Sulfated glycans on oral mucin as receptors for *Helicobacter pylori*

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*Helicobacter pylori* is able to colonize gastric epithelia, causing chronic active gastritis, gastric and duodenal ulcers and presumably gastric malignancies. Attempts to identify the natural reservoir for this microorganism other than the stomach have been unsuccessful. It is suspected that *H. pylori* can be transmitted orally, since the microorganism has been detected at various sites of the oral cavity. The aim of the present study was to determine whether *H. pylori* can bind to salivary mucins, which in vivo coat the oral epithelia, and characterize further the interaction. Binding of salivary mucins and of synthetic oligosaccharides was studied in ELISA and immunoblotting, using specific monoclonal antibodies, and synthetic neoglycoconjugates. *H. pylori* bound most avidly to a highly sulfated subpopulation of high molecular weight salivary mucins, secreted from the palatine salivary glands, and with less avidity to mucin species secreted by the sublingual and submandibular salivary glands, which are less sulfated. Binding was strongly enhanced upon decreasing pH from 6.0 to 5.0. Using synthetic polyacrylamide coupled oligosaccharides it was found that SO₃⁻-3-Gal and the SO₃⁻-3-Lewis antigen (Bore"n et al., 1989; Wood et al., 1991), sialyl-lactose (Evans et al., 1988), and the blood group-related Lewis antigen (Borén et al., 1993) in view of the suggested oral transmission route of *H. pylori*, we studied the interaction between salivary components and *H. pylori*. It was found that *H. pylori* bound preferentially to highly sulfated mucins secreted by the palatine glands, and that this interaction was enhanced upon lowering pH. Furthermore, we have identified a number of potential oligosaccharide receptors of *H. pylori*, including sulfated Lewis antigen and galactose-3-sulfate.

**Introduction**

*H. pylori* is a causative agent in chronic active gastritis, gastric and duodenal ulcers, and presumably gastric malignancies. No natural reservoir for this microorganism other than the stomach have been found as yet. *H. pylori* might be transmitted via the oral route, since the microorganism has been detected at various sites of the oral cavity, by culture as well as by polymerase chain reaction (Banatvala et al., 1993; Ferguson et al., 1993; Nguyen et al., 1993; Li et al., 1995; Namavar et al., 1995). Because the oral cavity functions as the port of entry to the gastro-intestinal system, defensive systems in saliva are relevant not just for maintenance of the oral health but for the entire organism. In this context, defining the factors involved in the attachment of this pathogen to components of the oral cavity is important. Human saliva contains several proteins implicated in the protection of the oral cavity against bacterial colonization, including slgA, lysozyme, proline-rich proteins, and the high- and low-Mₘ molecular weight salivary mucins, MG1 and MG2, respectively (LOOMIS et al., 1987; LAMKIN and OPPENHEIM, 1993; NIEUW AMERONGEN et al., 1995). Mucins are a family of highly glycosylated proteins (carbohydrate > 80%), which cover and protect epithelial tissues throughout the human body. High Mₘ salivary mucins are secreted mainly by the (sero)mucous submandibular, sublingual, and palatine glands. As main constituents of mucous layers investing the oral tissues, mucins are the first sites of attachment for microorganisms that colonize the oral cavity. On the other hand, salivary mucins, in particular MG2, acting as soluble receptor analogs for bacterial adhesins, promote oral clearance of microorganisms. High Mₘ mucins contain a wide spectrum of structurally different oligosaccharide side chains, some of which carry blood group active antigens (e.g., ABH, Lea, Leb) (Prakobphol et al., 1993), and may function as receptors for bacterial adhesins. For example, *H. pylori* binding to gastric mucins (PIOTROWSKI et al., 1991; TZOUVELEKIS et al., 1991) is mediated by sulfated carbohydrate side-chains, which have not been further characterized. In addition, a number of other structures have been implicated in the attachment of *H. pylori* to gastric epithelial cells, viz. phosphatidylethanolamine (Lingwood et al., 1992), GM3 ganglioside (Slomiany et al., 1989; Saitoh et al., 1991), sialyl-lactose (Evans et al., 1988), and the blood group-related Lewis antigen (Borén et al., 1993). In view of the suggested oral transmission route of *H. pylori*, we studied the interaction between salivary components and *H. pylori*. It was found that *H. pylori* bound preferentially to highly sulfated mucins secreted by the palatine glands, and that this interaction was enhanced upon lowering pH. Furthermore, we have identified a number of potential oligosaccharide receptors of *H. pylori*, including sulfated Lewis antigen and galactose-3-sulfate.

**Results**

To find out whether salivary components are involved in binding of *H. pylori*, we have tested which of the major salivary proteins can bind in vitro to *H. pylori*. Of the proteins tested only the high Mₘ salivary mucin MG1 was bound. The other salivary proteins, viz. α-amylase, lysozyme, proline-rich proteins, and cystatin S did not bind to *H. pylori*, either in ELISA or on immunoblots (data not shown).

The binding of high Mₘ salivary mucins (MG1) was further investigated in ELISA, in Western blots, and in immunoelectron microscopy. In the present study two strains of *H. pylori* have been used, *H. pylori* NCTC 11637 and strain 3B3, isolated from the subgingival dental plaque of a gastritis patient. Although in this article the results are shown that were obtained with strain NCTC 11637, essentially the same results were obtained with strain 3B3.

Figure 1 shows the binding of MG1 to *Helicobacter pylori*.
Binding of high molecular weight salivary mucins (MG1) to Helicobacter pylori at various ionic strength and pH values. After increasing the NiCl₂ concentration from 25 to 500 mM, a gradual decrease in binding of MG1 was observed (Figure 1A). On the other hand, lowering the pH from 6.0 to 5.0 resulted in a profound increase in binding (Figure 1B). These results indicate that under the physiological pH- and hypotonic conditions present in saliva, H. pylori can bind to the high molecular weight salivary mucin species MG1. Similar results were obtained when saliva or purified salivary mucins from donors with different blood groups (three blood group A, two secretors and one non-secretor; one blood group B, secretor) were tested, or when detection was conducted with another anti-MG1 mAb IN A (not shown). The ionic strength dependency suggested that the binding was governed by electrostatic interactions. No specific effect of calcium, magnesium, or sulfate ions, other than due to ionic strength, was observed (not shown). In contrast, divalent metal ions in low concentrations (1 mM) profoundly enhanced the MG1 binding to Helicobacter pylori at neutral pH values, in the order Ni²⁺ > Zn²⁺ > Fe²⁺ (Figure 1C). When binding was tested at different Zn²⁺ concentrations, it was found that maximal enhancing effect of Zn²⁺ ions occurred between 0.05 and 0.1 mM (not shown).

Binding of salivary mucins to H. pylori was confirmed by immunoblotting with mAbs F2 and E9. Figure 2 shows the immunoblots of the proteins extracted from H. pylori after incubation with saliva. In confirmation with the results of the ELISA (Figure 1), it was found that H. pylori bound to high M₃ mucins (Figure 2, lane 2). Probing with anti-mucin mAb E9 revealed that the mucin fraction that bound to H. pylori contained, relative to the starting material, only low amounts of the E9 epitope, i.e., sialylated Lewisa (compare Figure 2, lanes 4 and 5). These results, therefore, suggested that H. pylori preferentially bound to a mucin subpopulation that is enriched in F2 epitopes, i.e., sulfo-LewisX. The suggestion that binding of salivary mucins was mediated by receptors on Helicobacter pylori recognizing sulfated glycoconjugates was corroborated by the finding that mucin binding was inhibited best by sulfated biopolymers, including chondroitin sulfate and heparin (Figure 3). After removal of sulfate from salivary mucins by acid solvolysis, a 4-fold lowering in binding avidity was noted, again pointing to a role for sulfate residues in the binding (not shown). In contrast, sialic acid residues appeared not to be essential in this respect, since neither oxidation by sodium-periodate, nor mild acid hydrolysis of salivary mucins, which removed all sialic acid residues from the mucin, had an effect on binding (not shown).

Immunoelectron microscopy of H. pylori after incubation with purified mucins and labeling with an anti-MG1 mAb, is shown in Figure 4, illustrating that binding of mucin occurred to structures present at the periphery of the cell. To examine further the involvement of sulfated mucins, we conducted binding experiments with isolated salivary MG1 species with different sulfate contents. ELISAs with several purified MG1 species, isolated from segregated collected glandular secretions, indicated that the highly sulfated palatine MG1 species bound with the highest avidity (Figure 5), which is in agreement with the results of the immunoblots. At pH of 5.0, binding of palatine MG1 was saturated at a concentration as low as 0.2 μg/ml, whereas under these conditions virtually no binding of H. pylori to the low sulfated submandibular MG1 species could be demonstrated. However, for all MG1 species tested, including submandibular MG1, binding increased upon lowering the pH from 6.0 and 4.0. The order in binding affinities (palatine MG1 > sublingual MG1 > submandibular MG1)
Fig. 2. Immunoblotting of salivary mucins extracted from H. pylori after incubation with saliva. Saliva was 2-fold diluted in 50 mM sodium acetate buffer, containing 150 mM sodium chloride, pH 5.0; 10^7 H. pylori cells were incubated with the diluted saliva at 37°C for 2 h. Adhered salivary components were extracted, separated by electrophoresis (4-15% gradient polyacrylamide gels), transferred onto nitrocellulose filters and probed with mAb F2 (lanes 1-3) and mAb E9 (lanes 4-6). Lanes 1 and 4, whole saliva (starting material); lane 2 and 5, H. pylori extract, after incubation with saliva; lanes 3 and 6, Control extract, H. pylori, not incubated with saliva. Arrow indicates the position of the high Mr salivary mucin MG1.

Fig. 3. Effect of polyanions on binding of salivary mucins to Helicobacter pylori. Saliva was 2-fold serially diluted (in duplicate) in 50 mM sodium acetate buffer (pH 5.0), 150 mM NaCl, 0.5% Tween, containing 1 µg/ml of the indicated polyanion and incubated with Helicobacter pylori coated onto polystyrene microtiterplate wells for 2 h at 37°C. Bound mucins were detected with mAb F2.

Discussion
A number of studies (Banatvala et al., 1993; Ferguson et al., 1993; Nguyen et al., 1993; Li et al., 1995; Namavar et al., 1995) have demonstrated the presence of H. pylori at oral epithelia, such as the palate and the cheeks, in saliva and in dental plaque of patients suffering from gastric infections. These stud-
Table I. Characteristics of high-molecular weight salivary mucin preparations (MG1) from different salivary glands, used in this study

<table>
<thead>
<tr>
<th>Mucin species</th>
<th>Number of residues per molecule*</th>
<th>Reactivity towards mAbb</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Sialic acid</td>
<td>Sulfate</td>
</tr>
<tr>
<td>Sublingual</td>
<td>359</td>
<td>219</td>
</tr>
<tr>
<td>Submandibular</td>
<td>647</td>
<td>135</td>
</tr>
<tr>
<td>Palatine</td>
<td>194</td>
<td>1239</td>
</tr>
</tbody>
</table>

*Bolscher et al. (1995), assuming a molecular weight of 1,000,000 Da.


Donor blood group A, non-secretor.

ites suggested that the oral cavity harbors H. pylori and may be a source of (re)infection and transmission. In keeping with this, the present study showed that salivary mucins under the hypotonic conditions operating in vivo, can support binding of the gastric pathogen H. pylori, and that binding is enhanced upon lowering pH. Under physiological conditions, the pH in saliva fluctuates between 5.8 and 7.8 (Jenkins, 1978), but pH values as low as 4.0 have been measured in carbohydrate metabolizing dental plaque (Jensen and Schachtele, 1983). Thus, salivary mucins as constituents of mucous layers coating oral surfaces (Nieuw Amerongen et al., 1987; Bradway et al., 1992) can provide sites of attachment for H. pylori.

The present study indicates that in vitro H. pylori binds best to a highly sulfated subpopulation of salivary mucins (Figure 5).
The involvement of sulfated oligosaccharide side chains in the binding is further corroborated by the findings that sulfated neoglycoconjugates display highest binding and compete effectively with salivary mucins for the receptor on *H. pylori* (Figure 7). These results are in line with previous data showing that *H. pylori* bind to sulfated biomolecules, including sulfatides (Huesca et al., 1996; Kamisago et al., 1996), heparan sulfate (Ascencio et al., 1993) and a sulfated subpopulation of gastric mucins (Piotrowski et al., 1991).

From the difference in binding properties of sulfo-Lewis^b^ at one hand, and sialyl-Lewis^a^ and Lewis^a^ at the other, it can be inferred that the presence of the small negatively charged sulfate residue at the terminal galactose-residue of the Lewis^a^ antigen favors binding, and furthermore that sialic acid cannot substitute for sulfate in this respect. In the present study we did not find any support for a prevailing role of the Lewis^b^ antigen, which previously has been identified as a receptor of *Helicobacter pylori* in gastric epithelial cells (Borén et al., 1994), since *H. pylori* did hardly bind to synthetic Lewis^b^, whereas at the other hand salivary mucins from a non-secretor, missing the Lewis^b^ antigen, are readily bound (cf. Figure 1).

The presently found pH-dependency suggests that protonation of weakly acidic groups is critical for binding of salivary mucins to their receptors on *H. pylori*. The finding that metal ions, including nickel and zinc, enhance binding of *H. pylori* to salivary mucin points also to a role for cysteine and histidine residues, which often provide binding sites for these metal ions (Gilbert et al., 1995). Interestingly, *H. pylori* expresses several proteins displaying high affinity for nickel, including urease (Hawtin et al., 1991), membrane proteins implicated in nickel transport (Mobley et al., 1991) and heat shock proteins (Suerbaum et al., 1994) which have been implicated in sulfatide recognition (Huesca et al., 1996). Based on the present findings, it is speculated that nickel binding proteins play a role in the binding of *H. pylori* to its receptors.

**Materials and methods**

**Bacterial strains**

*H. pylori* strain NCTC 11637 and clinical strain 3B3 (Namevar et al., 1995) were grown in Brucella broth (Difco) supplemented with 5% fetal calf serum and incubated under microaerophilic condition at 37°C. After 48 h, cultures were harvested, washed twice with phosphate-buffered saline (PBS, pH 7.2), and resuspended to an OD_{500} of 0.1 and stored at −80°C until use.

**Antibodies**

Production and characterization of anti-mucin monoclonal antibodies F2 and E9 have been described previously (Rathman et al., 1990; Veerman et al., 1991). Antibody F2 recognizes (SO)_{3}-Lewis^a^ (Veerman et al., 1997). Antibody E9 is directed to the sialyl-Lewis^a^ antigen (E. Veerman, unpublished observations). In addition, salivary blood group A mucins were monitored with anti-MGl mAb INA, directed against the bloodgroup A antigen. Gastric mucins were monitored with mAb 19-OLE to H-type 2, obtained from Biosprobe, Amsterdam, The Netherlands. Production and characterization of the monoclonal antibody to salivary cystatin S has been described previously (Rathman et al., 1990). Rabbit polyclonal antibodies to lysozyme and amylase were from Sigma, St. Louis, MO. Polyclonal antisera against acidic proline-rich proteins was a kind gift from Dr. M. J. Levine, Buffalo, NY). Biotin labeled streptavidin was a kind gift from Dr. R. Negri, Brescia, Italy.

**Glycoconjugates**

Collection of whole saliva, segregated collection of submandibular, sublingual and palatine saliva, as well as isolation and immunochemical characterization of the high molecular weight mucin fractions thereof have been described previously (Veerman et al., 1992, 1995; 1996; Bolscher et al., 1995). Partial (immuno)chemical characterization of the isolated glandular mucin species are given in Table I. Gastric mucins were isolated with a method that previously has been described for isolation of salivary mucins (Veerman et al., 1989). In short, using a glass rod the mucous layer was gently scraped off from animal tissue obtained during surgery, and incubated in 50 mM Tris buffer (pH 7.2) containing 8 M urea, 0.5 M NaCl, 0.2 M KSCN, 10 mM EDTA, 10 mM benzamidine-HCl, and 1 mM PMSF at 4°C for 3 days. Then, undissolved material was removed by centrifugation at 50,000 x g for 20 min. The resultant supernatant was subjected to ultracentrifugation for 24 h at 100,000 x g. The obtained pellet was dissolved in water, dialyzed against distilled water, and freeze-dried.

Oxidation of salivary mucin carbohydrates was performed by incubation of purified mucins with 5 mM sodium metaperiodate (Veerman et al., 1995). Periodate treatment resulted in the complete loss of binding to mAb E9, whereas reactivity toward mAb F2 was not affected (Veerman et al., 1996). Mild acid treatment of salivary mucin was conducted by incubation of purified salivary mucins with 0.05 M HCl at 80°C for 1 h. Carbohydrate analysis showed that by this treatment virtually all sialic acid residues are removed, while the epitope recognized by mAb F2 remains intact (Veerman et al., 1995). Desulfation of mucins was conducted by acid solvolysis in anhydrous methanol, containing 0.05 M HCl at 32°C for 3 h. To enable subsequent detection in binding assays, the desulfated preparation and the parent native mucin preparation were labeled with digoxigenin (DIG) in simultaneous parallel incubations using the DIG-glycan labeling kit, according to the manufacturer's in-
neoglycoconjugates were omitted, indicated that direct binding of antibodies to LeLe' carriers, used in this study, in which neoglycoconjugates were coated directly to polystyrene wells with subsequent immunoenzymatic detection using anti-DIG peroxidase. Identical dose-response curves were obtained, justifying mutual comparison of signals obtained in binding studies.

Immunoblotting of salivary components extracted from H. pylori after incubation with saliva was conducted as follows: saliva was 2-fold diluted in 50 mM sodium acetate buffer, containing 150 mM sodium chloride, pH 5.0. A volume of 1 ml of diluted saliva was incubated with 107 H. pylori cells at 37°C for 2 h. After washing, bound antibodies were detected with horseradish peroxidase conjugated to anti-rabbit IgG. Antibodies (-1 μg/ml) or polyclonal antibodies (diluted 1:500). After washing, bound antibody was detected using alkaline-phosphatase conjugated to rabbit anti-mouse immunoglobulins, or to sheep anti-rabbit immunoglobulins.

Table II. Synthetic carbohydrates, multivalently bound to polycrylamide carriers, used in this study

<table>
<thead>
<tr>
<th>Name Structure</th>
<th>Name Structure</th>
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<tr>
<td>H1 antigen</td>
<td>Fuc1-2Galβ1-3GlcNACβ-R</td>
</tr>
<tr>
<td>Le'</td>
<td>Galβ1-3(Fucose-4GlcNACβ-R</td>
</tr>
<tr>
<td>Le</td>
<td>Fuc1-2Galβ1-3(Fucose-4GlcNACβ-R</td>
</tr>
<tr>
<td>Sialyl-Le'</td>
<td>Neu5Acα2-3Galβ1-4(Fucose-3GlcNACβ-R</td>
</tr>
<tr>
<td>Sulfot-Le'</td>
<td>SO3-3Galβ1-3(Fucose-4GlcNACβ-R</td>
</tr>
<tr>
<td>Sulfot-3-galactose</td>
<td>SO3-3GlcNACβ-R</td>
</tr>
<tr>
<td>Sulfonated N-acetylgalactosamine</td>
<td>SO3-5GlcNACβ-R</td>
</tr>
</tbody>
</table>

Structure of the synthetic biotinylated polymer (R) to which oligosaccharides are attached.

Immunelectron microscopy

H. pylori cells grown for 2 days were suspended in PBS to approximately 10^11 cells per ml. Cells were collected by centrifugation, and incubated with purified salivary mucin (0.5 ml, 100 μg/ml) for 1 h. Cells were washed three times to remove nonbound mucin and applied to Formvar carbon coated grids, which were subsequently incubated with mAb for 30 min. The reaction of mAb with bacterial cells was visualized using a colloidal gold (particle size 6 nm) conjugated goat-anti-mouse polyclonal serum (diluted 1:20 in PBS/glycine, obtained from Aurody, the Netherlands).

References


Helicobacter binding to an oral mucin receptor


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