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Adhesion of *Helicobacter pylori* strains to α-2,3-linked sialic acids

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Helicobacter pylori is a human pathogen associated with gastritis and peptic ulcer. Adhesion properties of *H. pylori* to various structures have been described in the literature, including evidence for sialic acid-binding. To study the specificity and frequency of sialic acid-binding, fourteen *H. pylori* strains were investigated using haemagglutination with derivatized erythrocytes carrying sialic acids only on defined glycans and using haemagglutination inhibition assays. From these studies *H. pylori* strains can be grouped into sialic acid-dependent and sialic acid-independent classes. The sialic acid-dependent strains require α-2,3-linked sialic acid for haemagglutination. The potential roles of sialic acid-dependent adhesions for *H. pylori*-related infections are discussed.

Keywords: sialic acid specificity, sialoside, haemagglutinin, *H. pylori* strains, derivatized erythrocytes

Abbreviations: Sia, sialic acids; Neu5Ac, *N*-acetyl-neuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Fm, *N*-formylneuraminic acid; Neu5TFA, *N*-trifluoroacetylneuraminic acid; RBC, human red blood cells (erythrocytes).

Introduction

Helicobacter pylori is a gastric pathogen which colonizes the mucus layer and epithelia of gastric tissue and is associated with chronic type B gastritis, peptic ulcer disease and even stomach cancer [1]. The attachment of *H. pylori* to gastric epithelial cells involves bacterial surface components with the ability to bind to cell surface molecules [2–4]. The identification of such bacterial adhesins is an important step to define specific interactions between host and pathogen, since they are considered to be crucial for the bacterial colonization of stomach. Therefore, the binding of *H. pylori* strains to various cell lines [5–8], gastric mucins [9], cholesterol [10] and extracellular matrix components [11–15] has been studied. Furthermore, different haemagglutination patterns have been obtained using erythrocytes from various species [16–17]. These reports have indicated the occurrence of haemagglutination activities binding sialic acids (Sia) on some *H. pylori* strains possibly specific for α-2,3-linked Sia based on haemagglutination inhibition studies. Although molecular characteristics of such adhesins have been described [16, 18], the requirement of Sia residues and the linkage specificity has remained inconclusive, since (1) sialidase treatment of erythrocytes did not abolish haemagglutination and (2) no inhibitors with well defined structures were used [17]. Since Sia-binding haemagglutinins may be important for the colonization of *H. pylori* on stomach epithelia, it was necessary to characterize Sia-specific haemagglutination activities on *H. pylori* strains in detail.

Since the patterns of sialylated glycans are cell type-dependent and developmentally regulated, it is likely that they influence cellular communication [19, also Schauer R and Kamerling JP, see Ref [A] added in proof]. Several pathogens use Sia as recognition determinants for adhesion to host cells. For example, S-fimbriated *E. coli* [20], *Streptococcus suis* strains [21], mycoplasma [22] and influenza virus [23–26] have been shown to possess

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surface haemagglutinins which specifically bind to terminal Sia residues of the host epithelial cells.

The different sialylated glycan structures on the surface of human erythrocytes (RBC) have been well characterized. Following sialidase treatment, RBC carrying one of these sialylated glycans (Table 1) can be obtained by resialylating the cells with purified sialyltransferases specific for one acceptor glycan [25]. In haemagglutination assays or in binding studies such derivatized RBC have been proven to be powerful tools for the determination of the binding specificity of bacterial [20–22], viral [24, 26] or mammalian adhesion molecules [27–28]. Therefore, this strategy was chosen to investigate the Sia-dependent adhesion of *H. pylori*.

The outcome of *H. pylori* infection varies greatly among individuals [29], which may be due to both strain and host differences. Whereas some people do not show any clinical symptoms, others develop chronic gastritis progressing to duodenal or gastric ulcers or even stomach cancer [30]. It is intriguing to speculate that these pathological differences may correlate with the expression of different haemagglutination activities on *H. pylori*. According to this proposal only some strains with certain characteristics, for example as Sia specificity, may influence the outcome of the disease. Therefore, we studied several *H. pylori* strains for the expression and specificity of Sia-dependent haemagglutinins.

Materials and methods

Materials

Sialidase (*Vibrio cholerae*) was purchased from Behringwerke AG (Germany), β-galactosidase (*E. coli*) from Boehringer Mannheim GmbH (Germany). Fetuin and α_1-acid glycoprotein (orosomucoid) were purchased from Sigma (St Louis Mo, USA), lactose from Merck (Germany), peanut (*Arachis hypogea*) agglutinin from E-Y Laboratories (La Jolla, USA). Q-Sepharose was from Pharmacia (Sweden). Sialyl(α-2,3)lactose and Neu5Ac(α-2,3)Gal(β-1,4)[Fuc(α-1,3)]Glc were obtained from IsoSep AB (Tullinge, Sweden) and sialyl(α-2,6)lactose was prepared as described [31]. CMP-[^{14}C]Neu5Ac (9.66 Bq pmol^{-1}) was from Amersham (Germany); CMP-Neu5Gc was a kind gift from Dr L. Shaw, Kiel University, Germany. CMP-Neu5Fm and CMP-Neu5TFA [32] were a kind gift from Dr H.-J. Groß and Dr R. Brossmer, University of Heidelberg. For resialylation experiments the following sialyltransferases were used to obtain cells containing exclusively Sia in the corresponding sialylated terminal oligosaccharide structures (Table 1). Gal(β-1,4)GlcNAc α-2,6-sialyltransferase (ST6GalI) giving Sia(α-2,6)Gal(β-1,4)GlcNAc (6-N) was purified from rat liver [33] and Gal(β-1,3)GalNAc α-2,3-sialyltransferase (ST3GalI) giving Sia(α-2,3)Gal(β-1,3)GalNAc (3-O) structures was purified from porcine liver [34]. Recombinant Gal(β-1,3/4)GlcNAc α-2,3-sialyltransferase (ST3GalIII) [35] giving Sia(α-2,3)Gal(β-1,3/4)GlcNAc (3-N) was kindly donated by Dr J.C. Paulson at Cytel Inc., La Jolla, USA.

Bacterial strains and culture conditions

Fourteen strains of *Helicobacter pylori* (6, 25, 32, 33, 52, 54, 66, 253, 915, 1139, 12225, 17874, 17875 and 19106) from the Culture Collection of the University of Gothenburg (Sweden) or clinical isolates from our collection (University of Lund) were studied [17]. Strains were stored at −80 °C in tryptic soy broth containing 15% (by vol) glycerol and were cultured under micro-aerophilic conditions at 37 °C for 3 days on GAB-CAMP agar with defibrinated horse blood (5% by vol) [36].

Enzyme treatment of glycoproteins

Asialofetuin and asialo-orosomucoid were prepared from the corresponding native glycoproteins by sialidase (*Vibrio cholerae*) treatment [37]. The glycoprotein (50 mg) was incubated with 2 U sialidase in 40 ml 50 mM sodium acetate buffer (pH 5.5) overnight at 37 °C in a dialysis bag against the same buffer. Then the reaction mixtures were dialysed against distilled water. Asialoglycoproteins were separated by ion-exchange chromatography on Q-Sepharose column (13 × 1.5 cm) eluting with 0.15 M NaCl. Enzyme was eluted from the column with 0.2 M NaCl. Collected fractions (2 ml) were assayed for protein at A_{280} and sialidase activity with 4-methylumbelliferyl-*N*-acetylneuraminic acid as substrate [38]. The fractions, containing glycoprotein, were dialysed against distilled water for 40 h and lyophilized. The product was analysed for Sia content using the orcinol/Fe^{3+}/HCl method [39]. Sialidase

Table 1. Sialylated glycans reconstituted on RBC using the sialyltransferases indicated

Glycan	*Abbreviation*	*Sialyltransferase*
Sia(α-2,6)Gal(β-1,4)GlcNAc-R	6-N	Gal(β-1,4)GlcNAc α-2,6-sialyltransferase
Sia(α-2,3)Gal(β-1,3/4)GlcNAc-R	3-N	Gal(β-1,3/4)GlcNAc α-2,3-sialyltransferase
Sia(α-2,3)Gal(β-1,3)GalNAc-R	3-O	Gal(β-1,3)GalNAc α-2,3-sialyltransferase

treatment released 95% of Sia residues from glycoproteins.

Asialofetuin (5 mg) was further treated with β-galactosidase (500 U) under the conditions described above [40]. The release of Gal was determined in haemagglutination inhibition assays of the Gal-specific peanut agglutinin with asialo-RBC using the enzyme-treated asialofetuin as inhibitor. The inhibitory potency of this asialo-agalactofetuin preparation was eight-fold lower compared to the asialofetuin corresponding to about 80% loss of terminal Gal residues in the glycoprotein.

Derivatization of erythrocytes

RBC (blood group A) were obtained by venipuncture from a healthy donor. Sialidase treatment and incorporation of Sia to give defined sialyloligosaccharide sequences on human erythrocytes was carried out as described [25]. Briefly, freshly harvested and washed human erythrocytes were incubated with *Vibrio cholerae* sialidase and then resialylated by incubation with CMP-[^{14}C]Neu5Ac, CMP-Neu5Gc, CMP-Neu5Fm or CMP-Neu5TFA and purified sialyltransferases, which form Sia(α-2,6)Gal(β-1,4)Glc NAc-R (6-N), Sia(α-2,3)Gal(β-1,3/4)GlcNac-R (3-N) and Sia(α-2,3)Gal(β-1,3)GalNAc-R (3-O), respectively (Table 1). The incorporation of Sia was estimated as described [28]. This analysis revealed for Neu5Ac the following incorporations with the corresponding sialyltransferases: 38 nmol ml^{-1} erythrocytes for 6-N, 58 nmol ml^{-1} erythrocytes for 3-N and 246 nmol ml^{-1} erythrocytes for 3-O. For 6-N and for 3-O glycans a similar total incorporation of Neu5Ac, Neu5Gc and Neu5Fm, but a lower incorporation of Neu5TFA (50–60% compared to Neu5Ac) was determined. For 3-N glycans a similar incorporation of all Sia derivatives used was obtained.

Haemagglutination assay

H. pylori cells were harvested in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl (PBS), washed once and suspended in PBS to a final concentration of 10^9 cells per ml. Equal volumes of two-fold dilutions of bacterial suspension and a 0.75% RBC suspension (25 μl) were mixed in wells of microtitre plates (U-shaped) and allowed to settle at room temperature for 1–2 h. The haemagglutination was visualized by the aggregated cells settling on the bottom of the well and forming a loose, even carpet. PBS was used as negative control [17].

Haemagglutination inhibition assay

Bacterial suspensions 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 25 μl of bacterial cell suspensions with 15 μl of a serial dilution of inhibitor for 1 h at room temperature after which 25 μl of RBC 0.75% suspension was added to each well and allowed to settle at room temperature for 1–2 h. Controls contained 15 μl PBS instead of the inhibitor solution.

Results

Sialic acid specificity of haemagglutination

The fourteen *H. pylori* strains studied could be grouped into two different classes on basis of their specificity to agglutinate RBC (Table 2). The haemagglutination of *H. pylori* strains 25, 52, 54, 1139 and 17874 was strongly reduced by removal of Sia from RBC. This indicates that these five strains (class I) express Sia-binding properties. In contrast, all other *H. pylori* strains investigated (class II) agglutinated sialidase-treated RBC as well or better than untreated cells. Therefore, these strains use Sia-independent sites on erythrocytes which could be terminal galactose (Gal) residues exposed after sialidase treatment.

Haemagglutination of resialylated erythrocytes

The specificity of *H. pylori* strains in class I for defined glycans was further examined by haemagglutination assays with human erythrocytes containing only one type of sialylated glycans (Table 3). All class I *H. pylori* strains bound to terminal α-2,3-linked Sia on both 3-N and 3-O glycans (Table 3). Only strain 54 recognized α-2,6-linked besides α-2,3-linked Sia. Besides Neu5Ac and Neu5Gc which occur on cell membranes and mucins, also two synthetic derivatives, *N*-formylneuraminic acid (Neu5Fm) and *N*-trifluoroacetylneuraminic acid (Neu5TFA), were incorporated into RBC in order to analyse whether these can serve as receptor determinants for *H. pylori*. For all

Table 2. Effect of sialidase treatment of human red blood cells (RBC) on haemagglutination by *H. pylori* strains (10^9 cells per ml)

	Haemagglutination titre	
H. pylori *strains*	*Native RBC*	*Asialo RBC*
25	16	1
17874	16	2
1139	16	1
52	16	0
54	4	0
915	0	16
19106	2	8
12225	2	8
32	1	4
6	1	1
66	1	1
253	1	1
17875	0	1
33	0	<1

Table 3. Haemagglutination ability of sialic acid specific *H. pylori* strains (10^9 cells per ml) with resialylated human erythrocytes

	Haemagglutination of H. pylori *strains*		
Glycans reconstituted on erythrocytes	*25,1139,17874*	*52*	*54*
Sia(α-2,6)Gal(β-1,4)GlcNAc (6-N) with			
Neu5Ac	–	–	+
Neu5Gc	–	–	+
Neu5Fm	–	–	+
Neu5TFA	–	–	+
Sia(α-2,3)Gal(β-1,3/4)GlcNAc (3-N) with			
Neu5Ac	+	+	+
Neu5Gc	+	–	+
Neu5Fm	+	+	+
Neu5TFA	+	+	+
Sia(α-2,3)Gal(β-1,3)GalNAc (3-O) with			
Neu5Ac	+	+	+
Neu5Gc	+	+	+
Neu5Fm	+	+	+
Neu5TFA	+	+	+

+, haemagglutination; –, no haemagglutination.

H. pylori strains the haemagglutination ability was independent of the Sia derivative incorporated. The exception was *H. pylori* strain 52 which did not agglutinate RBC containing Neu5Gc(α-2,3)Gal(β-1,3/4)GlcNAc sequence in N-linked oligosaccharides.

The class II *H. pylori* strains agglutinated RBC independent of derivatization with specific sialylated oligosaccharides (data not shown).

Haemagglutination inhibition assay

The specificity of *H. pylori* strains for sialic acids was further investigated by haemagglutination inhibition assays with different sialo- and asialoglycoconjugates. The haemagglutination of class I *H. pylori* strains was inhibited by fetuin and orosomucoid (Table 4). With the exception of strain 54, orosomucoid had a much lower inhibitory potency which could be explained by a higher content of the Neu5Ac(α-2,6)Gal sequence compared to the Neu5Ac(α-2,3)Gal isomer [41], whereas fetuin contains mainly α-2,3-linked terminal Neu5Ac [42]. The importance of the α-2,3-linked Sia residues for class I *H. pylori* strains was further supported by the finding that sialylated oligosaccharides, containing the Neu5Ac(α-2,3)Gal structure were good inhibitors for these strains. In addition, only haemagglutination by *H. pylori* strain 54 was inhibited by sialyl(α-2,6)lactose. However, compared to sialyl(α-2,3)lactose about 10-fold higher concentrations were needed. Although the inhibition by sialyllactose was significant, with both isomers residual haemagglutination was observed even in the presence of excess inhibitor. This could be explained by the contribution of two haemagglutinins specific for either sialyllactose isomer. In this case, the combination of both isomers at inhibitory concentrations would be expected to abolish this residual agglutination. However, the combination of 1.2 mM sialyl(α-2,6)lactose and 0.1 mM sialyl(α-2,3)lactose did

Table 4. Inhibition of haemagglutination of sialic acid specific *H. pylori* strains by sialoglycoconjugates

	Conc. (mM) causing 50% inhibition of H. pylori *strains:*				
Inhibitor	*25*	*17874*	*1139*	*52*	*54*
Fetuin	0.003	0.005	0.04	0.005	0.003
Neu5Ac(α-2,3)Gal(β-1,4)[Fuc(α-1,3)]Glc	1.5	2.0	2.0	0.7	0.2
Neu5Ac(α-2,3)Gal(β-1,4)Glc	0.9	1.2	0.9	0.4	0.1
Neu5Ac(α-2,6)Gal(β-1,4)Glc	> 2.4	> 2.4	> 2.4	> 2.4	1.2
Orosomucoid	0.1	0.1	0.1	0.05	0.007
Asialofetuin	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1
Lactose	> 100	> 100	> 100	> 100	> 100

not reduce the residual agglutination further. Asialofetuin and lactose had no inhibitory effect for these *H. pylori* strains, confirming the requirement of Sia for binding.

Haemagglutination of native RBC by class II *H. pylori* strains was not inhibited by any of the oligosaccharides at the concentrations used in this assay (Table 5a). For strains 32, 66 and 253 inhibition by fetuin and asialofetuin was observed at concentrations below 0.1 mM with similar concentrations for both glycoproteins (Table 5a) indicating the Sia-independence of binding. Only haemagglutination of native RBC by strain 19106 was Sia-dependent since fetuin was at least 400-fold more potent as an inhibitor than asialofetuin. Using sialidase-treated RBC in haemagglutination inhibition assays (Table 5b), for all strains including strain 19106 no significant difference in the inhibitory potencies of fetuin and asialofetuin was observed. Furthermore, β-galactosidase treatment of asialofetuin did not reduce the inhibitory potency. In contrast, for some strains, 19106, 12225, 17875 and 33, an enhanced inhibition was found after β-galactosidase treatment. No inhibition by asialoorosomucoid at concentrations up to 0.1 mM or by lactose up to 100 mM was observed with any of these strains (Table 5b).

Discussion

The objective of this study was to investigate the Sia-dependent haemagglutination activity of several *H. pylori* strains and to characterize its specificity towards defined sialylated glycans. The results presented demonstrate unambiguously that some *H. pylori* strains express Sia-binding adhesins.

The fourteen *H. pylori* strains investigated could be grouped into two different classes on the basis of their specificity to agglutinate RBC. The class I *H. pylori* strains (25, 52, 54, 1139 and 17874) require Sia on RBC for their haemagglutination. In this study experiments with resialylated RBC demonstrated that these strains recognize oligosaccharide chains with terminal α-2,3-linked Sia. Haemagglutination inhibition with oligosaccharides containing α-2,3-linked Sia confirmed this specificity. An additional fucose at the position α-1,3 to Glc in the oligosaccharide structure does not seem to influence the inhibitory potency. Only one *H. pylori* strain (strain 54) could also bind α-2,6-linked besides α-2,3-linked Sia, although with 10-fold lower affinity. No evidence was obtained that this is due to the presence of two independent haemagglutinins, since sialyl(α-2,3)lactose and sialyl(α-2,6)lactose did not show additive inhibitory potencies. Furthermore, the inhibition experiments with sialyl(α-2,3)lactose give evidence that the haemagglutinin of strain 54 has a higher affinity for Sia compared to the other class I strains. Therefore, it seems possible that the binding pocket of the haemagglutinin could accommodate α-2,6-linked Sia, although with a lower affinity than the α-2,3-linked isomer. The higher affinity of strain 54 to Sia could explain why this strain also agglutinated RBC containing α-2,6-linked Sia. Correspondingly, it cannot be excluded that the other strains also recognize α-2,6-linked Sia, but with an affinity too low to mediate agglutination of these RBC. Similar inhibitory effects have been observed also in the case of influenza A virus isolated from animals, which bind preferentially to α-2,3-linked Sia compared to α-2,6-linked Sia [23, 25, 41, 43].

Table 5a. Inhibition of haemagglutination of sialic acid-independent *H. pylori* strains by sialoglycoconjugates using native RBC

	Conc. (mM) causing 50% inhibition of H. pylori *strains:*					
Inhibitor	*19106*	12225	32	6	66	253
Fetuin	0.001	> 0.4	0.05	> 0.4	0.05	0.006
Asialofetuin	> 0.4	> 0.4	0.05	> 0.4	0.05	0.006
Orosomucoid	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1
Neu5Ac(α-2,3)Galβ1-4Glc	> 9.5	> 9.5	> 9.5	> 9.5	> 9.5	> 9.5
Neu5Ac(α-2,6)Galβ1-4Glc	> 9.5	> 9.5	> 9.5	> 9.5	> 9.5	> 9.5

Table 5b. Inhibition of haemagglutination of sialic acid-independent *H. pylori* strains by sialoglycoconjugates using asialo RBC

	Conc. (mM) causing 50% inhibition of H. pylori *strains:*								
Inhibitor	915	19106	12225	32	6	66	253	17875	33
Asialoorosomucoid	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1	> 0.1
Fetuin	0.1	0.1	> 0.4	0.4	> 0.4	0.2	> 0.4	0.2	0.01
Asialofetuin	0.08	0.1	0.1	> 0.2	0.2	0.2	0.1	0.1	0.08
β-galactosidase-treated asialofetuin	0.08	0.01	0.01	0.05	0.1	0.1	0.05	0.01	0.01
Lactose	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100

Besides *H. pylori*, other bacteria have been described to use specific sialylated glycans on cell surfaces for adhesion. For example, S-fimbriated *E. coli* bind to 3-O glycans selectively [20], whereas *S. suis* strains recognize only 3-N glycans preferentially of polylactosamine chains [21]. A similar specificity as for *S. suis* has been described for *Mycoplasma pneumoniae* [22]. Such specificities may regulate at which sites or cell type in a tissue (i.e. stomach) the bacteria or other pathogens colonize, since the patterns of the sialylated glycans are cell type- and tissue-specific. For example, it has been shown that the distribution of α-2,6- and α-2,3-linked Sia on bronchial epithelial cells and the mucin direct influenza A virus to ciliated cells as predominant sites of infection [43]. In order to better understand the significance of Sia-dependent haemagglutinins for *H. pylori* colonization, it is important to investigate the distribution of α-2,3- and α-2,6-linked Sia on stomach epithelia and mucin.

In contrast to class I strains, the haemagglutination by the other *H. pylori* strains investigated (class II) was not Sia-dependent. The increased haemagglutination titre observed with sialidase-treated RBC suggested that Sia protect the recognition sites for these haemagglutinins, for example terminal Gal residues. However, the inhibition studies using β-galactosidase-treated fetuin and lactose do not supply evidence that Gal is an important recognition determinant for these strains. In contrast, the data in Table 5b indicate that neither Sia nor Gal residues are required for the agglutination of RBC by class II strains. In summary, the haemagglutination inhibition observed with the fetuin preparations used are likely to be due to interactions with other sites which could be masked by Sia and Gal. These sites may be either the residual glycans or the protein backbone. Whereas this haemagglutination activity seems to be common to all class II strains, one strain (strain 19106) seems to express also a Sia-dependent haemagglutinin, since the inhibitory potency of fetuin with native RBC was dependent on the Sia residues of the glycoprotein.

Taken together, in this study we demonstrated that *H. pylori* strains are heterogenous with respect to their haemagglutination specificity. Only some strains appear to have Sia-specific receptors, which may be involved in the attachment of the bacteria to gastric epithelial cell surfaces of the host and are possibly relevant for their pathogenicity. A better understanding of the distribution of this haemagglutinin and the presence of sialylated glycans in stomach will be very helpful to elucidate this important aspect of *H. pylori* pathogenesis. Differences between infected and noninfected mucosal cells has been studied showing an increase of sialic acid-rich glycoproteins [44]. Recently, the detection of O-acetylated sialomucins in intestinal metaplasia and carcinoma of the stomach was described. Furthermore, this type of mucin in intestinal metaplasia was more likely, if *H. pylori* infection was identifiable in the adjacent gastric mucosa [45]. Therefore, one of the next steps will be to purify the Sia-dependent haemagglutinin(s) from these Sia-binding strains, further define its (their) specificity and frequency in *H. pylori* isolates.

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