > 1,000,000 [*46*, *65*, *66*]) and the low-molecular-weight mucin encoded by *MUC7* (MG2, M_r = 120,000 [*8*]). Studies of the purified mucins indicate that they are structurally distinct and heavily glycosylated (*39*, *51*). MG1, which consists of large, disulfide-linked peptide subunits, contains 19% protein and 81% carbohydrate (*39*, *64*), whereas MG2, which exists as a much smaller, single peptide chain, contains 30% protein and 68% carbohydrate (*39*, *51*, *53*). Our group has used mass spectrometry and nuclear magnetic resonance spectroscopy approaches to analyze the oligosaccharide structures that MG2 presents (*53*). We showed that the termini are composed of the T antigen (Galβ1→3GalNAc), sialyl-T antigen (Siaα2→3 Galβ1→3GalNAc), Lewis^x [Le^x, Galβ1→4-(Fucα1→3)GlcNAc], sialyl-Le^x [sLe^x, Siaα2→3Galβ1→4-(Fucα1→3)GlcNAc], and lactosamine (Galβ1→4 GlcNAc) determinants. In parallel studies we used the same combination of approaches to analyze the neutral and sialylated fractions of MG1 oligosaccharides (*64*). The results showed

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[1] Abbreviations: Ig, immunoglobulin; MG1, high-molecular-weight salivary mucin; MG2, low-molecular-weight salivary mucin; HEVs, high endothelial venules; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; BSA, bovine serum albumin; GSTs, glycosyl sulfotransferases.

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that MG1 carries several fucosylated determinants, including H type 1 and 2 (Fucα1→2Galβ1→3/4GlcNAc), Le^a [Galβ1→3(Fucα1→4)GlcNAc], Le^b [(Fucα1→2)Galβ1→3(Fucα1→4)-GlcNAc], Le^x, sLe^x, and Le^y [(Fucα1→2)Galβ1→4(Fucα1→3)GlcNAc]. In addition, we found that approximately 20% of MG1 saccharides are highly charged, suggesting the presence of sulfated species. Finally, our data indicate that the structures in the charged fraction are unusually complex, in that the average oligosaccharide contains 40 sugar residues.

Given the nature of salivary mucin glycosylation, it is assumed that carbohydrate residues form the outer facets on these molecules, ideally placed to interact with cells in the oral cavity. The importance of oligosaccharide-mediated interactions to cell adhesion is recognized in many normal and pathological processes (*35*, *61*). With regard to the latter, a variety of organisms have lectins that interact with carbohydrate receptors presented by host glycoproteins or glycolipids. For example, P-fimbriated *Escherichia coli*, which is implicated in human urinary tract infections, adheres via Galα1→4Gal sequences carried by glycolipids (*9*). *Helicobacter pylori*, a bacterium associated with gastritis, ulcers, and stomach cancer, has a specific adhesin, BabA, which recognizes the Le^b antigen carbohydrate sequence presented by the gastrointestinal mucosa of the host (*32*). Oral bacteria can also adhere via salivary carbohydrate receptors. For example, we showed that *Fusobacterium nucleatum*, a bacterium associated with periodontal disease, adheres to the highly glycosylated proline-rich glycoprotein via its N-linked saccharides that present lactosamine sequences in the context of the Le^y determinant (*22*). We also showed that *Streptococcus sanguis* strains adhere to MG2 via O-linked saccharides that carry the terminal sialyl-T antigen. Other bacteria (e.g., *Actinomyces naeslundii*) adhere to this molecule via T antigen and lactosamine sequences (*52*). In this regard, it is interesting to note that MG1 and MG2 are the major glycosylated components of the adsorbed pellicle that coats the tooth surface, whereas the proline-rich glycoprotein and salivary agglutinin are minor components (17). This suggests that these glycosylated molecules can serve as receptors for bacterial adherence to the tooth surface as well as for bacterial clearance from the oral cavity.

Carbohydrate receptors also play interesting roles in governing lymphocyte trafficking, the process whereby vascular endothelia, extracellular matrixes, and cells that reside in tissues present "traffic signals" that regulate lymphocyte movement (reviewed in refs *14*, *36*, *57*, and *62*). These adhesive interactions, which occur in a stepwise manner, involve the sequential actions of several molecular families, including lectins, chemokines, and integrins. Initially, lymphocytes extracted from the circulation begin rolling on endothelia, a Ca^{2+}-dependent process that is mediated by molecules with C-type lectin domains (e.g., leukocyte [L]-selectin that binds to carbohydrate ligands on the vasculature). In mice, lymph node high endothelial venules (HEVs) express sialomucins, such as GlyCAM-1 and CD34, that carry L-selectin ligands (*4*, *37*). In humans, four L-selectin glycoprotein ligands, including CD34 and podocalyxin, have been identified at the biochemical level (*6*, *55*, *59*). As expected, these molecules present carbohydrate-based recognition determinants. The exact nature of the naturally occurring carbohydrate structures that participate in high-affinity selectin interactions are still largely unknown. Analyses of the saccharides carried by GlyCAM-1 suggest that the sequences 6-sulfo sLe^x [Siaα2→3Galβ1→4(Fucα1→3)(SO_3→6)GlcNAc], 6′-sulfo sLe^x [Siaα2→3(SO_3→6)-Galβ1→4(Fucα1→3)GlcNAc] are involved (*28*).

Because of the inherent difficulty in isolating the amount of relevant oligosaccharides required for structural analyses, antibodies that recognize unique features of selectin ligands have been crucial to many advances in the field. The anti-peripheral node addressin monoclonal antibody termed MECA-79 is a prime example (*13*, *27*, *71*). This antibody, which is further defined as a high-affinity L-selectin carbohydrate ligand that contains a SO_3→6GlcNAc sequence (*71*), stains HEVs in both human and mouse lymph nodes (*33*, *63*). MECA-79 also perturbs function by blocking lymphocyte attachment to HEVs in vitro and short-term homing of lymphocytes to lymph nodes in vivo (*33*, *63*). To further elucidate the structural features of L-selectin ligands, other investigators tested the activity of synthetic saccharide mimetics. This approach revealed that sLe^x and several related structures are low-affinity L-selectin ligands (*3*, *16*); multivalency may increase binding affinity (*23*). In accord with these observations, we showed that MG2, which presents multiple sLe^x saccharide termini, supports tethering and rolling adhesion of leukocytes under shear stress (*53*). Interestingly, carbohydrate structures related to the MECA-79 determinant do not support bacterial adhesion (*52*). Together, these findings indicate that separate oligosaccharide determinants carried on MG2 mediate interactions with leukocytes and bacteria.

As reported here, the latter observation prompted us to investigate whether other salivary glycoproteins express the highly specialized, sulfated carbohydrate epitopes that constitute the high-affinity class of L-selectin ligands. In addition, we were interested in the role of MG1 oligosaccharides as receptors for bacteria that colonize the oral cavity. Given our current knowledge of the carbohydrate structures carried by highly glycosylated salivary proteins and the blood group ligand preferences of *H. pylori*, we were also interested in whether these molecules interact with this bacterium, which persistently infects the gastric mucosa of more than half of all people worldwide (*15*).

MATERIALS AND METHODS

Materials. Antibodies: Anti-Le^a and anti-Le^b were from Lab Vision Corp., Fremont, CA. Anti-sLe^a was from EMD Biosciences, Inc., San Diego, CA. Monoclonal antibodies that recognize L-selectin ligands (HECA-452 and MECA-79) were from BD Biosciences, San Jose, CA. The HECA-452 antibody recognizes sLe^x [Siaα2→3Galβ1→4(Fucα1→3)-GlcNAc] and related structures, including 6-sulfo sLe^x [Siaα2→3Galβ1→4(Fucα1→3)(SO_3→6)GlcNAc], 6′-sulfo sLe^x [Siaα2→3(SO_3→6)Galβ1→4(Fucα1→3)GlcNAc], and 6′,6-disulfo sLe^x [Siaα2→3(SO_3→6)Galβ1→4(Fucα1→3)-(SO_3→6)GlcNAc] (*43*). The MECA-79 antibody recognizes a high-affinity L-selectin ligand carbohydrate epitope containing SO_3→6GlcNAc (*71*). Anti-sLe^x was prepared by culturing the hybridoma cell line CSLEX-1, obtained from the American Type Culture Collection, Manassas, VA, as described (*21*). This antibody recognizes sLe^x and other related structures, excluding 6-sulfo sLe^x and 6′,6-disulfo sLe^x (*43*). Anti-gp-340 was a gift from Dr. J. R. Wright

(Duke University Medical Center, Durham, NC). This antibody specifically interacts with the salivary agglutinin (*54*). Anti-A, anti-B, and anti-O monoclonal antibodies were from Abcam, Inc., Cambridge, MA.

Other reagents: Brucella agar was purchased from BD Biosciences, San Jose, CA. The recombinant human L-selectin/Fc chimera was obtained from R&D Systems Inc., Minneapolis, MN. Horseradish peroxidase (HRP)-conjugated anti-mouse IgG, HRP-conjugated anti-mouse IgM, HRP-conjugated anti-rat IgM, and HRP-conjugated anti-human IgG were from Jackson Immuno Research Laboratories, West Grove, PA. Clear Blue Ovulation test kits were purchased from Unipath Diagnostics, Inc., Waltham, MA. Enhanced chemiluminescence (ECL) detection reagents, Hyperfilm ECL, and Hyperfilm βmax were obtained from Amersham Biosciences Corp., Piscataway, NJ. ^{35}S protein labeling mix was from Perkin-Elmer Life and Analytical Sciences, Inc., Boston, MA. Nitrocellulose membranes (0.45 μm) and nonfat dry milk were obtained from Bio-Rad, Hercules, CA. All other reagents were purchased from Sigma-Aldrich, St. Louis, MO.

Saliva Collection. Whole saliva samples were collected from 30 healthy donors (14 men and 16 women, ages 25−40). An additional sample was collected from a 65-year-old woman. The informed consent protocol for collecting the saliva samples was reviewed and approved by the Committee on Human Research, University of California, San Francisco. The samples, which were obtained between 10 a.m. and 11 a.m., were collected on ice. The donors were instructed to rinse their mouths with water before expectoration of 2−3 mL of saliva into a clean tube. In addition, saliva samples from two men, six women undergoing natural cycles, and one postmenopausal woman were collected every day for 31 days. The younger female donors had natural cycles. The day ovulation occurred was confirmed by using a Clear Blue Ovulation test kit. Additionally, saliva samples were collected from one donor during pregnancy (second and third trimesters) and lactation (months 2 to 6). All samples were diluted 1:1 with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and either subjected to electrophoretic separation immediately or stored at −20°C for later analyses.

Electrophoretic Separation and Western Blotting. Salivary components were separated under reducing conditions by SDS-PAGE (10% separating gel and 4% stacking gel) and transferred to nitrocellulose membranes. The membrane replicas were incubated in phosphate-buffered saline (PBS) with 0.1% Tween-20 (T-PBS) containing 5% nonfat dry milk (T-blotto) to prevent nonspecific binding prior to incubation with primary antibody as previously described (*53*). Immunoreactive bands were detected with HRP-conjugated secondary antibodies and visualized by using chemiluminescence. A replicate gel of the same samples was stained with Alcian blue and/or silver to confirm the equal loading of glycoproteins and proteins, respectively. Unless otherwise noted, this and all other experiments were performed at least three times.

Binding of Recombinant L-Selectin to Salivary Glycoproteins. Whole saliva samples were electrophoretically separated and transferred to nitrocellulose membranes as described above. Nonspecific binding was blocked by incubating the membrane in 5% T-blotto for 1 h at room temperature. A complex of human L-selectin/Fc chimeras (4 μg/mL) and HPR-conjugated anti-human IgG (1:500, vol/vol) was formed in 5% T-blotto by gentle mixing on a rotator (Cole-Parmer Roto-Torque Variable Speed Rotator) at room temperature for 20 min prior to overlaying on the membrane replicas and incubating on a rotator for 1 h. Then the membranes were washed three times with T-PBS. Protein bands that supported the binding of L-selectin were visualized by using chemiluminescence. As a control for specificity, an identical blot was incubated in the presence of 10 mM EDTA or MECA-79 antibody (4 μg/mL). In the latter case, the blot was preincubated with MECA-79 for 1 h prior to the start of the experiment.

Preparation of Radiolabeled Bacteria. *H. pylori* strains SMI65, SMI27, J99, 17874, 26695, and MO19 were employed. Their blood group antigen binding activity was previously characterized (*40*). The bacteria were maintained and grown on brucella agar plates (*32*). For metabolic labeling, ^{35}S protein labeling mix was diluted in PBS, and an aliquot (50 μCi/100 μl) was swabbed on the plate before streaking of the bacteria. After 36−48 h, the bacterial cells were harvested by gently rubbing the plate with a cotton swab, after which they were washed three times with PBS and finally resuspended at a concentration of 5×10^7 CFU/mL in PBS containing 5% bovine serum albumin (BSA) prior to the start of the experiment. The origin, growth, and metabolic labeling conditions of the following strains were as described (*52*): *S. sanguis* strains 804, 72-40, 10556; *Streptococcus anginosis* 27335; *Streptococcus salivarius* 13419; *Streptococcus oralis* 10557; *Streptococcus gordonii* 10558; *Streptococcus mutans* 25175; *Actinomyces viscosus* 19246, T-14V, T-14AV; *A. naeslundii* 12104; *F. nucleatum* FN2; *Fusobacterium periodonticum* 33612; *Capnocytophaga sputigena* 33612; *Capnocytophaga gingivalis* 33624; *Prevotella intermedia* 25261; and *Porphyromonas gingivalis* 33277.

Bacterial Adherence Assay. Adherence of bacterial strains to salivary components was examined by using a whole cell ligand binding approach and methods we have published (*45*). Briefly, nitrocellulose replicas of separated salivary components were incubated in 5% BSA in PBS for 1 h at room temperature to block nonspecific adherence and were then overlaid with a ^{35}S metabolically labeled bacterial cell suspension. The binding was allowed for 1 h at room temperature, before the membranes were gently washed three times with PBS. Bacterial adherence was detected by autoradiography. The binding characteristics of each strain were assessed at least twice.

RESULTS

MG1 Expresses Functional L-Selectin Carbohydrate Ligands. The data shown illustrate the range of glycosylation and/or sulfation patterns of proteins in the saliva samples that we analyzed. We incubated nitrocellulose replicas of electrophoretically separated salivary proteins from all donors with monoclonal antibodies that recognize L-selectin carbohydrate ligands. The patterns of L-selectin ligand expression of samples from six donors are shown in Figure 1A and B. As expected based on our previous findings (*53*), probing the membrane replicas with anti-sLe^x showed that most samples contained immunoreactive bands with the same electrophoretic mobility as MG1, MG2, and the salivary

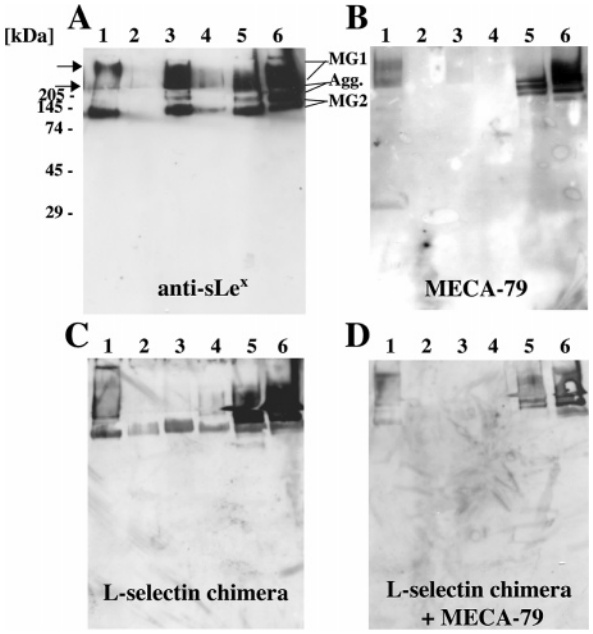

FIGURE 1: Salivary components express functional high-affinity L-selectin carbohydrate ligands. Whole saliva samples from six donors (lanes 1−6) were separated by SDS-PAGE and transferred to nitrocellulose. (A) MG1, MG2, and the salivary agglutinin (Agg.) were detected in most saliva samples when the nitrocellulose replica of the separated proteins was stained with anti-sLe^x, which recognizes sLe^x and related structures, excluding 6-sulfo sLe^x and 6′,6-disulfo sLe^x. (B) In contrast, only MG1 and the salivary agglutinin in some samples were detected when the replica was stained with the MECA-79 antibody, which recognizes an important class of sulfated, high-affinity L-selectin ligands. (C) When an identical replica was incubated with human L-selectin chimeras, the MG1 and salivary agglutinin bands that reacted with the MECA-79 antibody and the MG2 bands that reacted with anti-sLe^x supported the binding of L-selectin. (D) This interaction was significantly reduced in the presence of the MECA-79 antibody. Arrows indicate the top and bottom of the stacking gel.

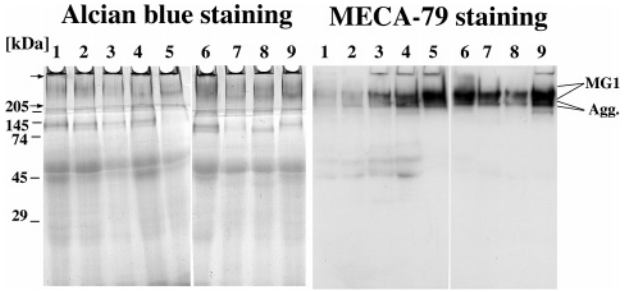

FIGURE 2: Salivary glycoproteins express the MECA-79 epitope during the window of receptivity, pregnancy, and lactation, suggesting hormonal regulation of the posttranslational processes that generate these specialized oligosaccharide structures. Whole saliva samples were collected from a single donor during the nonreceptive (lanes 1−3) and receptive (lanes 4 and 5) stages of the menstrual cycle. Additional samples were obtained from the same donor during pregnancy (lanes 6 and 7) and lactation (lanes 8 and 9). (Left) Samples were separated by SDS-PAGE and stained with Alcian blue, which allowed visualization of the glycoprotein bands in each lane. (Right) Gel of the same samples was transferred to nitrocellulose and stained with the MECA-79 antibody, which recognizes an important class of sulfated, high-affinity L-selectin ligands. Antibody reactivity dramatically increased as the window of receptivity approached. Likewise, antibody reactivity was high during pregnancy and lactation. Agg., the salivary agglutinin. Arrows indicate the top and bottom of the stacking gel.

agglutinin (Figure 1A). Consistent with the known genetic diversity in the expression of genes that regulate addition of the Lewis blood group antigens, there were many individual differences in staining intensities. Similar patterns, but higher immunoreactivity, were observed when a different blot was probed with the HECA-452 antibody, which recognizes sLe^x and related structures, including 6-sulfo sLe^x and 6′,6-disulfo sLe^x (data not shown). In contrast, when another replica was incubated with the MECA-79 antibody, which recognizes an important class of sulfated, high-affinity L-selectin ligands, MG1 and the salivary agglutinin were the major reactants (Figure 1B). In this case, fewer samples contained immunoreactive components.

To test the supposition that MECA-79 reactivity is synonymous with L-selectin ligand activity (*57*, *58*), we assayed the ability of the salivary glycoproteins/mucins that reacted with this antibody to support receptor binding. In these experiments, a different nitrocellulose replica of the same saliva samples was incubated with preformed complexes of human L-selectin/Fc chimeras and anti-human IgG under shear stress, which is required to reveal selectin−ligand interactions. As shown in Figure 1C, L-selectin bound strongly to the MG1 and salivary agglutinin bands that reacted with the MECA-79 antibody (compare Figure 1B and C). Consistent with our previous report (*53*), MG2, which presents oligosaccharides that react with sLe^x (Figure 1A) and HECA-452 (data not shown), also supported the binding of the L-selectin chimera (Figure 1C). In contrast, saliva samples with MG1 that reacted with anti-sLe^x and HECA-452, but not with MECA-79, failed to support L-selectin binding, suggesting masking of the sLe^x epitope which also serves as a ligand for L-selectin (*53*). The binding of L-selectin and its ligands was significantly reduced in the presence of the MECA-79 antibody (Figure 1D) or 10 mM EDTA (data not shown). This is consistent with the fact that selectin activity depends on the presence of calcium. The MECA-79 antibody also inhibited the binding of the L-selectin chimera to MG2 (Figure 1C and D). This observation was consistent with the results from additional experiments that showed that MG2 also expresses the MECA-79 epitope, albeit at much lower levels than those of MG1 and the salivary agglutinin (data not shown).

Cyclical Variations in Glycosylation of Salivary Proteins in Women. During the course of the experiments described above, we noticed day-to-day variations in the levels of MECA-79-reactive oligosaccharides in saliva samples obtained from female donors that were not observed in samples collected from male donors. Since our earlier studies showed that the MECA-79 epitope was also expressed on human uterine epithelial cells and the expression, which was low during the nonreceptive (follicular) phase, increased dramatically as the window of receptivity (luteal phase) approached (21), we theorized that glycosylation and/or sulfation of MG1 and the salivary agglutinin could also be hormonally regulated. We analyzed additional saliva samples collected every day from nine donors: seven women and two men. Six of the women had natural cycles; the day of ovulation was determined by measuring the luteinizing hormone surge using a Clear Blue Ovulation test kit.

The results of experiments in which we subjected a set of saliva samples from one female donor to immunoblot analysis with the MECA-79 antibody are shown in Figure 2. On day 1 after the start of menstruation, little immunore-

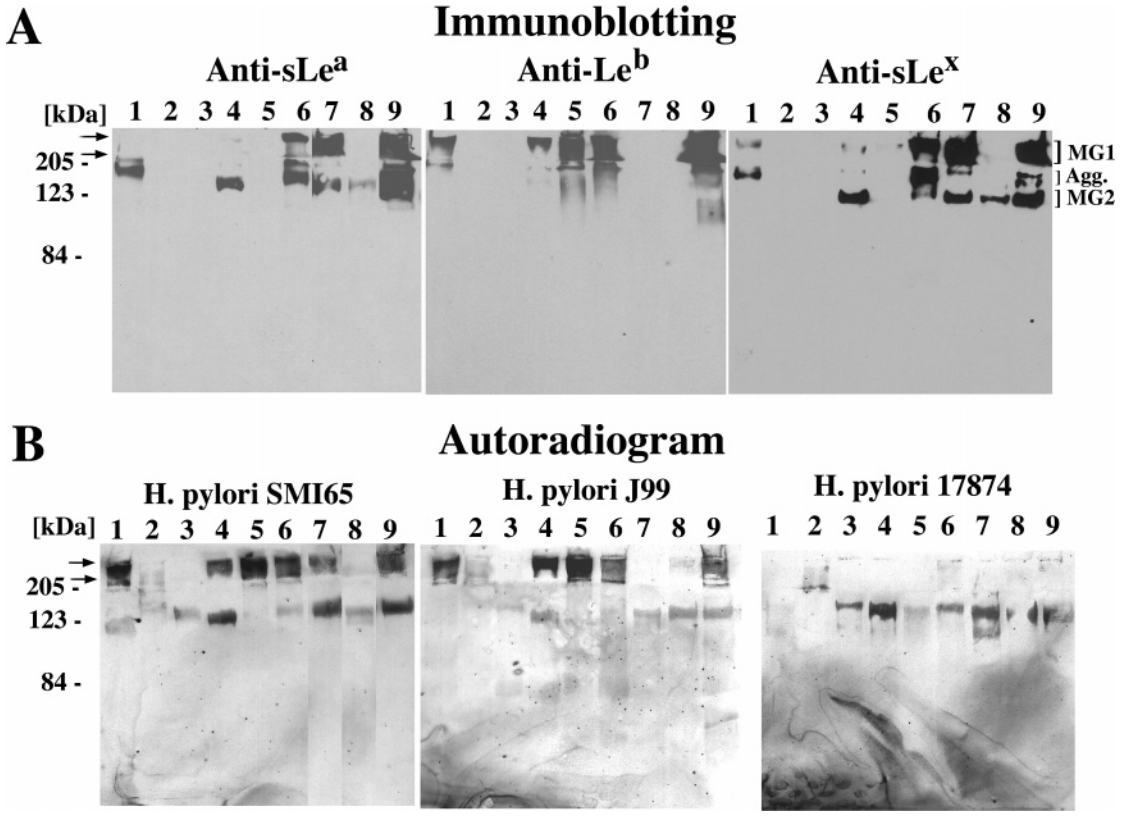

FIGURE 3: Human salivary glycoproteins express a diverse array of Lewis blood group antigens that interact with different *H. pylori* strains. (A) Whole saliva samples from nine donors (lanes 1−9) were separated by SDS-PAGE and transferred to nitrocellulose. The nitrocellulose replicas were stained with antibodies that recognize the sLe^a, Le^b, or sLe^x epitopes. Saliva samples from the majority of donors contained immunoreactive bands: MG1, the salivary agglutinin (Agg.), and/or MG2. Saliva from two donors (lanes 2 and 3) did not react with any of the antibodies tested. (B) Replicas of the same samples were overlaid with ^{35}S-labeled *H. pylori*. In most instances there was good agreement between the antibody reactivity and the bacterial adhesion data. Arrows indicate the top and bottom of the stacking gel.

activity was observed. But as the cycle continued, MECA-79 staining increased, with maximal reactivity a few days after ovulation, i.e., when the uterus was receptive to embryo implantation. The salivary agglutinin showed the same staining pattern. When the donor became pregnant, expression of the MECA-79-positive epitope remained high and continued to be high during lactation. Saliva samples from three other female donors showed the same pattern of cyclical variations in the expression of MECA-79-reactive oligosaccharides. Interestingly, expression of other carbohydrate epitopes (sLe^x, Le^a, Le^b, and the blood group antigens, e.g., A, B, H) on the salivary glycoproteins of these four donors did not change (data not shown). Samples from two other female donors had different patterns. One woman with a very long cycle (39 days) showed two peaks of MECA-79-reactivity—one before ovulation at day 16 and another after ovulation at day 32. The salivary glycoproteins of another woman did not express carbohydrate structures that were recognized by MECA-79. Likewise, samples from a postmenopausal woman and from two male donors, collected daily for 2 months, did not show day-to-day changes in MECA-79 reactivity. Together, these results are evidence that hormones can regulate, either directly or indirectly, the expression of carbohydrate modifications and that salivary glycoproteins have important biological roles.

MG1 Expresses Blood Group Antigens, Some of Which Support the Binding of H. pylori. First, we evaluated the expression of the Lewis blood group antigens in saliva samples from all of our donors. This allowed us to select for further study a subset of samples from nine donors who represented the entire range of blood group antigen expression patterns observed. Consistent with our previous results (*50*, *53*, *54*, *64*), MG1 and MG2 often reacted with anti-Le^a (data not shown), anti-sLe^a, anti-Le^b, and/or anti-sLe^x (Figure 3A). The salivary agglutinin, identified by anti-gp-340 reactivity, also stained with these antibodies. The salivary components of donors 2 and 3 had no immunoreactivity when evaluated with this panel of antibodies.

Next, we tested the ability of salivary components from these nine donors to support the adherence of bacteria that colonize the oral cavity. Interestingly, MG1 did not support the binding of any of the 18 strains of oral bacteria that we examined (data not shown), many of which interact with MG2 (*52*). Given the repertoire of blood group substances that MG1 carries, we were interested in the possibility that this mucin interacts with *H. pylori*. *H. pylori* strains SMI65, SMI27, J99, 17874, 26695, and MO19 were chosen because their binding activity with blood group antigens was previously characterized (*40*). The results are summarized in Table 1. The adherence patterns of *H. pylori* strains to nitrocellulose membrane replicas of salivary glycoproteins are shown in Figure 3B. SMI65, which interacts with sLe^a, Le^b, and sLe^x (Table 1), bound to MG1, MG2, and/or the salivary agglutinin in most of the samples. In many cases, there was concordance between the antibody reactivity and bacterial adhesion data. For example, few bacteria bound to the

Table 1: Binding of *H. pylori* to Soluble Fucosylated and Sialylated (Lewis) Antigen−Conjugates[a]

	binding of antigen-conjugates (%)		
H. pylori strain	sLe^a	Le^b	sLe^x
SMI65	49	33	60
J99	12	94	14
17874	4	2	47
SMI27	58	90	30
26695	>2	>2	>2
MO19	>2	>2	>2

[a] Adapted from ref 40.

salivary components of donors 2 and 3, which showed no antibody reactivity. Another *H. pylori* strain (SMI27), which interacts with the same oligosaccharide epitopes as SMI65, showed essentially the same adherence pattern (data not shown). Strain J99, which interacts with Le^b, primarily adhered to MG1 in samples where this mucin reacted with anti-Le^b. In accord with our previously published structural studies showing that MG2 of many individuals carries sLe^x, we found that *H. pylori* strains SMI65, SMI27, 17874, and J99 bound to this molecule. The binding intensities corresponded proportionately to the sLe^x binding activity previously characterized by using soluble antigen−conjugates (compare Figure 3B and Table 1). For example, SMI65 showed the highest level of binding and J99 the lowest. However, some exceptions were noted. For example, the salivary agglutinin of donor 2, which did not express the blood group antigens, weakly supported adherence of *H. pylori* strains; in other samples (donors 6, 7, and 9), the agglutinin that carried sLe^x did not interact with these bacterial strains. Additionally, no adherence to MG1 was detected, even in samples with strong anti-sLe^x reactivity (donors 6, 7, and 9), suggesting the possibility of steric hindrance. As expected, two *H. pylori* strains (26695 and MO19) that do not interact with Le blood group epitopes failed to bind to replicas of the nine saliva samples tested (data not shown). Finally, there was no correlation between expression of the MECA-79-reactive epitopes and *H. pylori* adherence (data not shown), suggesting that these structures do not serve as receptors for this bacterium. Together the results of these studies show that highly glycosylated salivary proteins, including MG1, MG2, and the salivary agglutinin, interact, through the carbohydrate structures they carry, with *H. pylori* strains that bind the relevant oligosaccharide species.

DISCUSSION

Previously we showed that MG2, which carries multiple sLe^x saccharide termini that are low-affinity L-selectin ligands, supports tethering and rolling of leukocytes under shear stress (*52*, *53*). This finding prompted us to investigate whether salivary glycoproteins also express the highly specialized sulfated carbohydrate epitopes that constitute the high-affinity class of L-selectin ligands. Accordingly, we analyzed saliva samples from 31 donors by probing nitrocellulose membrane replicas of salivary proteins with monoclonal antibodies that recognize different subsets of the carbohydrate portions of L-selectin ligands. Consistent with our previous studies (*52*−*54*), we found that MG1, MG2, and the salivary agglutinin stained with anti-sLe^x in most saliva samples. When an identical nitrocellulose membrane replica was stained with MECA-79, antibody reactivity, which was predominantly confined to MG1 and the salivary agglutinin, was detected in approximately half the samples. These results suggest that there is heterogeneity in glycosylation and/or sulfation of human salivary glycoproteins. This is not unexpected, since both posttranslational modifications require the coordinated actions of several transferase enzymes that are encoded by specific genes. For example, the glycosyltransferases responsible for the expression of Lewis blood group antigens on glycoproteins (e.g., salivary components) and glycolipids are encoded by a subset of fucosyl transferase genes (*30*, *50*). These enzymes catalyze the transfer of fucose to specific sites in precursor oligosaccharide chains.

Like glycosylation, sulfation requires sulfotransferase enzymes encoded by specific genes. Recently, a group of glycosyl sulfotransferases (GSTs) that generate the aforementioned sulfation modifications (Gal-6-SO_4 and GlcNAc-6-SO_4) were characterized at the molecular level (*12*, *18*, *25*, *29*). Thus far, six murine GSTs have been identified (GST-0−GST-5). Previous studies showed that, while GST-0, GST-1, GST-2, and GST-5 have broad tissue expression patterns (*18*, *31*, *67*−*69*), GST-3 is restricted to endothelial cells in HEVs. Accordingly, this enzyme is likely to be the L-selectin ligand sulfotransferase (*7*, *31*). In contrast, GST-4 was detected only in intestinal tissues (*38*). In humans, seven GSTs exist (*25*). We predict that the human homologues of GST-3 and -4 are also expressed in salivary glands.

We also investigated whether the MECA-79 epitope on salivary glycoproteins could support L-selectin binding. Our strategy exploited the observation that recombinant L-selectin chimeras can be used to detect their functional ligands (*7*, *19*, *49*). When nitrocellulose replicas of salivary proteins were incubated with human L-selectin chimeras and anti-human IgG under shear stress, L-selectin bound strongly to the MG1 and salivary agglutinin bands that expressed the MECA-79 epitope. Taken together, our results demonstrate for the first time that salivary glycoproteins express high-affinity endothelial L-selectin ligands. This result is in accord with our previous report that MG1 carries complex sulfated saccharides (*64*). We are in the process of characterizing these oligosaccharides in detail.

Concurrently, our group is investigating the repertoire of carbohydrate structures that are presented on the surface of human uterine epithelial cells. Interestingly, we detected both the Le blood group antigens and MECA-79 epitopes (*21*). Additionally, expression of the MECA-79 epitope, which was low during the nonreceptive (follicular) phase of the cycle, increased dramatically as the window of receptivity (luteal phase) approached. This result suggests that glycosylation and/or sulfation of uterine mucins is either directly or indirectly hormonally regulated. In the present study, we investigated whether this phenomenon occurred outside the uterus. Surprisingly, saliva samples collected daily from many of our female donors showed a marked upregulation of MECA-79-reactive oligosaccharide epitopes displayed by MG1 and the salivary agglutinin during the window of receptivity. This finding suggests the possible concordance of a portion of the carbohydrate structures expressed by highly glycosylated uterine and salivary glycoproteins. This discovery could explain the enigmatic association between

preterm labor and periodontal disease (*48*) in terms of shared carbohydrate receptors on oral and uterine surfaces that promote colonization of pathogenic bacteria.

Finally, we obtained new information about the carbohydrate receptors displayed by salivary proteins in terms of their bacterial receptor properties. In a previous study, we showed that MG2 supports adherence of several oral bacterial strains via saccharide termini carrying T antigen, sialyl-T antigen, and/or lactosamine determinants (*52*). In contrast, no interactions were detected with the sLe^x epitope that supports leukocyte rolling and tethering, suggesting that different oligosaccharide structures mediate adherence of different kinds of cells. Here we report that MG1 did not bind to any of the 18 strains of oral bacteria that we tested, including those that interact with MG2 (*52*). In the absence of data to support a role for MG1 in adhesion of oral species, we tested the hypothesis that this mucin serves as a receptor for *H. pylori*. The impetus was 3-fold. First, *H. pylori* interacts with the classes of fucosylated oligosaccharides that MG1 carries; different strains express adhesins that recognize specific determinants, e.g., sLe^a, Le^b, and sLe^x (*10*, *32*, *40*, *56*). Second, *H. pylori* adherence has great medical importance. This organism infects the gastric mucosa of about half of all people worldwide, causing peptic ulcer disease and increasing an individual's risk of gastric cancer (reviewed in ref. *34*). Third, *H. pylori*'s interactions with MG1 were previously reported (*11*, *70*). As expected, our results indicate that MG1 supports adherence of *H. pylori* strains and that there is a correlation between Lewis blood group status and *H. pylori* binding receptivity. Currently, oral−oral and oral−fecal routes are considered the most likely methods of transmission of *H. pylori*. Our results support the possibility of oral transmission, which could involve adherence in the mouth (*15*). Eradication of *H. pylori* infection with antibiotic treatment has proved to be difficult in patients who harbor *H. pylori* in the oral cavity/dental plaque (*44*). Thus, we note that our results also suggest new pharmacologic strategies for inhibiting infections by this organism. In contrast, both MG1 and agglutinin may serve as natural clearance agents for pathogenic bacteria such as *H. pylori*. Finally, it is interesting to consider the possibility that Lewis blood group antigens and L-selectin ligands are coexpressed in other parts of the gastrointestinal tract. Preliminary immunolocalization results suggest that this may occur in the stomach as well as the oral cavity. The implications with regard to *H. pylori* adhesion and leukocyte recruitment to site of infection remain to be determined but may likely involve an upregulation of sLe^x expression (*40*).

Taken together, our data suggest that MG1, MG2, and the salivary agglutinin have evolved to perform important, complementary functions within the oral cavity. Whereas MG2 presents low-affinity L-selectin ligands, MG1 and the salivary agglutinin, in some individuals, react with antibodies that specifically recognize the high-affinity species. Thus, salivary mucins can interact with leukocytes over a broad range of shear stress conditions. Likewise, these salivary components appear to play complementary roles in bacterial adhesion. The salivary agglutinin reacts with the widest array of oral bacteria (*54*), fewer species adhering via MG2. Interestingly, all three components can potentially interact with *H. pylori*. We speculate that individual differences in the carbohydrate structures presented by salivary proteins are important factors in determining the bacterial ecology of the oral cavity and, possibly, portions of the gastrointestinal tract.

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