Inhibition of *Helicobacter pylori* sialic acid-specific haemagglutination by human gastrointestinal mucins and milk glycoproteins

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Abstract

*Helicobacter pylori*, a human gastric pathogen causing chronic gastritis and duodenal ulcer disease, has been found in large amounts in gastric mucous gel layer. Mucin preparations, separated from human gastric juices and isolated from different colon regions, were examined for their ability to inhibit haemagglutination of *H. pylori* with the emphasis on evaluating the role of sialic acid-dependent haemagglutinins of the bacteria in colonisation of the stomach. The mucins showed high inhibitory activity for *H. pylori*, which was significantly decreased after the removal of sialic acids from the mucins. The inhibitory potencies using high molecular mass mucin-like components from bovine milk were comparable with those obtained for gastric mucins, suggesting their possible role in the prevention of *H. pylori* infection.

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1. Introduction

*Helicobacter pylori* is a human gastric pathogen which colonises the mucus layer and gastric epithelium and has been associated with type B gastritis and peptic ulcer disease [1]. The attachment of *H. pylori* to the human gastric mucosa is a complex process involving several specific structures recognised by the cell surface receptors [2,3]. *H. pylori* sialic acid-binding haemagglutinin(s) represent a group of such putative cell surface adhesins, proposed to be crucial for the bacterial colonisation of the stomach [4].

Gastrointestinal mucins, high molecular mass glycoproteins which are synthesised and secreted by mucus cells of the human stomach and intestine, show regional variations in composition and expres-
sion. The terminal region of O-glycosidically linked carbohydrate chains consist of sialic acid, fucose and N-acetylglalctosamine in addition to galactose and N-acetylglucosamine [5]. An increase in sialylation has been demonstrated for mucosal cells infected with *H. pylori* [6]. A possible association between the occurrence of O-acetylated sialic acids in intestinal metaplasia and *H. pylori* infection has also been reported [7]. Controversial results on the role of sialic acids and sulfate for *H. pylori* binding to gastric mucins have been reported. For example, in one study the influence of sulfation of gastric mucins for *H. pylori* binding to gastric mucosa has been shown excluding the role of sialic acids in haemagglutination inhibition assays [8]. In contrast, *H. pylori* binding to human gastric mucin in an enzyme-linked immunosorbent assay was demonstrated to be at least partly sialic acid-dependent [9]. Also for human salivary mucins the importance of both sialylated [10] and sulfated [10] components as receptor structures for *H. pylori* adhesion has been reported. In addition, the expression of different binding characteristics depending on growth conditions of the bacteria has been demonstrated. For example, two different sialic acid-binding adhesins were described for agar- and broth-cultured bacteria with the specificities for sialylated glycoproteins and sialylated glycosylceramides [11].

Recently, we have demonstrated that *H. pylori* strains can be classified into sialic acid-independent and -dependent strains specific for terminal α-2,3-linked sialic acids [12]. In this study we were interested to compare these strains with respect to their binding to gastric mucins to evaluate the role of sialomucins and sialic acid-specific adhesin(s) of *H. pylori* in human stomach infection by these bacteria.

2. Materials and methods

2.1. Materials

Human red blood cells (RBC) were obtained by venipuncture from a healthy donor. Sialidase (*Vibrio cholerae*) was purchased from Behringwerke AG (Germany). Fat globule membranes (MFGM-1) were prepared from bovine buttermilk obtained from a butter-making plant of Snow Brand Milk Products Co. Ltd. To remove casein by coagulation, rennet treatment was done at pH 6.2. HR-reennet (0.003% w/v) (Christian Hansen, The Netherlands) was added under mild agitation and then incubated at 30°C for 27 min until most of the casein was coagulated to form curd. After cutting the curd the mixture was heated slowly for 40 min to a final temperature of 40°C and kept at this temperature for a further 60 min. Subsequently, the curd was separated from whey containing MFGM using a filter cloth. Prepared whey solution was recirculated through a microfilter membrane (Carbosep M-14; 0.14 μm pore size, Techsep, France) to remove proteins and carbohydrates from the whey and to concentrate MFGM in the retentate. Diafiltration was performed by concentrating the whey to 30% of its original volume then washing with water 4 times the volume of the retentate under the following operating conditions: flow rate, 600 l min⁻¹; trans-membrane pressure, 2 kg cm⁻²; temperature, 50°C, and lyophilised and used as MFGM-1. This material was delipidated to give MFGM-2 by extraction with chloroform:methanol (1:1) followed by chloroform:methanol (2:1). Glyco-macropeptide (GMP) from bovine milk was prepared from bovine milk as described [13]. Sialyl(α-2,3)-lactose was prepared from bovine colostrum as described [14].

2.2. Mucin preparations

Human gastric juices, collected from individuals with blood type A, B, AB and O, were fractionated on lysine-Sepharose at pH 2.0. The adsorbed sulfate-rich glycoprotein fraction was eluted with 50 mM Tris-HCl buffer, pH 7.2, containing 1 M NaCl [15].

Human colonic mucins from different regions (cecum, ascending colon, sigmoid colon and rectum) were prepared as described previously [16]. Mucin preparations were analysed for sialic acid content using the orcinol/Fe³⁺/HCl method and for the diversity of sialic acids by fluorometric HPLC after mild acid hydrolysis [17,18]. For this 5 μl of each sample was lyophilised and hydrolysed in 100 μl of 2 M propionic acid at 80°C for 4 h [19]. After a 100 000×g centrifugation step in a Beckman TLA 45 rotor for 10 min, the supernatant was lyophilised
and the residue hydrolysed with 100 μl 0.1 M HCl at 80°C for 50 min and lyophilised. Both hydrolysates were combined in a minimum of water and lyophilised again. The samples were dissolved in 10 μl of 2 M acetic acid. After the addition of 49 μl 1,2-diamo-4,5-methylenedioxybenzene reagent, the mixture was heated for 1 h at 56°C in the dark. Separation was carried out on a RP18-column (Merck, LiChrospher 100, 250×5 mm i.d., particle size 5 μm) using methanol/acetonitrile/water (7:9:84, v/v) in isocratic mode (1 ml min⁻¹) for elution and detecting the corresponding quinoxalinones at an excitation wavelength of 373 nm and an emission wavelength of 448 nm. The peaks were identified by their retention times relative to the authentic standards Neu5Ac, 9-O-acetylated Neu5Ac (Neu5,9Ac2) and 7-O-acetylated Neu5Ac (Neu5,7Ac2).

2.3. Enzymatic and chemical treatment of mucins and milk glycoproteins

Desialylation was performed with V. cholerae sialidase incubating the samples (0.3 mg) with 5 mU sialidase in 100 μl 50 mM sodium acetate buffer (pH 5.5) containing 2 mM CaCl₂ at 37°C for 1 h. The enzyme was then inactivated at 96°C for 5 min. Control samples were treated in the same way without the addition of enzyme.

For acid hydrolysis of all sialic acids, the gastric mucins were incubated in 0.1 M HCl at 80°C for 1 h. After cooling on ice and neutralising with 1 N NaOH, the samples were dialysed against distilled water [18]. Beside acidic conditions, incubation in PBS (pH 7.2) was performed as a control.

For the de-O-acetylation of sialic acids, human colonic mucins were treated with 0.1 M NaOH for 1 h on ice followed by neutralising with an equal volume of 0.1 M HCl [18].

2.4. Bacterial strains and culture conditions

H. pylori strains 52 and 349/94 from the University of Lund collection were studied [12]. Strains were stored at −80°C in tryptic soy broth containing 15% (v/v) glycerol and were cultured under microaerophilic conditions at 37°C for 3 days on GAB-Camp agar with defibrinated horse blood (5% v/v) [20].

2.5. Bacterial samples

Bacteria were collected from the agar plates, washed once with 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS) and suspended in the same buffer to a final concentration of 10⁹ cells ml⁻¹. For the extraction of cell surface proteins including the sialic acid-binding activity, H. pylori cells harvested from agar plates (approx. 0.5 g wet weight) were washed once with PBS, treated with 2 ml deionised water containing 5 mM HEPES buffer (pH 7.2) for 30 min at room temperature and sedimented by centrifugation (10000×g at 4°C, 10 min). Prior to use, 1/10 vol. of 10×PBS was added to the supernatant.

2.6. Haemagglutination assay

Equal volumes of two-fold dilutions of H. pylori cell suspensions or cell surface protein extracts and 0.75% suspensions of human RBC (20 μl) were mixed in wells of microtitre plates (U-shaped) and allowed to settle at room temperature for 1–2 h. Haemagglutination was visualised by the aggregated cells settling on the bottom of the well forming a loose carpet [12].

2.7. Haemagglutination inhibition assay

Bacterial samples, bacterial suspensions or surface protein extracts were diluted with PBS to give 4 HAU (haemagglutinating units). One HAU was defined as the amount of bacteria which causes complete agglutination under the conditions described above.

Inhibition tests were performed by mixing 15 μl of bacterial samples with 15 μl of a serial dilution of inhibitor for 1 h at room temperature, after which 15 μl of a 0.75% RBC suspension was added to each well and allowed to settle at room temperature for 1–2 h.

3. Results

3.1. Haemagglutination inhibition by gastric mucins

Two H. pylori strains (52 and 349/94) were chosen
for our studies on the basis of their sialic acid-specific haemagglutination. The haemagglutination of \( H.\ pylori \) strain 52 is sialic acid-dependent by recognising oligosaccharide chains with terminal \( \alpha\)-2,3-linked sialic acid on RBC as demonstrated previously [12], whereas the \( H.\ pylori \) strain 349/94 agglutinates RBC independently of the presence of cell surface sialic acids.

The highly sulfated mucin preparations (approx. 55 \( \mu \)mol sulfate/100 mg mucin) from gastric juice eluted from lysine-Sepharose, contained 5–10 \( \mu \)mol sialic acid/100 mg mucin, depending on the origin of the sample. The sialic acid contents of unadsorbed and much less sulfated (approx. 6 \( \mu \)mol sulfate/100 mg mucin) mucin fractions were in the range of 4–5.5 \( \mu \)mol/100 mg mucin. Both these mucin fractions showed high inhibitory potency for \( H.\ pylori \) strain 52 (Table 1). No remarkable difference in the concentration causing 50% inhibition was observed when comparing the mucins from individuals with different blood types. After \( V.\ cholerae \) sialidase treatment, the inhibitory activity of the mucins was significantly decreased, since 3–10-fold higher concentrations were required for the same degree of inhibition (Table 1). Similar results were obtained when terminal sialic acids were hydrolysed with HCl. The haemagglutination inhibition activities of the control mucin samples, treated in a similar way but without sialidase or at neutral pH, were not impaired. The results obtained with extracted cell surface proteins of \( H.\ pylori \) sialic acid-dependent strain 52 were comparable with those for whole cells.

The mucin preparations showed inhibitory potency also for \( H.\ pylori \) strain 349/94, which was not abolished by the removal of sialic acids from mucins. Mucin concentrations necessary to cause 50% haemagglutination inhibition of this strain were comparable with those obtained for \( H.\ pylori \) strain 52 with sialidase-treated mucins.

### Table 1

Inhibition of haemagglutination of the sialic acid-dependent \( H.\ pylori \) strain 52 by human stomach mucins

| Mucin preparations from human gastric juice | Concentration causing 50% inhibition (\( \mu \)M sialic acid) |
|-------------------------------------------|-----------------------------------------------------|
| Lys-Sepharose bound fraction:             |                                                     |
| untreated                                 | 0.16, 0.84, 0.37, 0.12                               |
| sialidase-treated                         | 3.1\*\^a, 4.2\*\^a, 4.0\*\^a, 5.0\*\^a               |
| Lys-Sepharose unbound fraction:           |                                                     |
| untreated                                 | 0.9, 0.8, 0.7, 0.7                                   |
| sialidase-treated                         | 2.5\*\^a, 4.7\*\^a, 8.4\*\^a, 2.8\*\^a             |

\(^{a}\) In the case of sialidase-treated samples the sialic acid was present in free form. Our studies have shown that free sialic acid is not inhibitory up to 65 mM.

### Table 2

Inhibition of haemagglutination of the sialic acid-dependent \( H.\ pylori \) strain 52 by human colonic mucins

| Region from which mucin has been prepared | Sialic acid content of mucin (\( \mu \)mol/100 mg) | Conc. causing 50% inhibition (\( \mu \)M sialic acid) |
|------------------------------------------|-------------------------------------------------|--------------------------------------------------|
|                                          | Total sialic acids | \( O\)-acetylated sialic acids\(^{a}\) |                                                   |
| Cecum                                    | 74 | 13 | 7 |
| Cecum                                    | 23 | 7 | 1.5 |
| Ascending colon                          | 74 | 14 | 2.3 |
| Ascending colon                          | 21 | 7 | 0.7 |
| Ascending colon                          | 26 | 7 | 1.6 |
| Ascending colon                          | 19 | 5 | 1.7 |
| Sigmoid colon                            | 78 | 16 | 2.4 |
| Rectum                                   | 91 | 20 | 2.8 |
| Rectum                                   | 53 | 20 | 3.3 |

\(^{a}\) \( O\)-Acetylated sialic acids are the sum of Neu5,7Ac\(_2\) and Neu5,9Ac\(_2\).
3.2. Haemagglutination inhibition by colonic mucins

In human colonic mucins, there are various O-acetylated sialic acids beside Neu5Ac [21]. The total sialic acid content was in the range of 20–90 μmol sialic acid/100 mg mucin depending on the sample with 15–35% being Neu5,7Ac₂ and Neu5,9Ac₂. Also the colonic mucins were potent inhibitors for *H. pylori* strain 52 haemagglutination (Table 2). The alkaline treatment of the mucins for de-O-acetylation did not influence their inhibitory potencies for *H. pylori*-induced haemagglutination. In addition, no significant correlation between the ratio of O-acetylated sialic acids to total sialic acid content in mucin samples and their inhibitory potencies was found. However, it should be kept in mind that the level of O-acetylation in these mucins was not high enough to draw final conclusions on this aspect.

3.3. Haemagglutination inhibition by milk glycoproteins

Bovine MFGM were tested before and after delipidation. Both preparations inhibited haemagglutination of *H. pylori* strain 52 (Table 3) already at low concentrations. The finding that the delipidated material inhibited at least as well as the native fraction suggests that the mucin-like portion is most important for the inhibitory potency. GMP, a sialoglycoprotein from milk with defined sugar chains containing terminal Neu5Ac(α-2,3)-Gal residues, was an about 20-fold less potent inhibitor than MFGM, requiring sialic acid concentrations almost as high as sialyl(α-2,3)-lactose for 50% inhibition (Table 3).

4. Discussion

The aim of this study was to demonstrate the possible role of sialic acid residues in mucin and mucin-like preparations to be recognised by *H. pylori* sialic acid-specific haemagglutinin(s). Our results show high inhibitory potencies of these molecules for *H. pylori* sialic acid-specific haemagglutination. By far the best inhibitors were the gastric mucins (compare data in Tables 1–3). The inhibitory activities of gastric mucins were affected by sialidase treatment indicating the importance of sialic acids of these mucins for the recognition by *H. pylori* sialic acid-dependent strains. The residual inhibitory activities of the mucins after the removal of sialic acids could be explained by sulfate present in mucin preparations as binding sites for a corresponding adhesin of *H. pylori*. But obviously the presence of sialic acid residues on the gastric mucins strongly enhances the interaction with *H. pylori* strains expressing sialic acid-dependent adhesins.

O-Acetylation of sialic acids has been shown to be the essential receptor determinant for some viruses such as influenza C and corona viruses [22,23], whereas this sialic acid modification prevents the adhesion of other viruses such as influenza A or B [24]. Using human colonic mucins, which beside Neu5Ac contain also O-acetylated sialic acids, we did not obtain significant evidence for an influence of O-acetylation on *H. pylori* binding. This implies that this modification of sialic acids is not required for binding. However, it cannot be completely ruled out that O-acetylation can mask the binding sites for *H. pylori*, since only a part of the sialic acids in colonic mucins were substituted.

Roles of mucin-like components from milk in the prevention of bacterial adhesion have been reported for several pathogens [25,26]. In our studies, MFGM preparation showed a high inhibitory potency for *H. pylori*-induced haemagglutination. Furthermore, the lipid part of the preparation does not seem to be involved in the binding of bacteria, since delipidation did not change the inhibitory potency of the preparation. The results obtained with low molecular mass components from milk such as GMP with terminal α-2,3-linked Neu5Ac as well as with sialyl(α-2,3)-lactose showing an inhibitory effect against *H. pylori* at higher sialic acid concentrations compared to

| Inhibitor             | Concentration causing 50% inhibition (μM sialic acid) |
|-----------------------|-------------------------------------------------------|
| MFGM                  | 5.7                                                   |
| MFGM, delipidated     | 3.8                                                   |
| GMP                   | 110                                                   |
| Sialyl(α-2,3)-lactose | 400                                                   |

Table 3
Inhibition of haemagglutination of the sialic acid-dependent *H. pylori* strain 52 by milk glycoproteins

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S. Hirmo et al. / FEMS Immunology and Medical Microbiology 20 (1998) 275–281

FEMSIM 860 27-4-98
MFGM preparations (Table 3), and with free Neu5Ac not affecting the haemagglutination, demonstrate the relevance of a mucin-like presentation of the glycans for a pronounced inhibitory effect.

In our studies we demonstrate the importance of \textit{H. pylori} sialic acid-binding haemagglutinin(s) for the interaction with gastric mucins, facilitating the spreading of the bacteria in the host by leading them to specific epithelial cell targets to attach and to cause tissue damage. This does not exclude other possible role(s) of sialic acid-specific binding of \textit{H. pylori} in the colonisation process in the stomach. Furthermore, \textit{H. pylori} binding to the extracellular matrix component laminin was recently shown to be sialic acid-dependent [27]. Also the importance of sialylated oligosaccharides on human gastric epithelial cells as binding sites for \textit{H. pylori} has been described [28]. Since mucin-like high molecular mass components from milk showed high inhibitory potencies for \textit{H. pylori} adhesion comparable to gastrointestinal mucin preparations, these could serve as potent anti-infectious components against \textit{H. pylori}.

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