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Recognition of glycoconjugates by *Helicobacter pylori*: an apparently high-affinity binding of human polyglycosylceramides, a second sialic acid-based specificity*

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Helicobacter pylori has been reported to agglutinate erythrocytes and to bind to various other cells in a sialic acid-dependent way. The binding was inhibited by sialyllactose or fetuin and other sialylated glycoproteins. The specificity apparently requires bacterial growth on agar, since we found that it was lost after growth in the nutrient mixture Ham's F12. Instead, the bacteria bound with high affinity and in a sialic acid-dependent way to polyglycosylceramides of human erythrocytes, a still incompletely characterized group of complex glycolipids.

Bacteria grown in F12 medium were metabolically labelled with ^{35}S-methionine and analysed for binding to glycolipids on thin-layer chromatograms and to glycoproteins on blots after electrophoresis, with human erythrocyte glycoconjugates in focus. There was no binding to simpler gangliosides including GM3 or sialylparagloboside, or to a mixture of brain gangliosides. In contrast, polyglycosylceramides of human erythrocyte membranes bound at a pmol level. The activity was eliminated by mild acid treatment, mild periodate oxidation or sialidase hydrolysis. Erythrocyte proteins as well as a range of reference glycoproteins did not bind, except band 3, which was weakly active. However, this activity was resistant to periodate oxidation.

These results indicate a second and novel sialic acid-recognizing specificity which is expressed independently of the previously described specificity.

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

Abbreviations: PGCs, polyglycosylceramides; TLC, thin-layer chromatography; C, chloroform; M, methanol; EI/MS, electron impact ionization mass spectrometry, SDS PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; BSA, bovine serum albumin. 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 is a specific human pathogen which colonizes human gastric epithelium and is linked to important diseases in the upper gastrointestinal tract, such as gastric and duodenal ulceration and gastric carcinoma [1–4]. The bacterium adheres *in vitro* to different cultured cells [5–8] and agglutinates a variety of erythrocyte species [9–15], which is based on a multiplicity of adhesins of the bacterium [4]. The first detected binding specificity was shown to be dependent on sialic acid. Neuraminidase treatment of target cells or inhibition with various sialoglycoproteins or with sialyllactose considerably reduced bacterial binding or haemagglutination, and

*This paper is dedicated to Professor S.-i. Hakomori and is paper no. 1 from our research on *Helicobacter pylori*.

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NeuAcα3Gal was proposed as part of a colonization factor antigen for *H. pylori* [9]. Ganglioside GM3 (NeuAcα3-Galβ4Glcβ Cer) was reported as a possible candidate of cell surface receptor for this bacterium [15, 16]. Based on fetuin-binding activity of the expressed protein, a gene coding for a subunit of this adhesin was cloned and sequenced [17]. For expression of this adhesin *H. pylori* was grown on blood agar plates [9, 17].

During our studies of carbohydrate-binding specificities of *H. pylori* using various conditions for cultivation and assaying we have found a second sialic acid-dependent specificity, which is expressed in Ham's F12 liquid medium, a condition which does not induce the earlier described specificity. In human erythrocytes the only receptor-active glycoconjugate detected was polyglycosylceramide. Traditional gangliosides or glycoproteins were inactive. The present paper is a preliminary characterization of this specificity, which may be based on a novel sialylsaccharide epitope. We will report elsewhere a comparison of sialic acid-dependent specificities of *H. pylori* grown under the two conditions.

Materials and methods

Materials

A mixture of gangliosides GM1, GD1a, GD1b and GT1 (from bovine brain) was from Calbiochem (USA). Gangliosides GQ1b and GP1 (from human cerebellum) were obtained in the Department of Psychiatry and Neurochemistry of Göteborg University [18]. Other glycolipids were prepared in our laboratory [19]. Gangliosides from human erythrocyte membranes were separated into less polar and more polar fractions by Folch's partition (in C:M:H_2O, 8:4:3, by vol) [20]. Silica gel 60 TLC plates were from Merck (Germany), ceramide glycanase (from leech, *Macrobdella decora*) from Boehringer Mannheim GmBH (Germany), and sialidase (*Arthrobacter ureafaciens*) from Oxford GlycoSystems (England). Human glycophorin, calf fetuin, human orosomucoid (α1-acid glycoprotein), human lactoferrin, and human transferrin were purchased from Sigma (USA). NeuAcα3Galβ4Glcβ-CETE-BSA was a gift from J. Damén, Symbicom AB, Lund, Sweden, NeuAcα6Galβ4Glc-APD-BSA was obtained from BioCarb, Sweden (present supplier Accurate Chem. & Scien. Co, USA), and NeuAcα3Galβ4GlcNAcβ-DCP-BSA was from Dextra Laboratories (UK).

Bacterial strains

The following *H. pylori* strains were used: 002, 005, Ö10, F6, 032, C7050 (a gift from Dr D. Danielsson, Örebro Medical Center, Sweden), 4, 25, 52, 54, 57, 69, 73, 77, 176, 306, 1139, 11637, BH000334, (a gift from Dr T. Wadström, Department of Medical Microbiology, Lund University, Sweden), 604 (a gift from Dr G. Gosciniak, Wroclaw Medical University, Poland), 17874 and 17875 (strains from Culture Collection, Göteborg University). The two last strains are identical with National Culture Type Collection strains 11637 and 11638, respectively.

Isolation and identity of polyglycosylceramides from human erythrocyte membranes

PGCs (from blood group O donors) were obtained using a peracetylation procedure as described before [21]. The crude material isolated after Sephadex LH-20 chromatography was separated by Sephadex LH-60 chromatography, and the three fractions, characterized by hexose/sphingosine ratios of 18.8 (fraction 1), 12.3 (fraction 2) and 9.5 (fraction 3, see Fig. 1) were used for overlay studies. The PGCs had typical branched polylactosamine chains with terminal Fuc, terminal Gal and terminal NeuAc, as shown by combined analyses [21]. The results were in agreement with earlier reports on PGCs structure [22–24]. The identity of PGCs was confirmed by EI/MS [25] of the permethylated glycolipids before and after mild periodate oxidation and reduction. The EI/MS of the permethylated PGCs revealed a fragment ion at *m/z* 825 which indicated the presence of $NeuAc_1Hex_1HexNAc_1$. This fragment ion was replaced after mild periodate oxidation/reduction by ions at *m/z* 737 and 781 indicating a selective destruction of the sialic acid tail (data not shown). The PGCs contained on an average 1 sialic acid residue per circa 15 glycosyl units.

Isolation of polyglycosylceramides from other sources

PGCs were prepared from other human sources (placenta and colon mucosa) and from animal sources (rabbit small intestine and dog small intestine) using the techniques described earlier including criteria of identity [21].

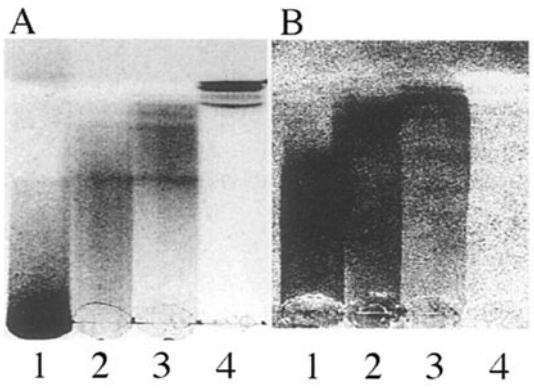

Figure 1. Binding of *H. pylori* to PGCs prepared from human erythrocytes. TLC plates were developed in propanol:0.25% KCl in water:M:C (7:5:1:0.5, by vol) (A) 4-methoxybenzaldehyde-stained plate; (B) autoradiogram after overlay with ^{35}S-labelled bacteria. Lanes 1, 2 and 3, PGC fractions of different degrees of complexity, 6 μg (2 nmols of total sialic acid) of each; Lane 4, reference mixture of brain gangliosides, 3 μg (about 3 nmol of total sialic acid).

Ceramide glycanase digestion of glycosphingolipids [26]

The incubation mixtures contained: 70 μg of the glycolipid fraction, 80 μg of sodium cholate and 0.7 mU of the enzyme in 60 μl of 50 mM acetate buffer, pH 5.0. The samples were incubated at 37 °C for 24 h after which 0.24 ml of water and 1.5 ml of C:M (2:1, by vol) were added to each test tube, and the tubes were shaken and centrifuged. The upper phases were collected, concentrated under nitrogen and analysed by TLC.

Desialylation of glycolipids

Chemical desialylation was performed in 1.5% acetic acid in water at 100 °C for 3 h. Enzymatic desialylation was done using sialidase from *Arthrobacter ureafaciens* according to the manufacturer's recommendations, except that sodium taurodeoxycholate was added to a final concentration of 1.5 $\mu g\,\mu l^{-1}$. The concentration of the substrate and the enzyme was 0.2 mM and 1 U ml^{-1}, respectively. The samples were incubated at 37 °C for 24 h.

Periodate oxidation of polyglycosylceramides

Mild periodate oxidation was performed in 1 mM $NaIO_4$ in 50 mM acetate buffer, pH 5.5 for 25 min on ice. The reaction was terminated by adding excess of $NaHSO_3$.

Preparation of red cell protein extracts, electrophoresis, electroblot, dot-blot and bacterial overlay on blotting membranes

Erythrocyte unsealed ghosts from fresh human red cells were prepared as described [27]. The ghosts were dissolved in 50 mM Tris-HCl (pH 8.0) buffer containing 2.5% SDS and 5% 2-mercaptoethanol for electrophoresis. SDS PAGE and Coomassie staining were carried out with Pharmacia PhastSystem™ according to the protocols of the manufacturer. Briefly, samples were heated to 95 °C for 5 min and centrifuged at 10 000 × **g** for 2 min before electrophoresis. A gradient gel of 8–25% or a homogenous gel of 12.5% was used and 2–4 μg protein was applied to each lane. After electrophoresis the gel was either stained with Coomassie R 350 (PhastGel™ Blue R, Pharmacia, Sweden) or electroblotted to a nitrocellulose membrane (0.45 μm) (Pharmacia, Sweden) according to the manual. The transfer buffer consisted of 20% methanol, 192 mM glycine, and 25 mM Tris at pH 8.3.

Periodate oxidation on the membrane blots was performed as follows. After electroblotting, the nitrocellulose membranes were washed twice in 50 mM sodium phosphate buffer, pH 6.5, and incubated in 20 mM $NaIO_4$ in 100 mM acetate buffer pH 5.5 for 30 min at room temperature, in the dark and then washed twice in phosphate buffer prior to blocking and binding studies as below.

Dot-blots were carried out with the Bio-Dot®Microfiltration Apparatus according to the protocols of the manufacturer (Bio-Rad Laboratories, USA) on PVDF-membrane (Immobilon-P, Millipore, USA). Briefly, a 2.5–5 μl sample dissolved in distilled water was added to each well and the samples were allowed to filter through the membrane by gravity flow. Each well was washed twice with water after which the membrane was removed from the apparatus and washed in 50 mM Tris-HCl and 200 mM NaCl, pH 8.0 prior to blocking and binding experiment.

The nitro-cellulose or PVDF membranes were pre-incubated in blocking solution, 3% BSA, 50 mM Tris-HCl, 200 mM NaCl, 0.1% NaN_3, pH 8.0 for 1.5 h. The membranes were then incubated with ^{35}S-labelled *H. pylori* in PBS. After 1.5–2 h the membranes were washed in 50 mM Tris-HCl, 200 mM NaCl, 0.05% Tween 20, pH 8.0, dried at room temperature, and exposed to autoradiography film overnight as below.

Other analytical methods

Hexose was determined according to Dubois *et al.* [28] and sialic acid according to Svennerholm [29].

H. pylori cultivation and labelling

Broth-grown *H. pylori* was obtained in the following way. The cultivation was started by inoculation of Gab Camp agar (GCA) plates [30], which were incubated for 2–3 days at 37 °C under humid (98%) microaerophilic conditions using Gas Pak 100 Anaerobic System (BBL, Cockeysville, USA). The plate-grown bacteria were tested microscopically to avoid contamination and coccal form cells and used to inoculate Ham's F12 nutrient mixture supplemented with 10% heat-inactivated fetal calf serum (SERA-Lab, UK), 10 ml per 25 cm^2 flask, 1×10^5 cfu ml^{-1}. To each flask 50 μCi of ^{35}S-methionine (Amersham, England) was added to label the cells metabolically, and the bacteria were grown overnight with mild agitation (50 rpm). The bacterial cultures were examined by phase-contrast microscopy to confirm morphology, motility and purity of the bacteria. The cells (positive for urease, catalase and oxidase and from the exponential growth phase) were harvested by centrifugation (1500 × **g**, 5 min), washed twice in PBS and resuspended in the same buffer. The final concentration of cells used for overlay assay was 1×10^8 cfu ml^{-1} (2000–3000 cpm μl^{-1}).

For cultivation of *H. pylori* on agar medium, a semi-solid Brucella agar (DIFCO Laboratories, Detroit, USA) supplemented with 10% heat-inactivated FCS and enriched with 0.5% IsoVitaleX was used. The agar medium was inoculated by streaking with bacterial material from the GCA plate, then 100 μCi of radioactive methionine per plate was sprinkled on the surface of agar. The incubation conditions, enzyme tests, microscopy and preparation of bacterial cells for overlay assay were done as described above.

Overlay of TLC plates

The overlay on TLC plates with ^{35}S-labelled *H. pylori* was performed essentially as described before [31]. The developed TLC plates were treated with 0.3% polyisobutylmethacrylate (Plexigum P28, Röhm GmbH, Darmstadt, Germany) in diethyl ether:n-hexane (3:1, by vol) for 1 min, dried and incubated in 2% BSA and 0.1% Tween in PBS for 2 h. The plates were then overlaid with radiolabelled bacteria suspended in PBS, and incubated at room temperature and under normal atmospheric conditions for additional 2 h. Control overlays showed no difference in binding when the plates were incubated under microaerophilic conditions. The plates were then washed five times with PBS, dried and exposed to Kodak X-OMAT AR films (Kodak Eastman Co., Rochester, NY) for 1–4 days.

Results

We will describe in detail in separate papers results from studies on the recognition by *H. pylori* of glycoconjugates other than PGCs. Based on the comparison with other bacteria studied by us and others *H. pylori* appears unusually complex both concerning the number of different carbohydrate-binding specificities and the variation in the expression of these with cultivation and assay conditions. The present paper, which is our first on *H. pylori*, is limited to an apparently novel sialic acid-dependent specificity restricted to PGCs. This specificity is expressed during cultivation in Ham's F12 liquid medium in which the specificity described by Evans *et al.* [9] and others is not expressed. *H. pylori* grown on agar apparently expresses both specificities. However, there is also a variation between clinical isolates in this respect.

The sialic acid-dependent specificities expressed on growth on agar will be described elsewhere, including haemagglutination studies. A wide range of acidic glycolipids and glycoproteins are receptor-active with characteristics previously reported. In the present paper only the specificity restricted to PGCs is described.

Binding of H. pylori to PGCs prepared from human erythrocyte membranes

PGCs separated on TLC plates were overlaid with ^{35}S-labelled *H. pylori*, and the binding was detected by autoradiography (Fig. 1). All fractions bound the bacteria strongly, resulting in dark streaks all along the lanes. The diffuse appearance in the lanes was due to the apparent microheterogeneity in the structure and number of sugars of these PGC fractions (compare gangliosides in lane 4). A definite binding of *H. pylori* was observed when 0.03 μg of the PGCs (corresponding to about 10 pmol of sialic acid) was applied to the plate (Fig. 2), indicating a high-affinity interaction. The binding activity disappeared after treatment of the PGCs with mild acid, mild periodate or neuraminidase. Figure 3 shows TLC of two PGC fractions after treatment with neuraminidase, and a corresponding autoradiogram after overlay with the radiolabelled bacteria.

Binding of H. pylori to PGCs prepared from other sources

PGCs of human leukocytes and human placenta bound strongly *H. pylori* on TLC plates in a sialic acid dependent manner (not shown). The PGCs obtained from human colon mucosa and dog small intestine showed also some binding activity, but the interaction was weak and less reproducible. PGCs from rabbit small intestine were inactive when tested under the same conditions.

Binding of H. pylori to simple glycosphingolipids prepared from human erythrocyte membranes

The gangliosides (from blood group O erythrocytes) recovered after Folch's partition were investigated for binding activity using overlay on TLC plates with ^{35}S-

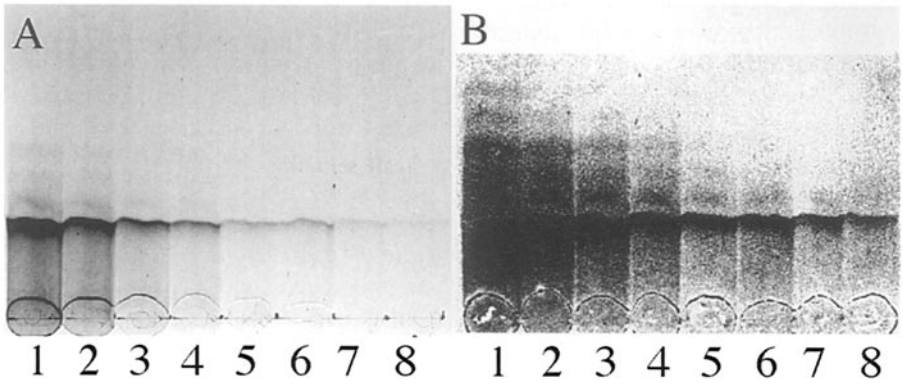

Figure 2. Analysis of detection level for PGCs (fraction 2, Fig. 1) on TLC plates. The plates were developed in C:M:water (50:55:19, by vol). (A) 4-methoxybenzaldehyde-stained plate; (B) autoradiogram after overlay with ^{35}S-labelled bacteria. The amounts of PGCs (lanes 1–8) were: 8.0, 4.0, 2.0, 1.0, 0.5, 0.25, 0.12, 0.06 and 0.03 μg. These amounts correspond to 2667, 1333, 666, 333, 166, 83, 42, 21 and 10 pmol of total sialic acid.

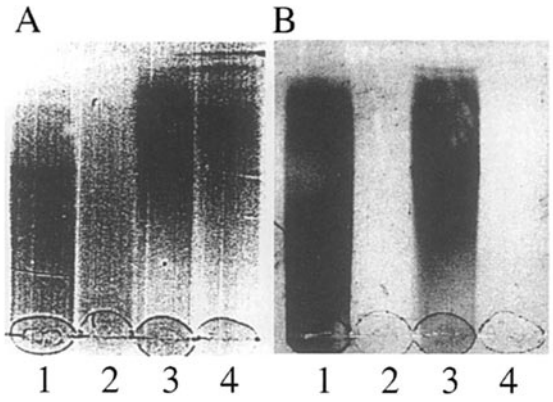

Figure 3. Overlay of sialidase-treated PGCs with ^{35}S-labelled *Helicobacter pylori*. TLC plates were developed as for Fig. 1 (A) 4-methoxybenzaldehyde-stained plate; (B) autoradiogram after overlay with ^{35}S-labelled bacteria. Lanes 1 and 3, PGC fractions (corresponding to fractions 2 and 3 in Fig. 1) 6 μg (2 nmol of sialic acid) of each; Lanes 2 and 4, the same fractions after sialidase treatment.

labelled bacterium. The lower fraction after solvent partition did not bind *H. pylori*. The main ganglioside species in the upper phase were also inactive, but there was a series of minor (hardly visible after chemical staining) bands with a very strong binding activity (Fig. 4, lane 2). The material was analysed in the same way using other chromatographic solvent systems and a negative binding to the major ganglioside species was confirmed (not shown). The receptor-active components migrated on TLC plates below the reference 7-sugar monosialoganglioside (lane 1) and overlapped the PGC fraction. The binding activity of these fractions disappeared after mild acid treatment (Fig. 4, lane 3).

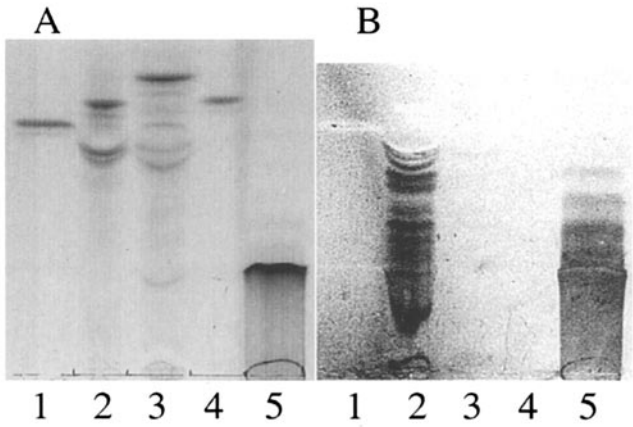

Figure 4. Binding of *H. pylori* to gangliosides of human erythrocyte membranes. The gangliosides were recovered from the upper (water) phase after Folch's partition. (A) 4-methoxybenzaldehyde-stained plate; (B) autoradiogram after overlay with ^{35}S-labelled bacteria. TLC plates were developed as for Fig. 2. Lane 1, Seven-sugar-containing monosialoganglioside of rabbit thymus, 2.2 μg (1.16 nmol of sialic acid); Lane 2, upper phase gangliosides, 30 μg; Lane 3, upper phase gangliosides after mild acid hydrolysis; Lane 4, NeuAcα3paragloboside, 2.2 μg (1.35 nmol of total sialic acid, see also Table 1); Lane 5, PGCs prepared from human erythrocytes, 6 μg.

Binding of H. pylori to different reference gangliosides

The *H. pylori* strains grown in Ham's F12 liquid medium did not bind to reference gangliosides when assayed on TLC plates (Table 1). Among tested gangliosides classified by us as negative in general were ganglioside GM3, brain gangliosides (GM1, GD1a, GD1b, GT1, GQ1b and GP1), NeuAcα3-paragloboside (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcβCer) and NeuAcα6-paragloboside (NeuAcα6Galβ4GlcNAcβ3Galβ4GlcβCer). There was a sporadic binding for some of these species but it was not reproducible and required larger amounts of the glycolipids.

Activity of different H. pylori strains

The positive binding to PGCs was found for *H. pylori* strains 25, 52, 54, 73, 032, 1139 and 11637 (NCTC), which constituted 33% of all strains tested. Strains 25, 52, 54, 73, 1139 and 11637 (NCTC) were reported earlier to

Table 1. Binding of *Helicobacter pylori* grown in Ham's F12 nutrient mixture to different glycoconjugates.

Preparation	*Binding*
Polyglycosylceramides (human erythrocytes)	++++
Neuα3Galβ4GlcβCer (GM3) (human liver)	–
NeuAcα3Galβ4GlcNAcβ3Galβ4GlcβCer (human erythrocytes)	–
NeuAcα6Galβ4GlcNAcβ3Galβ4GlcβCer (human meconium)	–
Brain gangliosides (GM1-GP1)	–
Band 3 region proteins (human erythrocytes)	+
NeuAcα3Galβ4Glcβ-BSA	–
NeuAcα6Galβ4Glcβ-BSA	–
NeuAcα3Galβ4GlcNAcβ-BSA	–
Glycophorin (human erythrocytes)[a]	–
Fetuin (calf serum)[a]	–
Orosomucoid (human serum)[a]	–
Lactoferrin (human milk)	–
Transferrin (human serum)	–

[a]Preparations previously shown to be active in haemagglutination inhibition assays [9, 32].

The binding activities were determined by overlay of TLC plates (glycolipids) or blots after electrophoresis (proteins) with ^{35}S-labelled *H. pylori* (032 strain). The amount of sialic acid in the analysed fractions were well above the detection level compared to Fig. 2.

agglutinate erythrocytes in a sialic acid-dependent manner [32]. Strain 032 was shown by us to agglutinate human erythrocytes with an inhibitory effect of fetuin (to be reported elsewhere). The PGC-negative strains (4, 005, 57, Ö10, 306, C7050, 11638 (NCTC) and BH000334 were tested in haemagglutination-inhibition assays) showed no or a weak haemagglutination effect.

Most of the experiments described in this paper were performed on *H. pylori* strains 032 and 11637 (NCTC). The frequency of binding of *H. pylori* 032 to PGCs recorded during a 2 year period (total number of plates >100) was >90%.

Binding of H. pylori to glycoproteins

H. pylori binding to SDS extracts of proteins prepared from human erythrocytes (Fig. 5) showed a weak binding to only one double-band with the same location on the gel as band 3 [33], and this binding did not disappear when the blots were treated with periodate (not shown). No binding was found to glycophorin in the extract or a purified sample (lane 1). Fetuin, orosomucoid, lactoferrin, and transferrin analysed in the same way also showed no binding (Table 1).

Binding of *H. pylori* to PGCs and two glycoproteins (fetuin and orosomucoid), known to inhibit haemmagglutination of erythrocytes by *H. pylori* [9, 32], were tested on dot-blots (Fig. 6). A clear binding to PGCs was observed, but no binding to the glycoproteins could be detected.

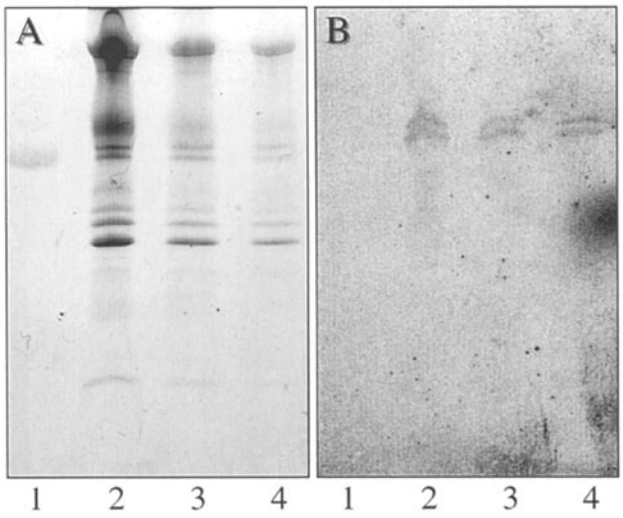

Figure 5. SDS PAGE of human erythrocyte protein extract on a 12.5% homogenous gel stained with Coomassie Brilliant Blue (A) and the corresponding autoradiogram after binding of ^{35}S-labelled *H. pylori* on nitro-cellulose membrane blot (B). Lane 1, purified glycophorin, 1.8 μg protein; Lanes 2–4, erythrocyte ghosts, 3.0, 1.5 and 0.75 μg total protein, respectively. Lane 2 was overloaded. The content of sialic acid in glycophorin and band 3 glycoprotein was well above the detection level compared to Fig. 2.

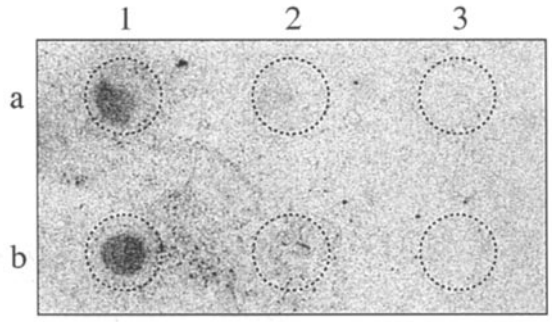

Figure 6. Autoradiogram after binding of ^{35}S-labelled *H. pylori* on dot-blot membrane. Row a, column 1, PGCs, 2.5 μg; column 2, fetuin, 5 μg; column 3, orosomucoid, 6 μg. Row b, as a, but with the double amount of samples applied.

Discussion

Assays based on agglutination of red cells of various animals are basic tools in the characterization of microbial adhesins. In case of the important pathogen *Helicobacter pylori* [4], agglutination of human erythrocytes was used to characterize a sialic acid-dependent binding by a fibrillar adhesin [9], the first detected specificity of this recently discovered bacterium. The agglutination was neuraminidase-sensitive and could be inhibited by sialyllactose, NeuAcα3Galβ4Glc, and by fetuin (but not by asialofetuin), and by several other sialylated glycoproteins. Similar characteristics have later been repeatedly documented for the binding of *H. pylori* to various cultured cells. A gene for a fetuin-binding protein was recently cloned and expressed in *E. coli* [17]. The sialic acid-specific adhesin on *H. pylori* cells appeared when the bacterium was grown on blood agar plates [17]. By the use of frozen tissue sections it was shown that the purified adhesin bound to the surface of epithelial cells of antrum of human stomach [34]. Sialyllactose or an antibody against the adhesin blocked this binding.

During our systematic studies of carbohydrate-binding specificities of *H. pylori* (results to be published) we made the observation that when Ham's F12 nutrient mixture was used for cultivation of the bacterium there was no binding to earlier reported receptor-positive glycoconjugates, including ganglioside GM3 and fetuin. Instead there was a high-affinity binding to PGCs of human erythrocyte membranes. This binding was absolutely dependent on sialic acid, since sialidase treatment completely eliminated detectable binding. Mild periodate also had the same effect. Mass spectrometry of PGCs after the periodate oxidation documented that only the tail of NeuAc had been cleaved. The elimination of binding in this way was in part a surprise to us, since PGCs are very complex and *H. pylori* displays several other carbohydrate-binding specificities (results to be published). A comparison of human PGCs with similar

preparations of non-human origin revealed that only human PGCs were strongly positive, although all of the preparations contained sialic acid and the core structures were very similar (to be published).

The trailing appearance of PGCs on TLC (Figs 1–4) was due to the microheterogeneity in structure with between 10 and at least 30 sugars in total including on an average 1.7 sialic acid residues per molecule (one sialic acid per ca 15 glycosyl units). Apparently, as there is a parallel chemical and autoradiographic staining, the binding epitope is present through this chromatographic interval.

The combined chemical analyses of PGCs indicate that they contain NeuAcα3(6)Galβ4GlcNAc, which is in agreement with previous findings concerning structures of polylactosamine-containing glycoconjugates of human erythrocytes [22–24, 35–37]. However, the carbohydrate sequences NeuAcα3Galβ4Glc, NeuAcα3Galβ4GlcNAc or NeuAcα6Galβ4GlcNAc were unable to bind *H. pylori* on TLC plates when present in simple linear glycolipids (Table 1). This suggests that other sequences or additional residues are necessary for the interaction with the bacterium. The same conclusion can be drawn from results of glycoprotein binding. The majority of tested sialoglycoproteins, including natural glycoproteins with NeuAcα3(6)Gal and NeuAcα3(6)Galβ4GlcNAc structures and neoglycoproteins carrying NeuAcα3(6)GalβGlc and NeuAcα3Galβ4GlcNAc carbohydrate sequences, did not bind *H. pylori* on blots after electrophoresis (Table 1). Moreover, there was no binding to sialoglycoproteins on dot-blots loaded with high amounts of the protein material. The only positive reaction on dot-blots was observed for PGCs, confirming the selectivity of the interaction of the broth-grown *H. pylori* for these glycolipids. Some of the tested glycoprotein fractions (fetuin, glycophorin and orosomucoid) were shown earlier to inhibit haemagglutination of erythrocytes by agar-grown *H. pylori* [9, 32]. Apparently, the haemagglutination of human erythrocytes found after bacterial growth in F12 medium is mainly based on the interaction with PGCs.

The nature of the binding epitope for *H. pylori* on band 3 glycoprotein is not known. This glycoprotein is the main protein carrier of polylactosamine chains in human erythrocyte membranes [35–37], and the similarity of core carbohydrate structures of this glycoconjugate and of PGCs is obvious. However, the binding was resistant to periodate oxidation (the conditions used were more drastic than required for selective oxidation of sialic acid) indicating that the sialic acid tail was not a part of the active structure. Moreover, the binding required relatively large amounts of the protein material and seemed to be of lower affinity than the binding to PGCs.

The results presented in this paper indicate that, depending on the growth conditions, *H. pylori* can express *in vitro* different sialic-acid related specificities. The sialylated epitope on PGC molecules seems to be recognized by a separate adhesin which is different from the sialyllactose/lactosamine-specific adhesin [9, 17]. The implication of sialylated epitopes in gastric colonization by *H. pylori* remains unknown. Also we do not know why only 1/3 of *H. pylori* strains are PGC-positive. The answer to these questions requires better knowledge of gastric mucosa glycoconjugate chemistry and a better understanding of rules which may regulate *H. pylori* variability *in vivo*. However, worth noticing is the high-affinity interaction (detection at a pmol level in Fig. 2) illustrated most strikingly in Fig. 4, where chemically undetectable complex glycosphingolipids in the upper phase of human erythrocyte gangliosides showed a strong binding by *H. pylori*.

We will describe separately a comparison of the two specificities using haemagglutination studies and different isolates and growth conditions. Important is the elucidation of the apparently human-specific and novel sialic acid containing binding epitope of PGCs. However, although there exists a rational method for the isolation of PGCs as a group [21], the extreme microheterogeneity will probably complicate the separation of PGCs into carbohydrate-homogenous species for a precise analysis of the binding epitope.

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References

1. deCross AJ, Marshall BJ (1993) *Am J Med Sci* **306**: 381–92.
2. Axon ATR (1993) *J Antimicrob Chemother* **32** Suppl A: 61–68.
3. Lin J-T, Wang J-T, Wang T-H, Wu M-S, Chen C-J (1993) *Hepato-Gastroenterol* **40**: 596–99.
4. Wadström T (1995) *Curr Opin Gastroenterol* **11**: 69–75.
5. Evans DG, Evans DJ, Graham DY (1989) *Infect Immun* **57**: 2272–78.
6. Armstrong JA, Cooper M, Goodwin CS, Robinson J, Wee SH, Burton M, Burke V (1991) *J Med Microbiol* **34**: 181–87.
7. Neman-Simha V, Megraud F (1988) *Infect Immun* **56**: 3329–33.
8. Fauchere JL, Rosenau A, Bonneville F (1989) *Gastroenterol Clin Biol* **13**: B59–64.
9. Evans DG, Evans DJ, Moulds JJ, Graham DY (1988) *Infect Immun* **56**: 2896–906.

10. Emödy L, Carlsson Å, Ljungh Å, Wadström T (1988) *Scand J Infect Dis* **20**: 353–54.
11. Huang J, Smyth CJ, Kennedy NP, Arbuthnott, JP, Keeling PWN (1988) *FEMS Microbiol Lett* **56**: 109–12.
12. Nakazawa T, Ishibashi M, Konishi H, Takemoto T, Shigeeda M, Kochiyama T (1989) *Infect Immun* **57**: 989–91.
13. Robinson J, Goodwin CS, Cooper M, Burke V, Mee BJ (1990) *J Med Microbiol* **33**: 277–84.
14. Huang J, Keeling PWN, Smyth CJ (1992) *J Gen Microbiol* **138**: 1503–13.
15. Slomiany BL, Piotrowski J, Samanta A, VanHorn K, Murty VLN, Slomiany A (1989) *Biochem Int* **19**: 929–36.
16. Saitoh T, Natomi H, Zhao W, Okuzumi K, Sugano K, Iwamori M, Nagai Y (1991) *FEBS Lett* **282**: 385–87.
17. Evans DG, Karjalainen TK, Evans DJ, Graham DY, Lee C-H (1993) *J Bacteriol* **175**: 674–83.
18. Miller-Podraza H, Månsson J-E, Svennerholm L (1991) *FEBS Lett* **288**: 212–14.
19. Karlsson K-A (1987) *Methods Enzymol* **138**: 212–20.
20. Folch J, Lees M, Sloane Stanley GH (1957) *J Biol Chem* **226**: 497–509.
21. Miller-Podraza H, Andersson C, Karlsson K-A (1993) *Biochim Biophys Acta* **1168**: 330–39.
22. Koscielak J, Miller-Podraza H, Krauze R, Piasek A (1976) *Eur J Biochem* **71**: 9–18.
23. Dejter-Juszynski M, Harpaz N, Flowers HM, Sharon N (1978) *Eur J Biochem* **83**: 363–73.
24. Zdebska E, Krauze R, Koscielak J (1983) *Carbohyd Res* **120**: 113–30.
25. Breimer M, Hansson GC, Karlsson K-A, Larson G, Leffler H, Pascher I, Pimlott W, Samuelsson BE (1980) In *Advances in Mass Spectrometry* (Quayle A, ed.) Vol 8 pp. 1097–108. London: Heyden & Son.
26. Ito M, Yamagata T (1989) *Methods Enzymol* **179**: 488–496.
27. Yang Z, Bergström J, Karlsson K-A (1994) *J Biol Chem* **269**: 14620–24.
28. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) *Anal Chem* **28**: 350–56.
29. Svennerholm L (1957) *Biochim Biophys Acta* **24**: 604–11.
30. Soltez V, Schalen C, Mårdh PA (1988) In *Proceedings of the Fourth International Workshop on Campylobacter Infections* (Kaijser B, Falsen E, eds) pp. 433–36. Kungälv, Sweden: Goterna.
31. Karlsson K-A, Strömberg N (1987) *Methods Enzymol* **138**: 220–32.
32. Lelwala-Guruge J, Ljungh Å, Wadström T (1992) *APMIS* **100**: 908–13.
33. Branton D, Cohen CM, Tyler J (1981) *Cell* **24**: 24–32.
34. Evans DG, Evans DJ, Jr (1995) *Methods Enzymol* **253**: 336–60.
35. Järnefelt J, Rush J, Li Y-T, Laine RA (1978) *J Biol Chem* **253**: 8006–09.
36. Fukuda M, Fukuda MN, Hakomori S-i (1979) *J Biol Chem* **254**: 3700–03.
37. Fukuda M, Dell A, Oates JE, Fukuda MN (1984) *J Biol Chem* **259**: 8260–73.