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Recognition of glycoconjugates by *Helicobacter pylori.* Comparison of two sialic acid-dependent specificities based on haemagglutination and binding to human erythrocyte glycoconjugates*

Halina Miller-Podraza‡, Jörgen Bergström, Maan Abul Milh and Karl-Anders Karlsson

Department of Medical Biochemistry, Göteborg University, Medicinaregatan 9A, S-413 90 Göteborg, Sweden

Helicobacter pylori **expresses separate binding characteristics depending on growth conditions, as documented by binding to human erythrocyte glycoconjugates. Cells grown in Ham's F12 liquid medium exhibited a selective sialic acid-dependent binding to polyglycosylceramides, PGCs (Miller-Podraza *et al.* (1996) *Glycoconjugate J* 13 : 453–60). There was no binding to traditional sialylated glycoconjugates like shorter-chain gangliosides, glycophorin or fetuin. However, cells grown on Brucella agar bound both to PGCs and other sialylated glycoconjugates. Fetuin was an effective inhibitor of haemagglutination caused by agar-grown cells, but had no or a very weak inhibitory effect on haemagglutination by F12-grown bacteria. PGCs were strong inhibitors in both cases, while asialofetuin was completely ineffective. The results indicate that *H. pylori* is able to express two separate sialic acid-dependent specificities, one represented by binding to fetuin, as described before, and another represented by a selective binding to PGCs.**

Keywords: ***Helicobacter pylori*****, human erythrocytes, sialic acid, polyglycosylceramides**

Abbreviations**: PGCs, polyglycosylceramides; TLC, thin-layer chromatography; SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; C, chloroform; M, methanol. The carbohydrate and glycosphingolipid nomenclatures are according to recommendations of IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids* (1977) 12 : 455–68; *J Biol Chem* (1982) 257 : 3347–51 and *J Biol Chem* (1987) 262 : 13–18).**

Introduction

Helicobacter pylori infection is believed to be one of the important causes of gastrointestinal diseases in humans [1–3]. Very little information exists on the mechanism of colonization at the target tissue, although several membrane components were described as possible receptors of this bacterium [4–9]. Studies from different laboratories indicate large differences in binding and adhesive properties between *H. pylori* strains [10–13], which was used to classify the bacteria according to haemagglutinating patterns [13]. Some strains were shown to agglutinate erythrocytes in a sialic acid-dependent manner, with fetuin being described as one of the most effective inhibitors. NeuAcα3Gal was proposed as part of the binding structure [14, 15] and ganglioside GM3 was shown to bind *H. pylori* on TLC plates [5, 8]. A subunit of the bacterial adhesin was reported cloned [15], but later this protein was proposed as an intracellular lipoprotein released upon lysis of the cells [16].

In our efforts to identify biological receptors for *H. pylori* we have used, in the first phase, human erythrocytes as a source of sialylated glycoconjugates. In a previous paper [17] we have shown that the bacterium displays a high-affinity, sialic acid-dependent binding to polyglycosylceramides (PGCs) isolated from these cells. Only a sporadic binding to other sialylated glycolipids was found. There was no binding to sialylated glycoproteins including fetuin and glycophorin. However this was observed only when the bacterium was cultivated in Ham's F12 liquid medium. After growth on Brucella agar plates, the bacterium bound to both PGCs and to other sialylated glycoconjugates. In the present work we investigated more carefully the adhesive properties of the F12- and agar-grown *H. pylori*, using solid phase binding assays and haemagglutination-inhibition tests.

*This is paper No. 2 in this series. The first paper is ref. [17].

‡To whom correspondence should be addressed. Tel: + 46 31 773 3154; Fax: + 46 31 41 3190.

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Materials and methods

Materials

PGCs of human erythrocytes were prepared according to the peracetylation procedure as described [18]. Crude gangliosides of human erythrocytes were obtained according to a conventional procedure [19] and Folch's partition [20]. Bovine brain gangliosides (a mixture of GM1, GD1a, GD1b and GT1) were purchased from Calbiochem (USA). Human glycophorin, bovine calf fetuin and taurodeoxycholic acid were from Sigma (USA). Neuraminidase (*Clostridium perfringens*) was purchased from Boehringer Mannheim GmbH (Germany). NeuAcα3Galβ4GlcNAcβ-DCP-BSA was from Dextra Laboratories (UK). Silica gel TLC aluminium plates were from Merck (Germany).

Bacterial strains

H. pylori strains NCTC 11637 obtained from the Culture Collection of Göteborg University, where it is labelled CCGU 17874, and 032 (a gift from Dr D. Danielson, Örebro Medical Centre, Sweden) were used in most of the experiments. The conditions of bacterial growth in Ham's F12 liquid medium and on Brucella agar plates, respectively, were exactly as described in a preceding paper [17].

Desialylation of glycolipids

Chemical desialylation was performed in 1.5% acetic acid in water at 100 °C for 3 h. For enzymatic digestion the glycolipids ($\sim 1\ \mu g\ \mu l^{-1}$) were incubated with neuraminidase ($1.5\ U\ ml^{-1}$) in 0.05 M acetate buffer (containing taurodeoxycholic acid, $0.75\ \mu g\ \mu l^{-1}$), pH 5.0, for 18 h at 37 °C. The glycolipids were analysed by TLC directly after digestion or were desalted using small Sephadex G-25 columns [21].

Solid phase binding assays

Overlay of TLC plates, blots after electrophoresis and dot-blots with ^{35}S-labelled *H. pylori* were performed as described [17].

Haemagglutination inhibition tests

Haemagglutination inhibition assays were done essentially according to [13]: 22.5 µl of inhibitor in PBS (in control tests pure PBS was used) were mixed with 12.5 µl of *H. pylori* suspension in PBS (4.9×10^{10} cells ml^{-1}) in a microtitre well and incubated at room temperature for 30 min. Then 25 µl of human erythrocytes (blood group O, 0.7% suspension in PBS) were added and the incubation was continued for 1.5 h. The degree of haemagglutination was evaluated under a microscope.

Results

Binding of *H. pylori* to sialylated glycoconjugates differed depending on cultivation conditions. When *H. pylori* was grown in Ham's F12 liquid medium there was a selective binding to PGCs with only a sporadic binding to other sialylated glycolipids. However, when grown on Brucella agar plates, the bacterium was bound by PGCs as well as other sialylated glycolipids and by glycoproteins.

Figures 1 and 2 illustrate the binding of *H. pylori* to a crude mixture of polar gangliosides separated under two

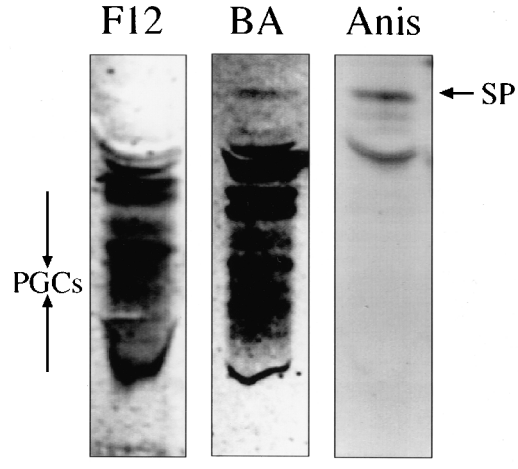

Figure 1. Binding of ^{35}S-labelled *Helicobacter pylori* (032), cultivated in F12 liquid medium (F12) and on Brucella agar plates (BA), to a crude polar ganglioside fraction prepared from human erythrocyte membranes. The TLC was developed with C/M/H_2O (50 : 55 : 19, by vol) solvent. To the right (Anis) the result after chemical staining with anisaldehyde (4-methoxybenzaldehyde) is shown. SP, sialylparagloboside; PGCs, polyglycosylceramides.

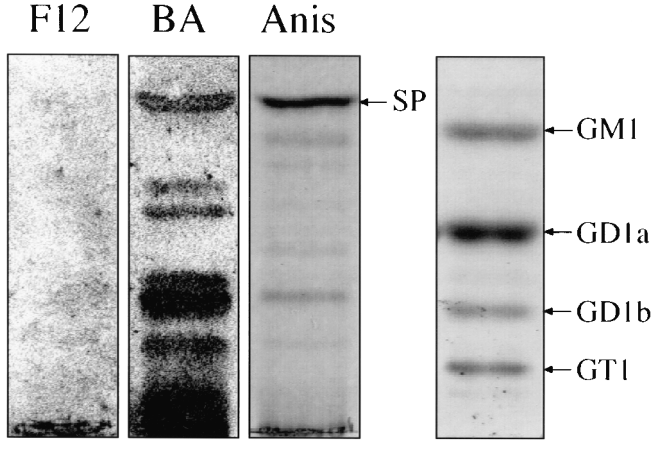

Figure 2. Binding of ^{35}S-labelled *Helicobacter pylori* (NCTC 11637), cultivated in F12 liquid medium (F12) and on Brucella agar plates (BA), to a crude polar ganglioside fraction prepared from human erythrocyte membranes. The TLC was developed with C : M : 0.25% KCl in H_2O (50 : 40 : 10, by vol) as solvent. The third lane (Anis) shows the result after chemical staining of the glycolipids with anisaldehyde (4-methoxybenzadehyde). To the right the chromatographic migration of reference brain gangliosides is shown. SP, sialyparagloboside.

Table 1. Comparison of binding to various glycoconjugates of *Helicobacter pylori* (NCTC 11637) cultivated in Ham's F12 liquid medium (F12) and on Brucella agar (BA)

Preparation	*F12*	*BA*
PGCs	+	+
PGCs, desialylated	−	−
Sialylparagloboside (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcβCer)	−	+
Ganglioside GM3 (human liver) (NeuAcα3Galβ4GlcβCer)	−	(−)[a]
Brain gangliosides (GM1-GP1)	−	(−)[a]
Band 3 region protein fractions (human erythrocytes)	(+)[b]	+
Glycophorin (human erythrocytes)[c]	−	+
Glycophorin (human erythrocytes), desialylated	−	−
Fetuin (calf serum)[c]	−	+
Fetuin (calf serum), desialylated	−	−
NeuAcα3Galβ4GlcNAcβ-DCP-BSA	−	+

The binding activities were determined by overlay of TLC plates (glycosphingolipids) or blots after elctrophoresis (proteins) with ^{35}S-labelled bacteria.
[a] irreproducible binding
[b] Binding insensitive to periodate oxidation.
[c] Preparations previously shown to inhibit haemagglutination of human erythrocytes by *H. pylori* [13, 14].

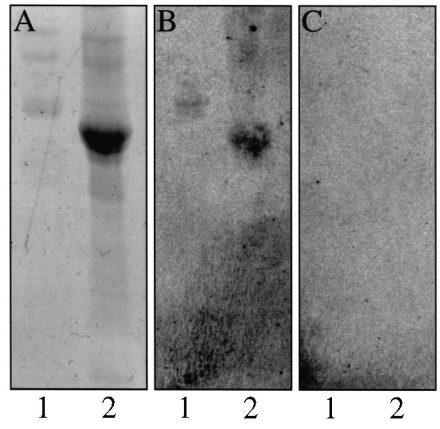

Figure 3. SDS PAGE of (1) human glycophorin, 1.8 μg and (2) calf fetuin, 2 μg. The 12.5% homogeneous gel was stained with Coomassie Brilliant Blue (A). In (B) and (C) the corresponding autoradiograms are shown after binding of ^{35}S-labelled *H. pylori* (NCTC 11637) grown on Brucella agar and in F12 liquid medium, respectively.

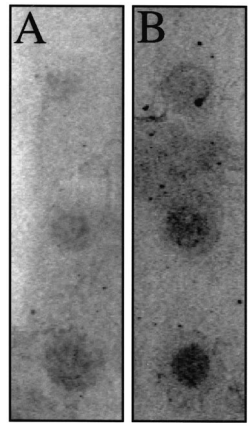

Figure 4. Autoradiograms after binding on dot-blot membranes of ^{35}S-labelled *H. pylori* (NCTC 11637) grown on Brucella agar. A, human glycophorin and B, calf fetuin. Amounts dotted on the membranes, from top, were 5, 10 and 20 μg, respectively. Five μg corresponds roughly to 1.3 μg (4.0 nmol) and 0.5 μg (1.6 nmol) of sialic acid of glycophorin and fetuin, respectively.

different chromatographic conditions. This fraction contained, in addition to shorter-chain gangliosides, very minor PGC components (Figure 1). These were bound very strongly, but the same amount was hardly visible by chemical staining. This was found for cells grown both in broth and on agar. However, shorter-chain gangliosides were positive only for cells grown on agar. The latter included sialylparagloboside (see sequence in Table 1) and several other gangliosides located further down, as shown by routine chromatography (Figure 2). The same result was obtained when purified sialylparagloboside and purified PGCs were assayed under the same conditions. The PGCs lost their binding activities after mild acid hydrolysis or neuraminidase treatment [17].

H. pylori grown on agar plates was bound to a range of gangliosides, as shown in Figures 1 and 2 and in Table 1. The frequency of binding of agar-grown *H. pylori* on TLC plates was about 90% for PGCs and sialylparagloboside. In the case of ganglioside GM3 and brain gangliosides the binding was irreproducible and was observed only sporadically for the NCTC 11637 strain. The frequency of binding of *H. pylori* to different glycolipids on TLC plates will be discussed in detail elsewhere.

Figures 3 and 4 and Table 1 show binding of *H. pylori* to sialoglycoproteins. The agar-grown bacterium bound to fetuin, glycophorin and neoglycoprotein with coupled α3-sialyllactosamine sequence, whereas broth-grown *H. pylori* did not bind to these glycoproteins. Fetuin was a much more effective receptor than glycophorin as shown by overlay of blots after electrophoresis and dot-blots (Figures 3 and 4), as related to sialic acid content. Asialofetuin and asialoglycophorin were totally inactive. PGCs were shown to be receptor-active for *H. pylori* grown in broth or on agar when tested on dot-blots (not shown).

Table 2 shows the results of haemagglutination inhibition tests performed on the NCTC 11637 *H. pylori* strain. The agar-grown bacteria generally displayed a stronger haemagglutination, although the strength of the reaction had a tendency to vary with different batches of bacterial cells. Fetuin had a definite inhibitory effect on haemagglutination

Table 2. Haemagglutination inhibition studies. The results are given as concentrations of fetuin and PGCs required for 50% inhibition of agglutination by *H. pylori* strain NCTC 11637 grown in Ham's F12 medium (F12) or on Brucella agar plates (BA). Values were calculated on the assumption that the mean mol wt of PGCs [18] and fetuin [28] were 5000 (9.7% of sialic acid) and 48 400 (6.3% of sialic acid according to SIGMA specification), respectively

Inhibitor	*F12* $\mu g\,\mu l^{-1}$ (μM)	*BA* $\mu g\,\mu l^{-1}$ (μM)
Fetuin	No or very weak	0.19 (3.9)
Fetuin as NeuAc	inhibition[a]	0.012 (38.8)[b]
Asialofetuin	No inhibition[a]	No inhibition[a]
PGCs	0.050 (10)	0.018 (3.6)
PGCs as NeuAc	0.0049 (15.8)[d]	0.0017 (5.5)[c]
Asialo-PGCs	No inhibition[a]	No inhibition[a]

[a] The highest concentration tested was 0.4 $\mu g\,\mu l^{-1}$.
[b] Mean of eight experiments.
[c] Mean of five experiments.
[d] Mean of three experiments.

caused by agar-grown bacteria, but had no or little influence on the haemagglutination caused by the F12-grown *H. pylori*. Human glycophorin was similar to fetuin (not shown). Asialofetuin was totally ineffective as an inhibitor of haemagglutination. PGCs were strong inhibitors in the case of both broth- and agar-grown bacteria and lost the activity after mild acid hydrolysis.

Discussion

The results indicate that *H. pylori* is able to express at least two sialic acid-dependent binding specificities. The first is represented by binding to fetuin and seems to be identical to the specificity described by Evans *et al.* [14, 15]. This activity was expressed clearly when the bacterium was grown on Brucella agar plates, as shown by overlay of blots after SDS PAGE and of dot-blots (Figures 3 and 4), and by haemagglutination inhibition tests (Table 2). Binding of *H. pylori* to sialylparagloboside on TLC plates seems to be a result of the same specificity. However, as will be discussed in detail elsewhere, the binding to other gangliosides including more complex ones, has to be interpreted with caution since internal epitopes not including sialic acid may also be recognized. The reproducible binding of agar-grown *H. pylori* to sialylparagloboside on TLC plates may indicate that NeuAcα3Galβ4GlcNAc is an important part of the sialic acid-containing epitope of fetuin. This occurs in N-linked carbohydrates of many proteins [22]. On the other hand NeuAcα3Galβ3GalNAc, present in O-linked short sugar chains of glycoproteins, seems to be of less importance, since glycophorin, despite its high content of this structure [23, 24], was a weaker receptor than fetuin (Figures 3 and 4).

The second sialic acid-dependent specificity of *H. pylori* is related to PGCs. This specificity was, in our studies, expressed alone on growth in Ham's F12 liquid medium, and therefore was apparently independent of the fetuin-binding activity. The binding was very strong and the detection level on TLC plates was 10 pmol as based on sialic acid content [17]. The nature of the epitope responsible for this activity remains, however, unknown. It may be a new sequence or a known structure (*eg* sialyl-*N*-acetyllactosamine) being receptor-active only in combination with other group(s) present on linear or branched chains. The strong binding to PGCs was also observed with agar-grown bacteria, and it is highly possible that these complex glycolipids carry both of these sialic acid-dependent epitopes. This is supported by the fact that PGCs were able to inhibit haemagglutination by *H. pylori* from both growth conditions, and that they were more potent inhibitors than fetuin in all our experiments (Table 2). Limited structural studies indicate that PGCs of human erythrocytes contain the same terminal trisaccharides [25] as sialylparagloboside ([26] see Table 1), and fetuin [27].

In our studies strong haemagglutination was observed for *H. pylori* strain 11637. However, there were variations between different strains in this respect, and in some cases fetuin also displayed some inhibitory effects in the case of F12-grown bacteria (not shown). Generally, F12-grown *H. pylori* showed a weaker haemagglutination than agar-grown bacteria, and for some strains these properties were completely lost in broth, which is in agreement with earlier reports by Evans *et al.* [14].

The conclusion from the present experiments, limited to interaction with human erythrocytes and glycoconjugates prepared therefrom, is that *H. pylori* may express two separate sialic acid-dependent binding specificities, and that the expression is dependent on cultivation conditions. The importance of this for the infection *in vivo* remains to be shown. Maybe local signals selectively induce receptor specificities which are essential in a particular situation of a complex environment (attachment to mucus, epithelial cells or white cells). Recently, low pH was reported to induce sulfatide binding by *H. pylori* [29]. In several reports in preparation we will describe sialic acid-related and other binding specificities of *H. pylori* in relation to stomach epithelium and white cells.

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