Helicobacter pylori and Neutrophils: Sialic Acid-Dependent Binding to Various Isolated Glycoconjugates

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Helicobacter pylori has been shown to agglutinate erythrocytes in a sialic acid-dependent manner. However, very few studies have examined relevant target cells in the human stomach. Neutrophils are required for the onset of gastritis, and the inflammatory reaction may be induced on contact between bacteria and neutrophils. In the present work, glycolipids and glycoproteins were isolated from neutrophils and were studied for binding by overlay with radiolabeled bacteria on thin-layer chromatograms and on membrane blots. There was a complex pattern of binding bands. The only practical binding activity found was sialic acid dependent, since treatment of glycoconjugates with neuraminidase or mild periodate eliminated binding. As shown before for binding to erythrocytes and other glycoconjugates, bacterial cells grown on agar bound to many glycoconjugates, while growth in broth resulted in bacteria that would bind only to polyglycosylceramides, which are highly heterogeneous and branched poly-N-acetyllactosamine-containing glycolipids. Approximately seven positive bands were found for glycoproteins, and the traditional ganglio- and globosides fraction showed a complex, slow-moving interval with very strong sialic-acid-dependent binding, probably explained by Fuc substitutions on GlcNAc.

Materials and Methods
Preparation of granulocyte cells. Human neutrophils were prepared fromuffy coats of venous blood of healthy donors as described (10). The procedure was a modification of the method of Bøyum (2) and included centrifugation of cells in Ficol medium, washing cells in phosphate-buffered saline (PBS)/glucose-gelatin solution, and sedimentation of the mixture in dextran solution. Erythrocytes remaining in the granulocyte fraction were removed by lysis in a 0.8% gelatin solution, and sedimentation of the mixture in dextran solution. Erythrocytes in the granulocyte fraction were removed by lysis in a 0.8% solution of NH4Cl in H2O. After incubation in NH4Cl for at least 10 min, the cells were centrifuged at 400 g for 10 min. The supernatant was discarded. The lysis and centrifugation were repeated until the preparation was free from erythrocytes. This procedure usually results in granulocyte fractions with neutrophil contents of greater than 95%. Cell fractions referred to in the text as total leukocytes and with a granulocyte content of 70 to 85% were prepared from unseparated buffy coats, which were lysed in NH4Cl solution (for the removal of erythrocytes) as described above. For comparison, we also obtained a smaller, highly pure, neutrophil (polymorphonuclear leukocytes, 100%) preparation from Claes Dahlgren, Institute of Medical Microbiology and Immunology, Göteborg University (18).

H. pylori strains. Bacterial strains used in these studies were 17874 and 17875 (CCUG) and 032 (a gift from Dan Danielsson, Örebro Medical Center, Sweden). H. pylori cultivation and labeling on agar plates or in broth were performed as described (26).

Preparation of glycosphingolipids. Gangliosides were prepared according to standard procedures (21). Ganglioside and carbohydrate nomenclature is according to recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature. The method included extraction of glycolipids from lymphoblastoid cells with mixtures of chloroform and methanol as well as alkaline degradation, dialysis, DEAE-cellulose column chromatography, and silicic acid column chromatography. The polyglycosylceramide fraction was isolated by the peracetylation method (26) as follows. The dry tissue residue (1.2 g) left after extraction of lipids and common glycolipids was peracetylated in formamide-pyridine-acetic anhydride (10:5:4, by volume; 22.8 ml) followed by extraction with an excess of chloroform (50 ml). After filtration, the extract was washed three times with water (17 ml each time), and the chloroform phase was evaporated to near dryness. The oil residue was applied to a Sephadex LH-20 column followed by Sephadex LH-60 chromatography. The crude polyglycosylceramide (PGC) prep-
aration was de-O-acetylated in 0.1 M NaOH in water overnight at room temperature and was diazoylated against distilled water for 2 days. The sample was next freeze-dried, extracted with 2-propanol–hexane-water (55:25:20, by volume; 2 ml), and centrifuged. Complex glycosphingolipids were recovered from the supernatant. The total sphingosine content in this fraction was 136 nmol.

**Extraction of proteins.** Membranes from fresh neutrophils were prepared by the method of Moore et al. (33). The outer membrane fraction was collected and dissolved in 25 mM Tris-HCl (pH 8.0) and 2.5% sodium dodecyl sulfate (SDS) and 1 mM EDTA, pH 8.0, heated to 95°C for 10 min and centrifuged at 10,000 × g for 10 min.

The protein concentration was determined by bichinchoninic acid protein assay reagent (Pierce, Rockford, Ill.). The sample was diluted to 2 to 4 mg of protein per ml, and 2-mercaptoethanol (5%) was added prior to SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Overlay on TLC plates with 35S-labeled bacteria.** Overlay of glycosphingolipids on thin-layer chromatography (TLC) silica gel plates with [35S]methionine-labeled bacteria was done essentially as described previously (22). Briefly, the silica gel plates with separated glycolipids were treated with 0.5% polysorbetyl-methacrylate (625 molecular weight) (Aldrich Chemical Company, Inc., Milwaukee, Wis.) in diethyl ether-n-hexane (3:1, by volume) for 1 min and were dried. The plates were then soaked in 2% bovine serum albumin (BSA) and 0.1% Tween in PBS for 2 h and were overlaid with radiolabeled cells. The plates were incubated under normal atmospheric conditions for an additional 2 h, were washed five times with PBS, were dried, and were exposed to Kodak X-OMAT AR films (Kodak Eastman Co., Rochester, N.Y.) for 1 to 4 days.

**Electrophoresis and bacterial overlay.** SDS-PAGE and Coomassie staining were carried out with Pharmacia PhastSystem according to the protocols of the manufacturer (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, samples were heated to 95°C for 5 min and were centrifuged at 10,000 × g for 2 min before electrophoresis. A homogeneous gel of 12.5% polyacrylamide was used, and 2 to 4 μg of protein was applied for each lane. After electrophoresis, the gel was either stained with Coomassie R 350 (PhastGel Blue R; Pharmacia) or was electrotoblotted to a polyvinylidene difluoride (PVDF) (0.2-μm) membrane according to manufacturers instructions. The transfer buffer consisted of 20% methanol, 192 mM glycine, and 25 mM Tris-HCl at pH 8.3.

The PVDF membrane was preincubated in blocking solution (3% BSA, 50 mM Tris-HCl, 200 mM NaCl, 0.1% NaN₃, pH 8.0) for 1.5 h. The membrane was then incubated with [35S]-labeled *H. pylori* in PBS. After 1.5 to 2 h, the membrane was washed five times in a solution of 50 mM Tris-HCl, 200 mM NaCl, and 0.05% Tween 20, pH 8.0, was dried at room temperature, and was exposed to Kodak film overnight.

**Ceramide glycanase digestion of glycolipids.** Ceramide glycanase (from the leech *Macrobdella decora* [55]; Boehringer Mannheim GmbH, Mannheim, Germany) digestion was performed at 37°C overnight. The reaction mixture contained 100 μg of PGCs, 75 μg of sodium cholate, and 0.5 μM of enzyme in 60 μl of 50 mM acetate buffer, pH 5.0. After digestion, the sample was mixed with 240 μl of water and 1,500 μl of chloroform–methanol (2:1, by volume), was shaken, and was centrifuged. The lower and upper phases contained free ceramides and free oligo- and polysaccharides, respectively. The hydrolysis was complete, and the recovery of the material after digestion was practically quantitative, as judged by TLC. Both phases were evaporated under nitrogen. The saccharides were desalted using a Sephadex G-15 column (Pharmacia, Uppsala, Sweden) which was packed and run in distilled water. The sugar-positive material (detected on TLC plates by anisaldehyde) was collected, evaporated, redissolved in a small volume of water, and used for TLC analysis, as described for polyglycosylceramides of human erythrocytes (28). The ceramides were redissolved in a small volume of 2:1 (by volume) chloroform–methanol and were used for TLC analysis (28). The released ceramides were further analyzed by fast atom bombardment mass spectrometry (29).

**Neuraminidase hydrolysis and periodate oxidation.** For neuraminidase (from Clostridium perfringens; Sigma Chemical Co., St. Louis, Mo.) treatment of glycoproteins on blots, the PVDF membranes were washed twice after blocking in 50 mM sodium acetate buffer (pH 5.5) containing 0.1% BSA and 5 mM CaCl₂, and were incubated in the same buffer (0.1 ml/cm²) with or without neuraminidase (50 mU/ml) at 37°C for 30 h (38).

Glycolipid desialylation and mild periodate oxidation were performed as described (26).

**Colorimetric tests.** Quantitative determination of hexose, sialic acid, and sphingosine was performed as described (30).

## RESULTS

Three groups of glycoconjugates of human neutrophils, gangliosides, PGCs, and glycoproteins, were tested for binding by radiolabeled *H. pylori* on TLC plates and membrane blots. The surfaces were overlaid with [35S]-radiolabeled bacteria, and the binding radioactivity was detected by autoradiography.

We use the term gangliosides to define a ganglioside fraction prepared by extraction with organic solvents and other standard procedures. More complex glycosphingolipids recovered from the extracted residue are referred to as PGCs.

**Binding to glycolipids.** Binding to gangliosides on TLC plates is shown in Fig. 1. This particular ganglioside fraction was prepared from total leukocytes (see Materials and Methods), but the same binding pattern was obtained for highly purified neutrophils. Polymorphonuclear leukocytes have been shown to contain a series of sialylated glycosphingolipids based on neolacto carbohydrate core chains, with predominant species located in three-, five-, and seven-sugar regions (16, 23, 34, 50). *H. pylori* recognized components in five- and seven-sugar regions, as well as in more complex fractions. Experiments with desialylated and periodate-oxidized gangliosides revealed a strict dependence of the binding on NeuAc, and the sialic-acid-independent *H. pylori* strain CCUG 17875 practically did not bind (Fig. 1, 75-ag). The weak reaction seen for CCUG 17875 in the seven-sugar region was not dependent on sialic acid and did not disappear after desialylation or mild oxidation and reduction. There was a preference of binding to NeuAc3GalβGlcNAc to NeuAc6GalββGlcNAc (16, 27), which is in agreement with results of other groups (15, 44). There was also a stronger binding to some complex fractions, confirmed by binding to a dilution series of gangliosides (not shown). The binding to S-3-PG and other gangliosides was strong after bacterial growth on solid media (agar plates). After growth in liquid media, this specificity was lost (26, 29) and only sialic-acid-dependent binding to PGCs was observed.

Figure 2 illustrates the binding to PGCs isolated from two cell fractions, total leukocyte fraction, panel A, and neutrophil fraction, panel B. Most of the components migrated below reference brain gangliosides. *H. pylori* from agar bound strongly to the whole chromatographic interval, including less polar components, while *H. pylori* from broth bound less strongly to less polar components. Three *H. pylori* strains were used in these studies, two classified as sialic-acid-dependent binders and one classified as a sialic-acid-independent binder. The sialic-acid-independent strain CCUG 17875 did not bind to PGCs, and the binding by the two other strains disappeared after desialylation of PGCs or after mild periodate oxidation.
and reduction, which shortens the sialic acid glycerol tail by one or two carbon atoms (31).

The glycosphingolipid nature of the PGC material was proved by ceramide glucanase digestion. Ceramide glucanase specifically cleaves the glycosidic bond between ceramides and carbohydrate chains in glycolipids, and the cleavage of leukocyte PGCs was monitored by TLC using polar solvent systems (28, 30).

The released oligo- and polysaccharides changed their speeds of migration compared to undigested PGCs. The released ceramides were analyzed separately by TLC with less-polar solvent (28). The digestion eliminated binding of H. pylori on TLC plates due to loss of ceramides, which are necessary for a hydrophobic anchoring of the saccharide chains in the plastic-treated silica gel during washing procedures. The structure of the saccharide part of the leukocyte PGC has not yet been analyzed. These compounds are not identical to myelolglycans, oligofucosylated gangliosides with up to 12 monosaccharides per core chain, described in human leukocytes by others (34, 49, 50). Glycolipids defined as myelolglycans are less polar than PGCs and are extracted together with gangliosides (16), in contrast to PGCs which remain in the residue after extraction (28). Our preliminary data from colorimetric tests and mass spectrometry showed the presence in leukocyte PGCs of ceramides (mainly d18:1-C16:0 and C24:1), hexose, hexosamine, sialic acid, and minor amounts of fucose. Electron impact ionization mass spectrometry analysis of the permethylated material revealed the presence of an abundant fragment ion corresponding to NeuAcHexHexNAc (m/z 825) (31). The same sequence is present in H. pylori-binding PGCs from human erythrocytes (31). We have shown using matrix-assisted laser desorption ionization–time of flight mass spectrometry (19) that PGCs of human erythrocytes contain sialylated molecules with more than 40 monosaccharides per ceramide. Mass spectrometry after degradation with endo-β-galactosidase indicated that the sialylated sequence is present entirely in the form of a three-sugar nonextended side branch (31a). A hydroxyl bond formation between the sialic acid glycerol tail and GlcNAcs of the neighboring branches may be necessary for the proper presentation of the binding epitope, as interpreted from the effect on binding of mild periodate and molecular modeling studies (1). Leukocyte PGCs are presently being analyzed in our laboratory. So far, the pattern of binding of the bacteria when grown on agar or in broth indicates that the binding epitope should be the same in leukocyte and erythrocyte PGCs.

**Binding to glycoproteins.** Binding of H. pylori to protein extracts obtained from fresh neutrophil outer membranes is shown in Fig. 3. Recognition of at least seven protein bands could be observed and these were sensitive to neuraminidase treatment (compare 74-ag and 74-ag-n in panel A). This binding was only observed for strains grown on agar and known to bind sialic acid, as illustrated by the control experiment performed on calf fetuin (Fig. 3, panel B). The sialic-acid-binding strain CCUG 17874 and the nonbinding strain CCUG 17875 were used for comparison.

**DISCUSSION**

Human leukocytes apparently contain a variety of sialylated glycoconjugates with high potency to bind H. pylori. Binding on artificial surfaces was demonstrated for glycolipids and glycoproteins isolated from mixtures of human leukocytes as well as for neutrophils. We will report elsewhere (in collaboration with C. Dahlgren and A. Karlsson) the binding to glycoconjugates isolated from subcellular fractions of neutrophils.

The simplest binding-active molecule was shown elsewhere (16, 27) to be a five-sugar monosialoganglioside, S-3-PG, NeuAca3Galβ4GlcNAcβ3Galβ4GlcCer (Fig. 1), having sialyl-N-acetyllactosamine as the terminal trisaccharide. Sialyl-N-acetyllactosamine is structurally related to the sialyllactose (NeuAcα3Galβ4Glc) present in the negative three-sugar glycolipid (3s in Fig. 1). Other groups using various methods have, however, reported this saccharide or glycolipid to be active (6, 7, 15, 25, 43, 44, 46). A stronger binding was observed by us for some complex gangliosides, and this could depend on the presence of extended carbohydrate chains with repeated lactosamine units and/or fucose branches. Neolacto carbohydrate chains with Fuca3GlcNAc substitutions have been shown to be present in human leukocytes, in both glycolipids (34, 49, 50) and glycoproteins (48).

Binding to PGCs apparently represents a separate sialic...
the corresponding autoradiograms after binding of 35S-labeled acrylamide homogeneous gel stained with Coomassie brilliant blue (Coom) and blots (Neutr, panel A) and of calf fetuin (Fetuin, panel B) on a 12.5% polyacrylamide gel. The agar-dependent binding may be to NeuAcGalβ4GlcNAc, as indicated in the original paper (6), and the PGC content was about 0.8 nmol per 10^8 cells (calculation based on sphingosine content). The PGCs are, therefore, less abundant than gangliosides in terms of concentration but may provide efficient multivalent epitope structures. The effectiveness of binding to glycolipids may be improved by the formation of plasma membrane microdomains with locally concentrated receptor structures, as reported for lymphocytes (47) and other cells (12, 41, 45, 51).

The biological significance of the sialic acid-dependent binding of neutrophil glycoconjugates by H. pylori is still unclear. Although established reference bacterial strains may be positive or negative binders of sialic acid, it is of interest that fresh clinical isolates expressed a consistent binding (44). Future experiments may test if a sialic acid-mediated interaction between bacterial cells and neutrophils is essential for a strong inflammation. Noteworthy in this respect is our finding of a very low content of sialylated glycoconjugates of gastric epithelium (data not shown). Therefore, the expression of a sialic-acid-binding adhesin(s) may support bacterial homing to neutrophils rather than to epithelial cells. An adhesin related to sialylactose-inhibitable hemagglutinin was reported in 1993 (8). However, later reports claimed that this cloned protein was not an adhesin but rather a lipoprotein (17, 37). Therefore, the adhesin(s) responsible for the bindings reported in this work remains to be identified.

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