Lactotetraosylceramide, a novel glycosphingolipid receptor for Helicobacter pylori, present in human gastric epithelium

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Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M201113200

Published: 2002-01-01

Link to publication

Citation for published version (APA):
Lactotettraosylceramide, a Novel Glycosphingolipid Receptor for Helicobacter pylori, Present in Human Gastric Epithelium

The binding of Helicobacter pylori to glycosphingolipids was examined by binding of 35S-labeled bacteria to glycosphingolipids on thin-layer chromatograms. In addition to previously reported binding specificities, a selective binding to a non-acid tetraglycosylceramide of human meconium was found. This H. pylori binding glycosphingolipid was isolated and, on the basis of mass spectrometry, proton NMR spectroscopy, and degradation studies, were identified as Galβ3GlcNAcβ3-Galβ4Glcβ1Cer (lactotetraosylceramide). When using non-acid glycosphingolipid preparations from human gastric epithelial cells, an identical binding of H. pylori to the tetraglycosylceramide interval was obtained in one of seven samples. Evidence for the presence of lactotetraosylceramide in the binding-active interval was obtained by proton NMR spectroscopy of intact glycosphingolipids and by gas chromatography-electron ionization mass spectrometry of permethylated tetrasaccharides obtained by ceramide glycanase hydrolysis. The lactotetraosylceramide binding property was detected in 65 of 74 isolates (88%). Binding of H. pylori to lactotetraosylceramide on thin-layer chromatograms was inhibited by pyrothine with lactotetraose but not with lactose. Removal of the terminal galactose of lactotetraosylceramide by galactosidase hydrolysis abrogated the binding as did hydrazinolysis of the acetamido group of the N-acetylgalcosamine. Therefore, Galβ3GlcNAc is an essential part of the binding epitope.

Adhesion of microorganisms to target cells is regarded as a first step in pathogenesis of infections, where the specificity of the adhesins of the infectious agent on the one hand, and the receptor structures expressed by the epithelial cells of the host target organ on the other are important determinants of the host range and the tissue tropism of the pathogen (1).

The human gastric pathogen Helicobacter pylori is an etiologic agent of chronic active gastritis, peptic ulcer disease, and gastric adenocarcinoma (2, 3). This Gram-negative bacterium has a very distinct host range and tissue tropism, i.e. it requires human gastric epithelium for colonization (4). In the human stomach most of the bacteria are found in the mucus layer (5), but selective association of the bacteria to surface mucous cells has also been shown (4, 6).

Several different binding specificities of H. pylori have previously been demonstrated. Thus, the binding of the bacterium to such diverse compounds as phosphatidylethanolamine and gangliotetraosylceramide (7), the Leb blood group determinant (8), heparan sulfate (9), the GM3 ganglioside and sulfatide (10, 11), and lactosylceramide (12), has been reported. A sialic acid-dependent binding of H. pylori to large complex glycosphingolipids (polyglycosylceramides) has also been documented (13). However, only one H. pylori adhesin, the Leb binding BabA adhesin, has been identified to date (14).

In the present study a number of different H. pylori strains were labeled with [35S]methionine and examined for binding to a panel of different naturally occurring glycosphingolipids separated on thin-layer plates. Two distinct binding specificities were repeatedly detected by autoradiography. As previously described in detail, H. pylori bound to lactosylceramide, gangliotetraosylceramide, and gangliotetraosylceramide (12). The only binding activity initially detected in human gastrointestinal material was to a compound in the tetraglycosylceramide region of the non-acid fraction of human meconium. The isolation and structural characterization of this H. pylori binding glycosphingolipid and the identification of the same compound in human gastric epithelial cells are described in the present paper.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Labeling—The bacteria used and their sources are described in Table I. In most of the experiments four strains, the type strains CCUG 17874 and 17875 (obtained from Culture Collection, University of Goteborg, (CCUG), Sweden), and the clinical isolates S-002, and S-032, were used in parallel.

1 The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (CBN for Lipids: Eur. J. Biochem. (1977) 79, 11–21; J. Biol. Chem. (1982) 257, 3347–3351; and J. Biol. Chem. (1987) 262, 13–18). It is assumed that Gal, Glc, GlcNAc, GalNAc, NeuAc, and NeuGc are of the D-configuration, Fuc of the L-configuration, and all sugars present in the pyranose form. In the shorthand nomenclature for fatty acids and bases, the number before the colon refers to the carbon chain length, and the number after the colon gives the total number of double bonds in the molecule. Fatty acids with a 2-hydroxy group are denoted by the prefix h before the abbreviation, e.g. h16:0. For long chain bases, d denotes dihydroxy, and t denotes trihydroxy. Thus d18:1 designates sphingosine (1,3-dihydroxy-2-aminooctadecane), and t18:0 designates phytosphingosine (1,3,4-trihydroxy-2-aminooctadecane).
Table I

Heliobacter pylori isolates used in binding assays

<table>
<thead>
<tr>
<th>Strains</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>I-VII</td>
<td>Obtained from the following sources.</td>
</tr>
<tr>
<td></td>
<td>I. Strains were obtained from the Culture Collection University of Göteborg (CCUG) at Göteborg, Sweden.</td>
</tr>
<tr>
<td></td>
<td>II. Strains were obtained from the Department of Medical Microbiology and Immunology, Örebro Medical Centre Hospital, Sweden.</td>
</tr>
<tr>
<td></td>
<td>III. Strains were obtained from the Department of Medical Microbiology, University of Lund, Sweden.</td>
</tr>
<tr>
<td></td>
<td>IV. Strains were obtained from the Department of Microbiology, Medical University of Wrocław, Poland.</td>
</tr>
<tr>
<td></td>
<td>V. These strains were a kind gift of Dr. Thomas Borén, Umeå University, Sweden.</td>
</tr>
<tr>
<td></td>
<td>VI. These strains were a kind gift of Dr. Rainer Haas, Ludwig-Maximilians-Universität, Munich, Germany.</td>
</tr>
</tbody>
</table>

Bacterial strains

1. 15816, 17135, 17874, 17875, 18430, 18943, 20649, 20985, 20986, 20996, 20999, 30991, 30993, 41936
2. S-002, S-005, S-032, F6, O10, C7050
3. 4, 15, 17, 48, 51, 56, 62, 65°, 69, 73, 77, 78, 80, 81, 88, 95, 176°, 185, 188, 191, 198°, 214, 225, 239°, 244, 263, 266, 269°, 271°, 273°, 275, 287, 306, 313, 1139, BH00031
4. BH000324, BH000325, BH000331, BH000332, BH000334
5. IV. 1, 177, 408, 604, 609
6. V. P468, MO19, J99, 17875babA2
7. VI. P1, P1-140
8. VII. S51, SS1 (Abpla)

*No binding to lactotetraosylceramide was obtained with these strains.

The conditions used for culture and 35S-labeling of the bacteria have been described previously (12). For binding assays, the bacteria were suspended to 1 × 10^9 colony forming units/ml in phosphate-buffered saline (PBS) or pH 7.4. The specific activities of the suspensions were ~1 cpmp/100 H. pylori organisms.

Thin-layer Chromatography—Thin-layer chromatography was performed on glass- or aluminum-backed silica gel 60 high performance thin-layer chromatography plates (Merck) using chloroform/methanol/water (60:35:8, by volume) as the solvent system. Chemical detection was accomplished by anisaldehyde (15).

Chromatogram Binding Assay—The chromatogram binding assay was done as described (16). Mixtures of glycosphingolipids (20–80 µg/lane) or pure compounds (1–4 µg/lane) were separated on aluminum-backed silica gel plates (Merck) using chloroform/methanol/water (60:35:8, by volume) as the solvent system. Chemical detection was accomplished by anisaldehyde (15).

Reference Glycosphingolipids—Acid and non-acid glycosphingolipid fractions from the sources given in the legend of Fig. 1 and in Table III were obtained by standard procedures (17). The individual glycosphingolipids were isolated by acetylation of the total glycosphingolipid fractions and repeated chromatography on silicic acid columns. The identity of the purified glycosphingolipids was confirmed by mass spectrometry (18), proton NMR spectroscopy (19), and degradation studies (20, 21). Galβ3GlcNAcβ3Galβ4Glc1Cer (No. 3 of Table III) was generated from Galβ3GlcNAcβ3Galβ4Glc1Cer (No. 2) by treatment with anhydrous hydrazine, as described (12).

Isolation of the H. pylori Binding Tetracylosylceramide from Human Meconium—A total non-acid glycosphingolipid fraction (262 mg) was obtained from 17 pooled meconia by standard methods (17). The non-acid glycosphingolipids (240 mg) were first separated by HPLC on a 2.2 × 30-cm silica column (YMC SH-044–10, 10 µm particles; Skandinaviska Genetec, Kungsbacka, Sweden) eluted with a linear gradient of chloroform/methanol/water 65:25:4 to 40:40:12 (by volume) for 180 min and with a flow of 2 ml/min. Aliquots of each 2-ml fraction were analyzed by thin-layer chromatography using anisaldehyde as the staining reagent. The tetracylosylceramides were collected in tubes 78–88, and after pooling of these fractions, 14.2 mg was obtained. This material was acetylated and further separated by HPLC on a YMC SH-044–10 column eluted with a linear gradient of chloroform/methanol (95:5, by volume) in chloroform for 90 min and with a flow of 2 ml/min. After deacetylation, aliquots from each 1-ml fraction were analyzed by anisaldehyde staining on thin-layer chromatograms, and the glycosphingolipid-containing fractions were examined for H. pylori binding activity. Most of the H. pylori binding glycosphingolipid was collected in tube 62, and this fraction (2.4 mg) was used for structural characterization.

Isolation of Non-acid Glycosphingolipids from Human Gastric Epithelium—Stomach tissue (10 × 10 cm pieces) were obtained from the fundus region from patients undergoing elective surgery for morbid obesity. After washing with 0.9% NaCl (w/v), the mucosal cells were gently scraped off and kept at −70 °C. The material was lyophilized, and acid and non-acid glycosphingolipids were isolated as described (17). Acid glycosphingolipids were also isolated from non-mucosal residues. The blood group of the patients and the amounts of glycosphingolipids obtained from each specimen are given in Table II.

The non-acid glycosphingolipids from case 4 (2.9 mg) were separated by HPLC on a 1.0 × 25-cm silica column (Kromasil-Sil, 10-µm particles, Skandinaviska Genetec) using a gradient of chloroform/methanol/water 65:25:4 to 40:40:12 (by volume) over 180 min with a flow rate of 2 ml/min. Aliquots from each fraction were analyzed by thin-layer chromatography using anisaldehyde as the staining reagent. The tetracylosylceramides were collected in tubes 12–17. Tubes 12–14 also contained a compound with mobility in the triglycosylceramide region on thin-layer chromatograms and, after pooling of these three fractions, 0.2 mg was obtained (designated fraction 4-I). The fractions in tubes 15–17 were pooled separately, giving 0.5 mg of tetraglycosylceramides (designated fraction 4-II).

Separation of 10.0 mg of the non-acid glycosphingolipid fraction from case 5 was done using the same system as above, with a gradient of chloroform/methanol/water 60:35:8 to 40:40:12 (by volume). The fraction collected in tube 11 (designated fraction 5-I) contained triglycosylceramides and tetraglycosylceramides (0.1 mg), whereas only tetraglycosylceramides were obtained in tubes 12 and 13. Pooling of the latter two fractions resulted in 0.3 mg (designated fraction 5-II).

EI Mass Spectrometry—Before mass spectrometry, the glycosphingolipids were permethylated, as described (22). The tetraglycosylceramide isolated from human meconium was analyzed on a VG ZAB 2F/9F mass spectrometer (VG Analytical, Manchester, UK) using the in-beam technique (23). Analytical conditions were electron energy 45 eV, trap current 500 µA, and acceleration voltage 8 kV. Starting at 250 °C, the temperature was elevated by 6 °C/min.

The tetraglycosylceramides from the mucosal cells of human stomach were analyzed by the same technique on a JEOL SX-102A mass spectrometer (JEOL, Tokyo, Japan). Analytical conditions were electron energy 70 eV, trap current 300 µA, and acceleration voltage 10 kV. The temperature was raised by 15 °C/min, starting at 150 °C.

Degradation Studies—The permethylated glycosphingolipid from human meconium was hydrolyzed, reduced, and acetylated (20, 21), and the partially methylated alditol and hexosaminitol acetates obtained were analyzed by gas chromatography-EI mass spectrometry on a Trio-2 quadrupole mass spectrometer (VG Maslab, Altrincham, UK). The Hewlett Packard 5890A gas chromatograph was equipped with an on-column injector and a 15 m × 0.25-mm fused silica capillary column, DB-5 (J&W Scientific, Rancisco Cordova, CA), with a 0.25-µm film thickness. The samples were injected on-column at 70 °C (1 min), and the oven temperature was increased from 70 to 170 °C at 50 °C/min and from 170 °C to 260 °C at 8 °C/min. Conditions for mass spectrometry were electron energy 40 eV and trap current 200 µA. The components were identified by comparison of retention times and mass spectra of partially methylated alditol acetates obtained from reference glycosphingolipids.

Proton NMR Spectroscopy—Proton NMR spectra were acquired at 7.05 Tesla (300 MHz) on a Varian VXR 300 (Varian, Palo Alto, CA) and at 11.75 Tesla (500 MHz) on a JEOL Alpha-500 (JEOL, Tokyo, Japan). Data were processed off-line using NMR1 (NMRI, Syracuse, NY). The deuterium-exchanged glycosphingolipid fractions were dissolved in di-
methyl sulfoxide-\textsubscript{d}\textsubscript{6}/\textsubscript{D}\textsubscript{2}O (98:2, by volume), and spectra were recorded at 30 °C with a 0.4-Hz digital resolution. Chemical shifts are given relative to tetramethylsilane.

Ceramide Glycosphingolipid from Human Gastric Epithelium—The procedure of Hansson et al. (24) was used for the enzymatic hydrolysis. Briefly, 100 µg of fraction 4-II from case 4, fraction 5-II from case 5, reference globoside from human erythrocytes (25), reference lactotetraosylceramide from human meconium, and reference lactoneotetraosylceramide (obtained by sialidase treatment of sialyl-lactoneotetraosylceramide from human erythrocytes; Ref. 26) were dissolved in 100 µl of 0.05 M sodium acetate buffer, pH 5.0, containing 120 µg sodium cholate and sonicated briefly. Thereafter, 1 milliliter of ceramide glycanase from the leech, Macrobdella decora (Roche Molecular Biochemicals) was added, and the mixtures were incubated at 37 °C (Roche Molecular Biochemicals) was added, and the mixtures were incubated at 37 °C for 24 h. The reaction was stopped by the addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharide-containing upper phase thus obtained was separated by thin-layer chromatography. One chromatogram was stained with anisaldehyde, and duplicate chromatograms were monitored by binding of radiolabeled \textsuperscript{35}S-labeled \textit{H. pylori}. By subsequent autoradiography only a few bands were visualized, as shown in Fig. 1B. The binding in lane 4 (gangliotriasoylceramide) and lane 7 (gangliotetraosylceramide) was judged to correspond to the “gangli binding specificity” of \textit{H. pylori} described previously in detail (12).

In addition, selective binding of \textit{H. pylori} to a compound migrating in the tetraglycosylceramide region in the non-acid glycosphingolipid fraction of human meconium was detected (Fig. 1B, lane 6). This binding was only obtained when detergent (Tween 20 or deoxycholic acid) was present in the coating buffer. Solution 4 (2% bovine serum albumin and 0.1% Tween 20 in PBS) was therefore subsequently utilized as standard coating procedure. The binding-active tetraglycosylceramide from human meconium was isolated by HPLC and characterized by mass spectrometry, proton NMR, and gas chromatography-EI mass spectrometry after degradation as follows.

Chemical Structure of the \textit{H. pylori} Binding Glycosphingolipid from Human Meconium—The binding-active tetraglycosylceramide was isolated from 240 mg of total non-acid glycosphingolipids. By HPLC of the native glycosphingolipid fraction, 14.2 mg of tetraglycosylceramides were obtained. The tetraglycosylceramide fraction was acetylated and further separated by HPLC, giving 2.4 mg of pure binding-active glycosphingolipid. Each step during the preparative procedure was monitored by binding of radiolabeled \textit{H. pylori} on thin-layer chromatograms.

Structural characterization identified lactotetraosylceramide (Galβ3GlcNacβ3Galβ4Glcβ1Cer) as the binding-active component. This basis was concluded on the following observations.

EI mass spectrometry of the permethylated tetraglycosylceramide (Fig. 2) demonstrated a carbohydrate chain with Hex-HexNAc-Hex-Hex sequence and d18:1 and d18:0 long chain bases combined with both hydroxy and non-hydroxy fatty acids of mainly 22 and 24 carbon atoms. A type 1 chain (Hexβ3HexNAc) was indicated by the absence of a fragment ion at \textit{m/z} 182, which is a dominating ion in the case of 4-substituted HexNAc (30, 31).

The binding positions between the carbohydrate residues were obtained by degradation of the permethylated tetraglycosylceramide, i.e. the sample was subjected to acid hydrolysis followed by reduction and acetylation. The resulting partially methylated alditol acetates were analyzed by gas chromatography-EI mass spectrometry. The reconstructed ion chromatogram thus obtained had four carbohydrate peaks (A shown). The acetate of 2,3,4,6-tetramethylgalactitol identified a terminal galactose, whereas the presence of the acetate of 4,6-di-methyl-2-N-methylacetamidoglucitol (3-substituted N-acetylglucosamine) indicated a type 1 chain. The two remaining peaks, acetates of 2,4,6-trimethylgalactitol and 2,3,6-trimethylglucitol, were derived from 3-substituted galactose and 4-substituted glucose, respectively. In combination with the data

\begin{table}[h]
\centering
\caption{Blood group data and amounts of glycosphingolipids isolated from human stomach specimens}
\begin{tabular}{|l|l|l|l|}
\hline
Case No. & blood group & Tissue & Non-acid glycosphingolipids & Acid glycosphingolipids \\
\hline
1. ORh\(^{-}\) & Mucosal cells & 7.0\(^{a}\) (11.9)\(^{b}\) & 8.5\(^{a}\) (14.4)\(^{b}\) & \\
2. ARh\(^{+}\) & Mucosal cells & 2.7 (6.0) & 22.0 (48.8) & \\
3. ARh\(^{+}\) & Mucosal cells & 3.6 (18.0) & 10.5 (53.5) & \\
4. ARh\(^{+}\) & Mucosal cells & 6.4 (14.5) & 2.9 (6.6) & \\
5. ARh\(^{+}\) & Mucosal cells & 6.0 (24.0) & 4.8 (19.2) & \\
6. ARh\(^{-}\) & Mucosal cells & 23.0 (38.0) & 5.5 (9.2) & \\
7. Unknown & Mucosal cells & 4.9 (18.1) & 8.2 (30.4) & \\
8. Non-mucosal residue & Mucosal cells & 2.5 (15.6) & 7.5 (46.8) & \\
9. Non-mucosal residue & Mucosal cells & 4.3 (8.7) & 7.6 (15.5) & \\
\hline
\end{tabular}
\end{table}

\(^{a}\) The weight is given in mg. \\
\(^{b}\) Expressed as mg/g dry tissue weight.
from mass spectrometry, a carbohydrate chain with the sequence Gal1–3GlcNAc1–3Gal1–4Glc1 was deduced.

The anomeric region of the proton NMR spectrum (Fig. 3) contained five large β-doublets ($J_{1,2} \approx 6$ Hz). The glucose anomeric proton signal (4.20 ppm, $J_{1,2} = 7.2$ Hz) was split into two signals, as is often the case due to ceramide head group differences. At 4.28 ppm ($J_{1,2} = 7.2$ Hz), the Galβ anomeric proton appeared, which is indicative of a substitution at the 3-position. The internal GlcNAcβ anomer was seen at 4.79 ppm ($J_{1,2} = 8.0$ Hz) with its N-acetylated methyl protons resonating at 1.82 ppm. Finally, the terminal Galβ signal was found at 4.15 ppm ($J_{1,2} = 6.8$ Hz), indicating a 1→3 linkage. All anomeric chemical shifts were thus in agreement with published results for lactotetraosylceramide (32).

Thus, the H. pylori binding glycosphingolipid from human meconium was identified as Galβ3GlcNAcβ3Galβ4Glcβ1Cer, i.e. lactotetraosylceramide, which has previously been described from the same source (33).

Comparison with Isoceptors—Several pure glycosphingolipids structurally related to lactotetraosylceramide were examined for H. pylori binding activity using the chromatogram binding assay (exemplified in Fig. 4). The results are summarized in Table III. The only binding-active glycosphingolipid was lactotetraosylceramide (No. 2; Fig. 4, lane 2), whereas all the substitutions tested abolished the binding. Thus, the addition of an α-fucose in the 2-position (No. 4; Fig. 4, lane 3), an α-galactose (No. 6), or an α-N-glycolylneuraminic acid (No. 7) in 3-position, or an α-N-acetyleneuraminic acid in 6-position of the terminal galactose or an α-fucose in 4-position of the N-acetylgalactosamine (No. 5; Fig. 4, lane 4) was not tolerated. No binding to GlcNAcβ3Galβ4Glcβ1Cer (No. 1; Fig. 4, lane 1) was obtained, demonstrating the importance of the Galβ3GlcNAcβ part. The acetamido group at 2-position of the penultimate N-acetylgalactosamine contributed substantially to the interaction, since removal of this moiety (No. 3) completely abolished the binding.

Inhibition Experiments—The ability of soluble oligosaccharides to interfere with the binding of H. pylori to glycosphingolipids on thin-layer plates was examined by incubating the bacteria with free lactotetraose or lactose before binding on chromatograms. The results are shown in Fig. 5. Thus, incubation with lactotetraose (0.1 mg/ml) inhibited the binding of H. pylori to lactotetraosylceramide, whereas incubation with lactose had no inhibitory effect.

Binding of H. pylori to Non-acid Glycosphingolipids of Whole Human Stomach—To examine the expression of binding-active glycosphingolipids in the target tissue of the bacteria, the binding of H. pylori to glycosphingolipids isolated from whole human stomach was first investigated. The tetraglactosyleramide region of these non-acid fractions was dominated by globoside (Fig. 6A, lane 4), which at least for human small (34) and large intestine (35), is derived from the non-epithelial part. No bind-
When using the non-acid glycosphingolipid fraction isolated from the stomach of a blood group A (Rh+/H11001) individual (36), which lacked the galactosyltransferase responsible for the conversion of lactosylceramide to globotriaosylceramide (37) and consequently was devoid of globoside (Fig. 6A, lane 3), a binding of *H. pylori* in the tetraglycosylceramide region was detected (Fig. 6B, lane 3). The tissue in this case was obtained after surgery for peptic ulcer disease. Because of limited amounts available, no chemical characterization of this binding-active tetraglycosylceramide was possible.

**Binding of *H. pylori* to Glycosphingolipids of Human Gastric Epithelium—**Next we examined the binding of *H. pylori* to glycosphingolipids isolated from the epithelial cells of human
stomach. Because non-neoplastic tissue rarely is excised during normal surgical procedures, glycosphingolipids were isolated from specimens from the fundus region obtained from patients undergoing surgery for obesity. In total, glycosphingolipids were isolated from mucosal scrapings from seven individuals and, in two cases, also from the non-mucosal residues.

The major components of acid glycosphingolipid fractions migrated on thin-layer chromatograms as sulfatide and the GM3 ganglioside. No binding of \textit{H. pylori} to these acid glycosphingolipids was obtained (not shown). No binding of the bacteria to the non-acid glycosphingolipids from the non-epithelial stroma was observed.

The non-acid glycosphingolipid fractions isolated from the gastric epithelial cells from five of the seven individuals are shown in Fig. 7A. In one of the seven samples a binding of \textit{H. pylori} in the tetraglycosylceramide region was obtained (Fig. 7B). The fraction containing the binding-active tetraglycosylceramide (case 4) and one non-binding fraction (case 5) were separated by HPLC, and the isolated tetraglycosylceramides from each case (shown in Fig. 8) were characterized by $^1$H NMR, EI mass spectrometry, and gas chromatography-EI mass spectrometry of permethylated tetrasaccharides obtained by ceramide glycanase hydrolysis as follows.

Proton NMR of the Tetracylosylceramide Fractions from Human Gastric Epithelium—The proton NMR spectrum of fraction 4-II isolated from case 4 (data not shown) was dominated by globoside with its anomeric signals appearing at 4.81 ppm (Galβ), 4.52 ppm (GalαGalβ), 4.26 ppm (Galαβ), and 4.20/4.17 ppm (Glcβ). However, a small peak on the base of the Galα signal revealed that another glycosphingolipid was also present in this fraction. This signal was consistent with Glcβ1→3Galβ1→4Glcβ1Cer (lactotetraosylceramide), the potential other signals being buried under the globoside resonances. However, the GalαH1 of globotetraosylceramide would also have a very similar chemical shift. The exact shifts varies with temperature and other factors. To resolve this we compared the reference spectra of lactotetraosyl-, globotetraosyl-, and globotriaosylceramide run under similar conditions at 400 MHz. A reference mixture of lactotetraosylceramide and globotetraosylceramide was also prepared and run at 500 MHz. These comparisons...
Novel H. pylori Binding Glycosphingolipid

EI-Mass Spectrometry of the Tetracycosylceramide Fractions from Human Gastric Epithelium—The mass spectra (not shown) obtained by direct inlet EI mass spectrometry of the permethylated derivatives of fraction 4-II and 5-II from cases 4 and 5, respectively, were very similar. In both spectra the ions at m/z 260 and 228 (260 minus 32) were prominent, demonstrating a terminal HexNAc, whereas no ion indicating a terminal Hex at m/z 219 was found. Terminal HexNAc-Hex was shown by an ion at m/z 464. A fragment ion at m/z 945 (944 + 1) containing the whole carbohydrate chain and part of the ceramide indicated a HexNAc-Hex-Hex carbohydrate sequence.

Thus, by EI mass spectrometry only the major compound of the two samples, most likely globoside, was identified, whereas the minor compounds of the fractions indicated by the proton NMR experiments could not be discerned. However, the increased resolution obtained by combining chromatographic methods and mass spectrometry permitted the identification of these minor compounds, as described in the following paragraph.

High Temperature Gas Chromatography-EI Mass Spectrometry of Permethylated Tetrasaccharides from Human Gastric Epithelium—Fraction 4-II from case 4 and fraction 5-II from case 5 were hydrolyzed with ceramide glycanase, and the released tetrasaccharides were permethylated and analyzed by gas chromatography and gas chromatography-EI mass spectrometry. The results are summarized in Figs. 9 and 10. Each chromatographic peak was resolved in α- and β-conformer.

The tetrasaccharides of the stomach epithelium of the H. pylori binding case 4 were resolved into two peaks, as shown in Fig. 9, Run B. The dominating peak eluted at the same retention time as the saccharide from reference globoside, whereas the minor peak eluted at the retention time of the saccharide from reference lactotetraosylceramide.

The tetrasaccharides of the stomach epithelium of the non-binding case 5 (Fig. 9, Run C) were also resolved into two peaks, with the major peak at the same retention time as the saccharide from reference globoside. The smaller peak in this case eluted at the retention time of the saccharide from reference lactotetraosylceramide.

To further substantiate the differences in the tetracycosylceramide fractions from the H. pylori binding case 4 and the non-binding case 5, mass spectra of the permethylated oligosaccharides were obtained (Fig. 10).

The spectra of the dominant peaks of both cases were in agreement with that of standard globoside (not shown). However, the spectra of the minor tetrasaccharides of the H. pylori binding case 4 (Fig. 10, III) and the non-binding case 5 (Fig. 10, IV) showed some dissimilarities. Fragment ions demonstrating a terminal Hex-HexNAc-Hex carbohydrate sequence were seen at m/z 187 (219 – 32), 219, 432 (464 – 32), 464, and 668 in both spectra. However, in the spectrum of the late-eluting peak of case 5, the fragment ion at m/z 182 was prominent, as it was in the reference spectrum Fig. 10, II. In contrast, this ion was absent in the spectrum of the late-eluting peak of case 4 as well as in the reference spectrum Fig. 10, I. The fragment ion at m/z 182 is characteristic for type 2 carbohydrate chains, Galβ4GlcNAcβ (30, 31). The fragment ion at m/z 432 (464 minus 32) was also prominent in the spectrum of the saccharide from case 5 as in the spectrum of reference lactotetraosylceramide (Fig. 10, II), indicating that methanol is more readily eliminated from Galβ4GlcNAcβ chains than from Galβ3GlcNAcβ chains, most probably from C2-C3. The saccharide from case 4 gave a strong fragment ion at m/z 228. This ion was also predominant in the spectrum of reference lactotetra-
The glycosphingolipid composition of the human gastric epithelium has not been well characterized. In a study of glycosphingolipids of the mucosal cells and submucosal tissue of the human gastrointestinal tract (40), an enrichment of sulfatides in the fundic and antral mucosa of the stomach was reported. The major non-acid glycosphingolipids migrated as galactosylceramide, lactosylceramide, globotriaosylceramide, and globoside on thin-layer plates, whereas the main gangliosides migrated as GM3, GM1, and GD3. *H. pylori* binding lactosylceramide with phytosphingosine and hydroxy fatty acids has also been characterized in the human gastric epithelium (12).

In addition, the blood group Cad-active ganglioside (GalNAcβ4(NeuAcα3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer) has been identified in the fundus region of human stomach (41), whereas it was not found in the pyloric region (42), indicating a differential expression of glycosphingolipids in different regions of the human stomach.

Because of limited access to human gastric tissue, we initially concentrated on the *H. pylori* binding glycosphingolipid detected in human meconium, which is the first sterile feces of the newborn and consists mainly of extruded mucosal cells from the developing gastrointestinal tract. After isolation, this *H. pylori* binding glycosphingolipid was characterized by mass spectrometry, proton NMR, and methylation analysis as Galβ3GlcNAcβ3Galβ4Glcβ1Cer (lactotetraosylceramide). The tissue distribution of this glycosphingolipid is very limited. Until recently lactotetraosylceramide had only been identified in human meconium (33) in the small intestine of an individual previously resected according to Billroth II (34) in normal human gastric mucosa and in human gastric cancer tissue (43). However, the “normal” mucosa in 4 of the 5 cases described in the latter report was obtained by antrectomy due to duodenal or gastric ulcer. Immunohistochemical studies, using the monoclonal antibody K-21 demonstrated a selective expression of the Galβ3GlcNAc sequence in superficial human gastric mucosa of non-secretor individuals (44) coinciding with the localization of *H. pylori* binding to tissue sections (4, 6). An immunohistochemical study utilizing polyclonal antibodies binding to the Galβ3GlcNAc sequence showed the presence of lactotetraosylceramide in the brush border cells of human jejunum and ileum of blood group OLe(a–b–) non-secretor individuals and also of one individual with the blood group OLe(a+b+) non-secretor (45).

The relevance of the lactotetraosylceramide binding specificity was substantiated by the binding of *H. pylori* to the non-acid glycosphingolipid region of the non-acid glycosphingolipids isolated from the target epithelial cells of human stomach. By proton NMR and gas chromatography-mass spectrometry of permethylated tetrasccharides obtained by ceramide glycanase hydrolysis, it was demonstrated that the binding-active fraction contained lactotetraosylceramide. This binding-active lactotetraosylceramide was only found in one of seven individuals analyzed, which is suggestive in view of the fact that although infection with *H. pylori* and the associated chronic gastritis are very common, only a small fraction of those infected develops any further consequences such as peptic ulcer or gastric adenocarcinoma (46). The presence of lactotetraosylceramide on the gastric epithelial cells may be one of the

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**FIG. 9.** Reconstructed ion chromatograms of permethylated oligosaccharides released by ceramide glycanase. Run A was a reference mixture of globoside, lactotetraosylceramide, and lactoneotetraosylceramide, whereas run B was the tetraglycosylceramides from the gastric epithelium of case 4 of Table II, and run C was the tetraglycosylceramides from the gastric epithelium of case 5 of Table II. The analytical conditions are described under “Materials and Methods.” The oligosaccharides of the reference mixture (Run A) have been marked.

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**Frequency of Lactotetraosylceramide Binding among H. pylori Strains**—The frequency of expression of the lactotetraosylceramide binding property was estimated by analyzing the binding of the 74 *H. pylori* isolates listed in Table I to glycosphingolipids on thin-layer chromatograms. For the binding assays the bacteria were grown from stock cultures and examined for binding of lactotetraosylceramide of human meconium by the chromatogram binding assay. A positive binding indicated a pattern identical to that seen in lane 6 of Fig 1B. The strains that failed to bind were re-cultured twice from storage and re-assayed by the chromatogram binding assay, i.e. no binding to lactotetraosylceramide was detected in three consecutive assays of the strains assigned as non-binding. By these criteria, 9 of the 74 isolates analyzed (strains 15, 65, 176, 198, 239, 269, 271, and 272 and BH000334 of Table I) were non-binding, whereas 65 isolates (88%) expressed the lactotetraosylceramide binding capacity. A further notation was that lactotetraosylceramide was recognized also by the mutant strains lacking the Leb binding adhesin (babA2 mutant strain; Fig. 4D) or lacking the Alp protein (strain P1—140; Fig. 4C) or lacking the HpA protein (strain SS1(AhpA); not shown).

**DISCUSSION**

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co-factors necessary for the development of the severe consequences of the infection. An interesting notation in this context is that the stomach of the blood group A(Rh+/H11001) individual, where *H. pylori* binding in the tetraglycosylceramide region was observed, was obtained after surgery for peptic ulcer disease.

Serologic typing using erythrocytes and saliva demonstrated that the blood group status of case 4 was ALe(a+b)/H11002 non-secretor (data not shown), and this is in agreement with the presence of *H. pylori* binding-unsubstituted lactotetraosylceramide in the gastric epithelium of this individual. The non-secretor status of this individual is interesting in view of the increased prevalence of duodenal ulcer among non-secretors (47–49). One study (50) has demonstrated that non-secretion is not associated with increased susceptibility to infection with *H. pylori*. However, one may speculate that the secretor status determines the outcome of the colonization, *i.e.* that the increased liability of non-secretors to develop peptic ulcer disease may be due to the presence of the *H. pylori* binding lactotetraosylceramide on the gastric epithelium of these individuals.

Lactotetraosylceramide is also known as the Le\(^{a}\) antigen, present in red cell Lewis-negative ABH non-secretors (for review, see Ref. 51). However, to our knowledge no studies of the frequency of *H. pylori* infection among Le(a-b-)non-secretor individuals have been reported.

Among the 74 *H. pylori* isolates analyzed in this study, 65 strains (88\%) were found to express the lactotetraosylceramide binding specificity, whereas 9 strains were non-binding. The high prevalence of the lactotetraosylceramide binding property among the *H. pylori* isolates demonstrates that it is a conserved property of this gastric pathogen and may, thus, represent an important virulence factor.

Under the experimental conditions of the present study, *H. pylori* did not bind to the glycosphingolipids tentatively identified as sulfatide and the GM3 ganglioside in the acid fractions from human gastric epithelium. The binding of *H. pylori* to lactotetraosylceramide was not affected by changing the growth conditions, since this binding was obtained both when the bacteria were grown on agar and in broth. Also, binding to lactotetraosylceramide was obtained both with bacteria grown for 12 and 120 h. The binding to lactotetraosylceramide was inhibited by incubating the bacteria with free univalent lactotetraose but not with lactose.
Huesca et al. (52) report that upon treatment of H. pylori with acidic pH or heat the binding of this bacterium to sulfatide is induced. In our hands, when the chromatogram binding assay was conducted at pH 5, the bacteria failed to bind to any glycosphingolipid, including gangliotetraosylceramide, sulfatide, lactotetraosylceramide, and the Leb hexaglycosylceramide (data not reproduced). Alternatively, a binding to a multitude of glycosphingolipids with diverse sequences was observed. However, the pH gradient of the human gastric mucus layer ranges from pH 2 on the luminal side to almost pH 7 on the epithelial cell surface (53), suggesting that binding assays conducted at pH 7.3 may be of relevance for attachment of H. pylori to epithelial receptors.

The Leb determinant (Fuco2Galβ3(Fuco4GlcNAcβ) is based on the type 1 disaccharide unit, which is the terminal part of lactotetraosylceramide. Binding to lactotetraosylceramide was, however, also obtained with strains devoid of Leb binding activity, as e.g. the CCUG 41936 strain (identical to the 26695 strain) and the MO19 strain (14). Furthermore, inactivation of the babA gene coding for the Leb binding adhesin (14) did not abolish the binding of lactotetraosylceramide. Thus, the binding of H. pylori to the Leb determinant and to lactotetraosylceramide represents two separate binding specificities and not a cross-binding.

This was further substantiated by inspection of the minimum energy molecular model of lactotetraosylceramide in comparison with the Leb-6 glycosphingolipid, as shown in Fig. 11. In trying to discern the important parts making up the binding epitope of lactotetraosylceramide, two observations, the non-binding of lactotetraosylceramide (GlcNAcβ3Galβ4Glcβ1Cer) and of lactotetraosylceramide in which the acetamido moiety had been converted to an amine (Galβ3GlcNHβ3Galβ4Glcβ1Cer), indicate that the terminal disaccharide Galβ3GlcNAcβ3 constitutes the epitope. The non-binding of the latter structure further indicates either that an intact acetamido group is essential for binding to occur or that an altered conformation results since an amine no longer may participate in hydrogen bond interactions with the 2-OH group of the internal Galβ4. A combination of these two effects is also possible. Moreover, extension of the terminal Gal of lactotetraosylceramide by Gala3 or Fuco2 or substitution of the penultimate GlcNAc by Fuco4 yields structures that are inactive, suggesting that the major part of the terminal disaccharide Galβ3GlcNAcβ3 is directly involved in interactions with the adhesin responsible for binding. In the Leb structure, the GlcNAcβ3 residue is inaccessible, and the penultimate Galβ3 partly so since they are covered by the two fucoses, as seen in the top view of Fig. 11. Furthermore, since the binding of H. pylori to Leb is inhibited by the free oligosaccharide of the Leb isostructure (8), the GlcNAcβ3 residue of Leb is not essential for binding to this compound. Alignment of the minimum energy structures of the terminal tetrascacharide part of Leb-6 and Leb-6 shows that the only difference is an ~180° turn of the GlcNAcβ3 residue, thus proving the non-requirement of the acetamido moiety of the GlcNAcβ3 residue (or even more likely the whole residue) in the Leb structure, whereas in lactotetraosylceramide the opposite is true. It may be further noted that the angle between the ring plane of the terminal Galβ3 in lactotetraosylceramide and the corresponding plane in the Leb structure is close to 40°, due to the crowding caused by the two additional fucose units, affording an additional reason as to why these structures should be regarded as separate receptors for H. pylori.

In summary, the adherence of H. pylori to the mucosal cells of human stomach appears to be a multicomponent system where several bacterial adhesins recognize and bind to different receptors in the target tissue. This study identifies yet another H. pylori binding compound, i.e. lactotetraosylceramide. The distribution of this glycosphingolipid is very limited, and hitherto it has only been found in the human gastrointestinal tract. In other human tissues lactotetraosylceramide is substituted with fucose or sialic acid and thereby non-binding under the assay conditions used.
Acknowledgments—We thank Dr. Ivar Pedajas, Södersjukhuset, Stockholm, Sweden, for help with the blood group typing. Special thanks to Dr. Maan Abul Milh for help with culture of the bacteria and for providing the strains listed under IV in Table I.

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