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Lactotetraosylceramide, a Novel Glycosphingolipid Receptor for *Helicobacter pylori*, Present in Human Gastric Epithelium*

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The binding of *Helicobacter pylori* to glycosphingolipids was examined by binding of ^{35}S-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 *H. pylori* isolates (88%). Binding of *H. pylori* to lactotetraosylceramide on thin-layer chromatograms was inhibited by preincubation with lactotetraose but not with lactose. Removal of the terminal galactose of lactotetraosylceramide by galactosidase hydrolysis abolished the binding as did hydrazinolysis of the acetamido group of the *N*-acetylglucosamine. 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 Le^b blood group determinant (8), heparan sulfate (9), the GM3[1] 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 Le^b binding BabA adhesin, has been identified to date (14).

In the present study a number of different *H. pylori* strains were labeled with [^{35}S]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, gangliotriaosylceramide, 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 Göteborg, (CCUG), Sweden, and the clinical isolates S-002, and S-032, were used in parallel.

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[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-aminooctadecene), and t18:0 designates phytosphingosine (1,3,4-trihydroxy-2-aminooctadecane).

TABLE I
Helicobacter pylori isolates used in binding assays

Strains I-VII were obtained from the following sources. I. Strains were obtained from the Culture Collection University of Göteborg (CCUG), Sweden. II. Strains were obtained from the Department of Medical Microbiology and Immunology, Örebro Medical Centre Hospital, Sweden. III. Strains were obtained from the Department of Medical Microbiology, University of Lund, Sweden. IV. Strains were obtained from the Department of Microbiology, Medical University of Wroclaw, Poland. V. These strains were a kind gift of Dr. Thomas Borén, Umeå University, Sweden. VI. These strains were a kind gift of Dr. Rainer Haas, Ludwig-Maximilians-Universität, Munich, Germany. VII. These strains were a kind gift of Dr. Ingrid Bölin, Göteborg University, Sweden.

Bacterial strains
I. 15816, 17135, 17874, 17875, 18430, 18943, 20649, 30985, 30986, 30988, 30990, 30991, 30993, 41936
II. S-002, S-005, S-032, F6, Ö10, C7050
III. 4, 15[a], 17, 48, 51, 56, 62, 65[a], 69, 73, 77, 78, 80, 81, 88, 95, 176[a], 185, 188, 191, 198[a], 214, 225, 239[a], 244, 263, 266, 269[a], 271[a], 272[a], 275, 287, 306, 1139, BH00031, BH000324, BH000325, BH000331, BH000332, BH000334[a]
IV. 1, 177, 480, 604, 608, 609
V. P466, MO19, J99, 17875/*babA2*−
VI. P1, P1–140
VII. SS1, SS1 (Δ*hpa*A)

[a] No binding to lactotetraosylceramide was obtained with these strains.

The conditions used for culture and ^{35}S-labeling of the bacteria have been described previously (12). For binding assays, the bacteria were suspended to 1×10^8 colony forming units/ml in phosphate-buffered saline (PBS),[2] pH 7.4. The specific activities of the suspensions were ~1 cpm/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 assays were 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. The dried chromatograms were soaked for 1 min in diethyl ether/*n*-hexane (1:5, by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich). After drying, the chromatograms were coated to block unspecific binding sites. Initially different coating conditions were tested, *e.g.* 1% polyvinylpyrrolidone (w/v) in PBS (Solution 1), 2% gelatin (w/v) in PBS (Solution 2), 2% bovine serum albumin (w/v) in PBS (Solution 3), 2% bovine serum albumin (w/v) and 0.1% (w/v) Tween 20 in PBS (Solution 4), or 2% bovine serum albumin (w/v) and 0.2% (w/v) deoxycholic acid in PBS (Solution 5). The most consistent results were obtained with Solution 4, which subsequently was used as the standard condition. Coating was done for 2 h at room temperature. Thereafter, a suspension of ^{35}S-labeled bacteria (diluted in PBS to 1×10^8 colony forming units/ml and $1–5 \times 10^6$ cpm/ml) was gently sprinkled over the chromatograms and incubated for 2 h at room temperature. After washing six times with PBS and drying, the thin-layer plates were autoradiographed for 3–120 h using XAR-5 x-ray films (Eastman Kodak Co.).

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). Galβ3GlcNH$_2$$\beta$3Gal$\beta$4Glc$\beta$1Cer (No. 3 of Table III) was generated from Galβ3GlcNAcβ3Galβ4Glcβ1Cer (No. 2) by treatment with anhydrous hydrazine, as described (12).

Isolation of the H. pylori Binding Tetraglycosylceramide 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, and the fractions positive for anisaldehyde staining were further tested for binding of *H. pylori* using the chromatogram binding assay. The *H. pylori* binding fractions were collected in tubes 78–88, and after pooling of these fractions, 14.2 mg were 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). In two cases glycosphingolipids were also isolated from the 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 tetraglycosylceramides 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 tube 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/HF 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 Masslab, 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, Ranco Cordova, CA), with 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-

[2] The abbreviations used are: PBS, phosphate-buffered saline; Hex, hexose; HexNAc, *N*-acetylhexosamine; EI, electron ionization; HPLC, high performance liquid chromatography; BSA, bovine serum albumin.

TABLE II
Blood group data and amounts of glycosphingolipids isolated from human stomach specimens

Case No. blood group	Tissue	Non-acid glycosphingolipids	Acid glycosphingolipids
1. ORh−	Mucosal cells	7.0[a] (11.9)[b]	8.5[a] (14.4)[b]
	Non-mucosal residue	2.7 (6.0)	22.0 (48.8)
2. ARh+	Mucosal cells	3.6 (18.0)	10. (53.5)
3. ARh+	Mucosal cells	6.4 (14.5)	2.9 (6.6)
4. ARh+	Mucosal cells	6.0 (24.0)	4.8 (19.2)
5. ARh+	Mucosal cells	23.0 (38.0)	5.5 (9.2)
6. ARh−	Mucosal cells	4.9 (18.1)	8.2 (30.4)
7. Unknown	Mucosal cells	2.5 (15.6)	7.5 (46.8)
	Non-mucosal residue	4.3 (8.7)	7.6 (15.5)

[a] The weight is given in mg.
[b] Expressed as mg/g dry tissue weight.

methyl sulfoxide-d_6/D_2O (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 Glycanase Treatment of Tetraglycosylceramides 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 milliunit of ceramide glycanase from the leech, *Macrobdella decora* (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 from ceramides and detergent on a Sep-Pak C_{18} cartridge (Waters, Milford, MA). The eluant containing the oligosaccharides was dried under nitrogen and under vacuum and thereafter permethylated as described (22).

High Temperature Gas Chromatography and Gas Chromatography-EI Mass Spectrometry of the Permethylated Oligosaccharides—The analytical conditions were essentially the same as described in Karlsson *et al.* (27). Capillary gas chromatography was performed on a Hewlett Packard 5890A gas chromatograph using a fused silica column (10 m x 0.25-mm internal diameter) coated with 0.03 μm of cross-linked PS 264 (Fluka, Buchs, Switzerland) and with hydrogen as carrier gas. The permethylated oligosaccharides were dissolved in ethyl acetate, and 1 μl of sample was injected on-column at 70 °C (1 min). A two-step temperature program was used, 70 °C to 200 °C at 50 °C/min followed by 10 °C/min up to 350 °C.

Gas Chromatography-EI mass spectrometry was performed on a Hewlett Packard 5890-II gas chromatograph coupled to a JEOL SX-102A mass spectrometer. The chromatographic conditions as well as the capillary column were the same as for the analyses by gas chromatography, and the conditions for mass spectrometry were interface temperature 350 °C, ion source temperature 330 °C, electron energy 70 eV, trap current 300 μA, and acceleration voltage 10 kV.

Inhibition with Soluble Oligosaccharides—As a test for possible inhibition of binding by soluble sugars ^{35}S-labeled *H. pylori* strains S-002 and S-032 were incubated for 1 h at room temperature with various concentrations (0.05, 0.1, and 0.2 mg/ml) of lactotetraose (Accurate Chem. and Sci. Corp., Westbury, NY) or lactose (J. T. Baker Inc.) in PBS. Thereafter the chromatogram binding assay was performed as described above.

Molecular Modeling—Minimum energy conformations of the various glycosphingolipids listed in Table III were calculated within the Biograf molecular modeling program (Molecular Simulations Inc., Waltham, MA) using the Dreiding-II force field (28) on a Silicon Graphics4D/35TG work station. Charges were generated using the charge equilibration method (29), and a distance-dependent dielectric constant of 3.5 was used for the Coulomb interactions. In addition, a special hydrogen bonding term was used in which D_{hb} was set to −4 kcal/mol (28).

RESULTS

Binding to Mixtures of Reference Glycosphingolipids—A number of well characterized glycosphingolipid mixtures representing a large variety of carbohydrate sequences were separated by thin-layer chromatography. One chromatogram was stained with anisaldehyde, and duplicate chromatograms were used for binding of ^{35}S-labeled *H. pylori*. By subsequent autoradiography only a few bands were visualized, as shown in Fig. 1*B*. The binding in *lane 4* (gangliotriaosylceramide) and *lane 7* (gangliotetraosylceramide) was judged to correspond to the "ganglio binding specificity" of *H. pylori* described previously in detail (12).

In addition, selective binding of *H. pylori* to a compound migrating in the tetraglycosylceramide region in the non-acid glycosphingolipid fraction of human meconium was detected (Fig. 1*B*, *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 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 *H. pylori* on thin-layer chromatograms.

Structural characterization identified lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer) as the binding-active component. This conclusion was based 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 t18:0 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 *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 (not shown). The acetate of 2,3,4,6-tetramethylgalactitol identified a terminal galactose, whereas the presence of the acetate of 4,6-dimethyl-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

TABLE III
Results from binding of Helicobacter pylori to pure glycosphingolipids on thin-layer plates

No. trivial name	Glycosphingolipid structure[a]	Binding[b]	Source
1. Lactotri	GlcNAcβ3Galβ4Glcβ1Cer	−	Malignant melanoma
2. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	Human meconium
3.	Galβ3GlcNH$_2$β3Galβ4Glcβ1Cer	−	Human meconium[c]
4. H5–1	Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−	Human meconium
5. Le[a]-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−	Human intestine
6.	Galα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−	Monkey intestine[d]
7.	NeuGcα3Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−	Rabbit thymus
8.	NeuAcα6Galβ3GlcNAcβ3Galβ4Glcβ1Cer	−	Commercial[e]
9. Globoside	GalNAcβ3Galα4Galβ4Glcβ1Cer	−	Human erythrocytes

[a] The Galβ3GlcNAcβ parts have been underlined.
[b] + marks a significant darkening on the autoradiogram when 2 μg was applied on the thin-layer plate, while − marks no darkening.
[c] Glycosphingolipid No. 3 was produced from Galβ3GlcNAcβ3Galβ4Glcβ1Cer from human meconium (No. 2) by treatment with anhydrous hydrazine (12).
[d] Glycosphingolipid No. 6 was generated from Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glcβ1Cer from monkey intestine by incubation in 0.05 M HCl at 80 °C for 2 h.
[e] Glycosphingolipid No. 8 was purchased from Wako Chemicals GmbH, Neuss, Germany.

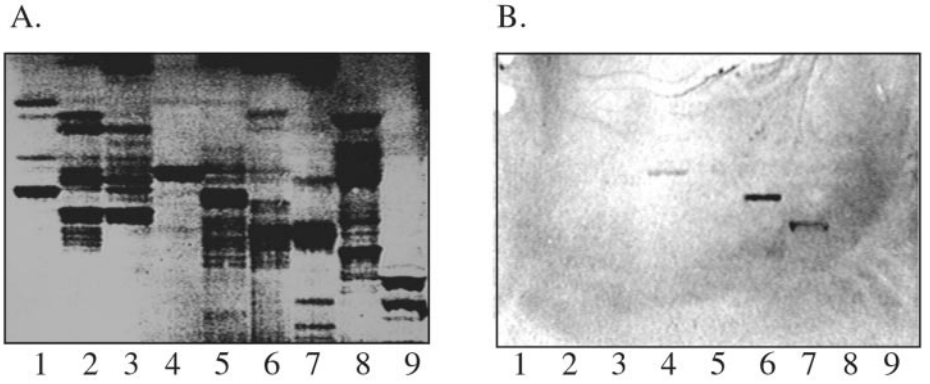

FIG. 1. **Detection of a *H. pylori* binding glycosphingolipid in the non-acid glycosphingolipid fraction of human meconium.** *A*, glycosphingolipids detected with anisaldehyde. *B*, glycosphingolipids detected by autoradiography after binding of radiolabeled *H. pylori* strain CCUG17875. The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8, by volume) as the solvent system, and the binding assay was performed as described under "Materials and Methods." The autoradiogram in *B* was obtained after coating of the thin-layer chromatogram with 2% BSA and 0.1% Tween 20 in PBS. The lanes contained non-acid glycosphingolipids of human blood group A erythrocytes, 40 μg (*lane 1*); non-acid glycosphingolipids of dog small intestine, 40 μg (*lane 2*); non-acid glycosphingolipids of guinea pig small intestine, 40 μg (*lane 3*); non-acid glycosphingolipids of guinea pig erythrocytes, 40 μg (*lane 4*); non-acid glycosphingolipids of rat small intestinal epithelium, 40 μg (*lane 5*); non-acid glycosphingolipids of human meconium, 40 μg (*lane 6*); non-acid glycosphingolipids of mouse feces, 40 μg (*lane 7*); acid glycosphingolipids of human blood group O erythrocytes, 40 μg (*lane 8*); bovine brain gangliosides, 40 μg (*lane 9*). Autoradiography was for 12 h.

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 ($J_{1,2} \approx 8$ 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 Galβ4 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*-acetamido methyl protons resonating at 1.82 ppm. Finally, the terminal Galβ signal was found at 4.15 ppm ($J_{1,2} = 6.6$ Hz), indicating a 1-to-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 Isoreceptors—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*-acetylneuraminic acid in 6-position of the terminal galactose or an α-fucose in 4-position of the *N*-acetylglucosamine (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*-acetylglucosamine 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 tetraglycosylceramide region of these non-acid fractions was dominated by globoside (Fig. 6*A*, *lane 4*), which at least for human small (34) and large intestine (35), is derived from the non-epithelial part. No bind-

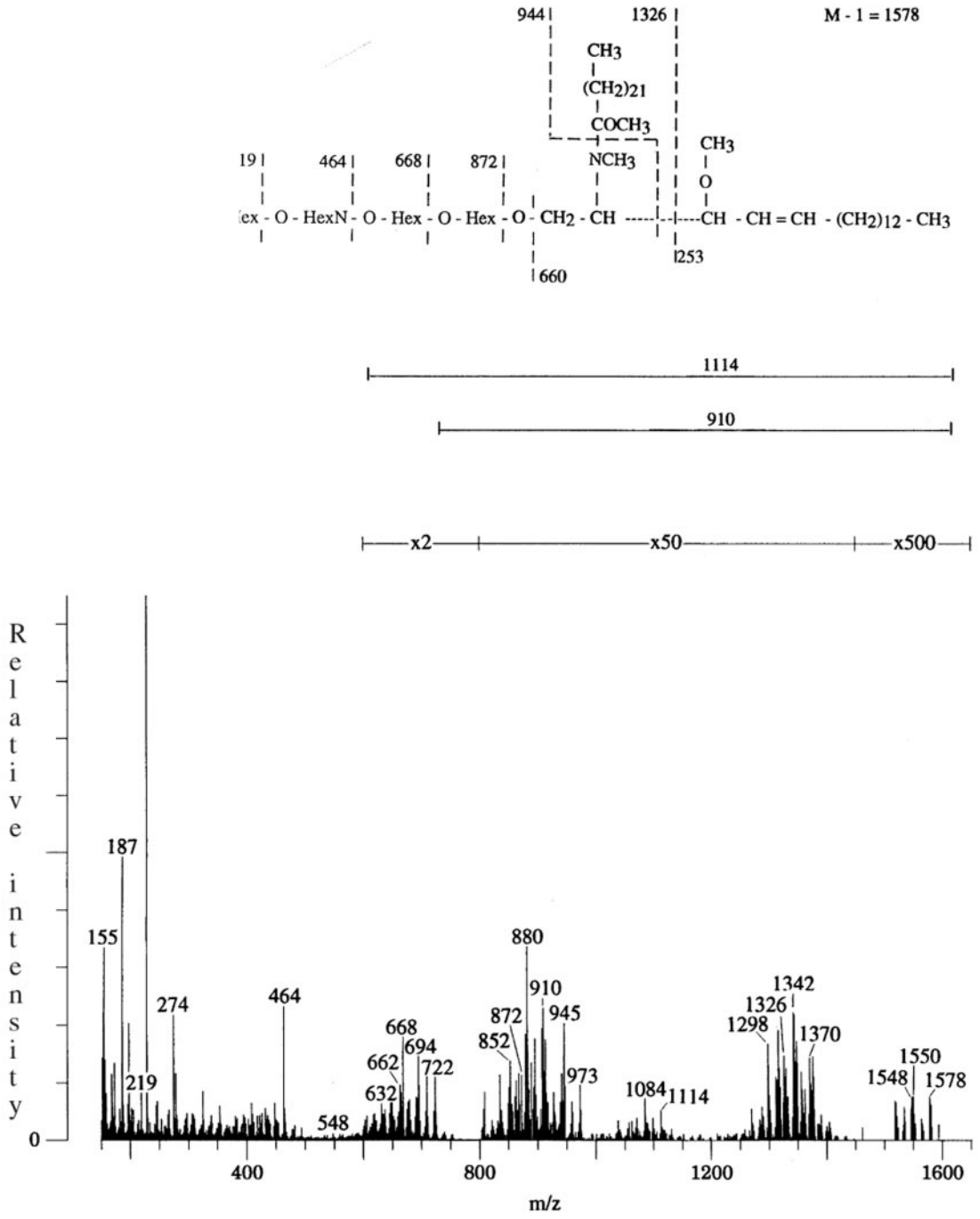

FIG. 2. **EI mass spectrum of the permethylated *H. pylori* binding tetraglycosylceramide from human meconium.** The spectrum was recorded at 300 °C. Above the spectrum is a simplified formula for interpretation, representing the species with sphingosine and hydroxy 24:0 fatty acid. The carbohydrate sequence ions at *m/z* 219 and 187 (219 − 32), 464, 668, and 872 demonstrated a tetraglycosylceramide with Hex-HexNAc-Hex-Hex sequence. This was supported by the fragment ion at *m/z* 945 (944 + 1), which consisted of the whole carbohydrate chain and part of the fatty acid. Molecular ions corresponding to the species with d18:1–24:0, d18:1-h22:0, and d18:1-h24:0 ceramides were found at *m/z* 1548, 1550, and 1578, respectively. Loss of the terminal parts of the carbohydrate chain from the molecular ions were also seen (explained below the formula for the species with d18:1-h24:0 ceramide). Immonium ions, containing the complete carbohydrate chain together with the fatty acid, were found at *m/z* 1298 and 1326 and also gave evidence of a carbohydrate part composed of 3 Hex and 1 HexNAc combined with h22:0 and h24:0 fatty acids. The ions at *m/z* 1342 and 1370 also indicated a compound with 3 Hex and 1 HexNAc and phytosphingosine with h22:0 (1582 − 241) and h24:0 (1610 − 241) fatty acids. Additional information about the ceramide composition was given by the series of ions at *m/z* 548–722, demonstrating a mixture of species ranging from d18:1–16:0 to t18:0-h24:0.

ing to these fractions was obtained (Fig. 6*B*, *lane 4*). However, when using the non-acid glycosphingolipid fraction isolated from the stomach of a blood group A(Rh+)p individual (36), which lacked the galactosyltransferase responsible for the conversion of lactosylceramide to globotriaosylceramide (37) and consequently was devoid of globoside (Fig. 6*A*, *lane 3*), a binding of *H. pylori* in the tetraglycosylceramide region was detected (Fig. 6*B*, *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

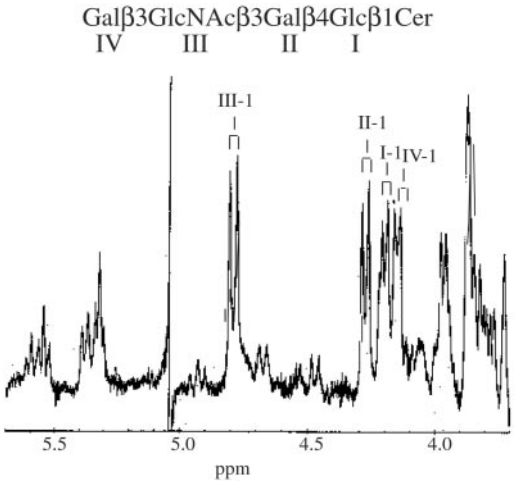

FIG. 3. **The anomeric region of a 300-MHz proton NMR spectrum of the *H. pylori* binding glycosphingolipid from human meconium.** 4000 scans were collected at a probe temperature of 30 °C. The large dispersion-like signal at 5.04 ppm is an instrumental artifact.

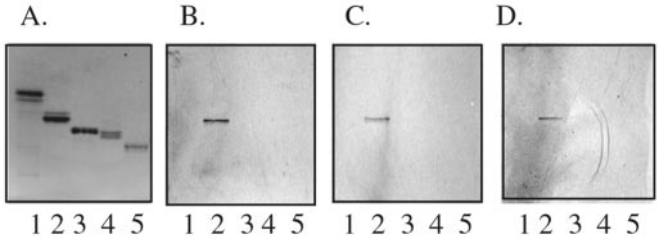

FIG. 4. **Binding of *H. pylori* to pure glycosphingolipids separated on thin-layer plates.** *A*, chemical detection by anisaldehyde. *B–D*, autoradiograms obtained by binding of ^{35}S-labeled *H. pylori* strain CCUG 41936 (*B*), P1–140 (*C*), and the *babA2* mutant strain (*D*). The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8, by volume) as solvent system, and the binding assay was performed as described under "Materials and Methods" using 2% BSA and 0.1% Tween 20 in PBS as the coating buffer. The lanes were GlcNAcβ3Galβ4Glcβ1Cer (lactotriaosylceramide), 4 μg (*lane 1*); Galβ3GlcNAcβ3Galβ4Glcβ1Cer (lactotetraosylceramide), 4 μg (*lane 2*); Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer (H5 type 1 glycosphingolipid), 4 μg (*lane 3*); Galβ3(Fucα4)GlcNAcβ3Galβ4-Glcβ1Cer (Le^a-5 glycosphingolipid), 4 μg (*lane 4*); and Fucα2Galβ4(Fucα3)-GlcNAcβ3Galβ4Glcβ1Cer (Y-6 glycosphingolipid), 4 μg (*lane 5*). Autoradiography was for 12 h.

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 compounds of acid glycosphingolipid fractions migrated on thin-layer chromatograms as sulfatide and the GM3 ganglioside. No binding of *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. 7*A*. In one of the seven samples a binding of *H. pylori* in the tetraglycosylceramide region was obtained (Fig. 7*B*). 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.

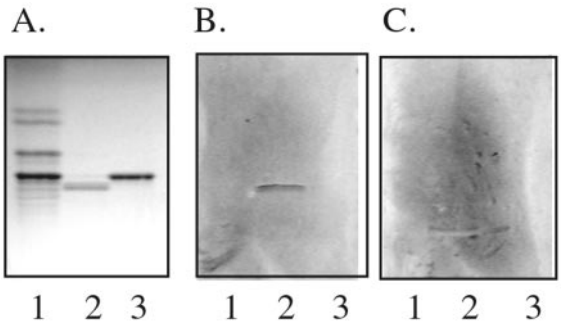

FIG. 5. **Effect of preincubation of *H. pylori* with oligosaccharides.** Radiolabeled *H. pylori* strain CCUG 17875 was incubated with lactose (0.2 mg/ml) or lactotetraose (0.1 mg/ml) in PBS for 1 h at room temperature. Thereafter the suspensions were utilized in the chromatogram binding assay. Thin-layer chromatogram stained with anisaldehyde (*A*), binding of *H. pylori* incubated with lactose (*B*), and binding of *H. pylori* incubated with lactotetraose (*C*). The *lanes* were non-acid glycosphingolipids of human blood group AB erythrocytes, 40 μg (*lane 1*); Galβ3GlcNAcβ3Galβ4Glcβ1Cer (lactotetraosylceramide), 4 μg (*lane 2*); and GalNAcβ3Galα4Galβ4Glcβ1Cer (globoside), 4 μg (*lane 3*). The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8, by volume) as the solvent system, and the binding assay was performed as described under "Materials and Methods" using 2% BSA and 0.1% Tween 20 in PBS as the coating buffer. Autoradiography was for 12 h.

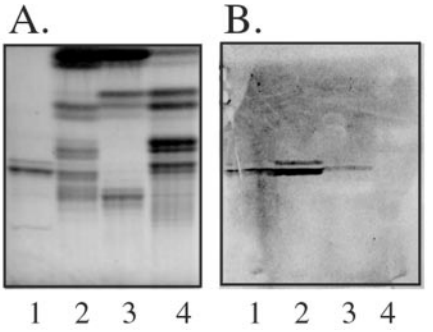

FIG. 6. **Binding of *H. pylori* to non-acid glycosphingolipids of whole human stomach.** Thin-layer chromatogram of separated glycosphingolipids detected with anisaldehyde (*A*) and autoradiogram obtained by binding of ^{35}S-labeled *H. pylori* strain S-002 (*B*). The *lanes* were lactotetraosylceramide of human meconium, 4 μg (*lane 1*); non-acid glycosphingolipids of human meconium, 40 μg (*lane 2*); non-acid glycosphingolipids of human stomach of a blood group A(Rh+)p individual, 40 μg (*lane 3*); and non-acid glycosphingolipids of human stomach of a blood group A(Rh+)P individual, 40 μg (*lane 4*). The glycosphingolipids were separated on aluminum-backed silica gel plates using chloroform/methanol/water (60:35:8, by volume) as the solvent system, and the binding assay was done as described under "Materials and Methods." The coating buffer contained 2% BSA and 0.1% Tween 20 in PBS. Autoradiography was for 5 h.

Proton NMR of the Tetraglycosylceramide 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 (Galα), 4.52 ppm (GalNAcβ), 4.26 ppm (Galβ), and 4.20/4.17 ppm (Glcβ). However, a small peak on the base of the Galα H1 signal revealed that another glycosphingolipid was also present in this fraction. This signal was consistent with GlcNAcβ H1 of lactotetraosylceramide, the potential other signals being buried under the globoside resonances. However, the Galα H1 of globotriaosylceramide 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

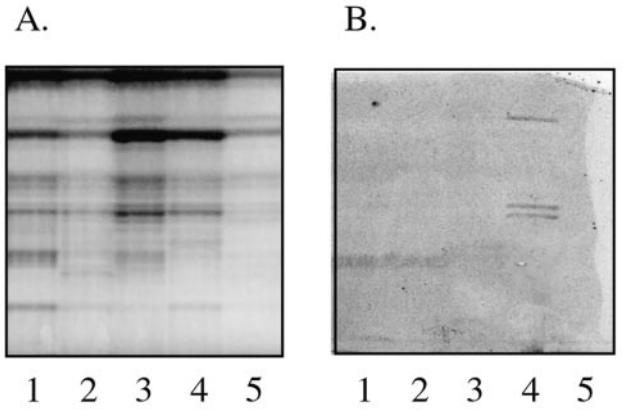

FIG. 7. **Binding of *H. pylori* to non-acid glycosphingolipids from human gastric epithelium.** *Lanes 1–5* were non-acid glycosphingolipids (80 μg/lane) of human gastric epithelium of five individuals (cases 1–5 of Table II). (*A*) Chemical detection with anisaldehyde. (*B*) Autoradiogram obtained by binding of ^{35}S-labeled *H. pylori* strain S-032. The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water (60:35:8, by volume) as solvent system, and the binding assay was performed as described under "Materials and Methods," using 2% BSA and 0.1% Tween 20 in PBS as coating buffer. Autoradiography was for 12 h.

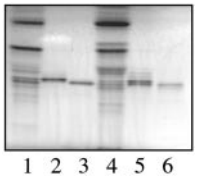

FIG. 8. **Thin-layer chromatogram showing the tetraglycosylceramide-containing fractions obtained from gastric epithelium of cases 4 and 5 of Table II.** The lanes were total non-acid glycosphingolipids of gastric epithelium of case 4, 80 μg (*lane 1*); fraction 4-I from case 4, 4 μg (*lane 2*); fraction 4-II from case 4, 4 μg (*lane 3*); total non-acid glycosphingolipids of gastric epithelium of case 5, 80 μg (*lane 4*); fraction 5-I from case 5, 4 μg (*lane 5*); and fraction 5-II from case 5, 4 μg (*lane 6*). The glycosphingolipids were separated on glass-backed silica gel plates using chloroform/methanol/water (60:35:8, by volume) as solvent system and stained with anisaldehyde.

clearly showed that the signal at 4.79 ppm belonged to a β-anomeric proton from the *N*-acetylglucosamine of lactotetraosylceramide. This was further corroborated when analyzing the more early eluting tetraglycosylceramide-containing fraction (4-I) from case 4. Here two non-overlapping α-anomeric signals from galactose, one corresponding to the internal Galα H1 of globotetraosylceramide (4.81 ppm) and the other corresponding to terminal Galα H1 of globotriaosylceramide (4.78 ppm) were found.

The presence of lactotetraosylceramide should also give rise to a different methyl signal from the *N*-acetamido glucose (38) compared with the *N*-acetamido galactose of globotria- and globotetraosylceramide. The GalNAc methyl signal was seen at 1.85 ppm, and the methyl signal of the GlcNAc in lactotetraosylceramide was seen at 1.82 ppm, which is identical to our reference spectra and in close agreement with the values reported in Clausen *et al.* (39). From the intensities of the methyl signals it was estimated that fraction 4-II contained ~5% lactotetraosylceramide.

The early eluting tetraglycosylceramide-containing fraction (5-I) from case 5 contained both globotria- and globotetraosylceramide, as evidenced by α-anomeric signals at 4.81 and 4.78 ppm, respectively. The more late-eluting tetraglycosylceramide-containing fraction (5-II) also contained a β-doublet at 4.65 ppm corresponding to GlcNAcβ of lactoneotetraosylceramide (39). The *N*-acetamido glucose of this glycosphingolipid had a methyl signal at 1.82 ppm, in agreement with earlier data on lactoneotetraosylceramide (38).

EI-Mass Spectrometry of the Tetraglycosylceramide 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-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 of reference lactoneotetraosylceramide.

To further substantiate the differences in the tetraglycosylceramide 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 lactoneotetraosylceramide (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 lactotetrao-

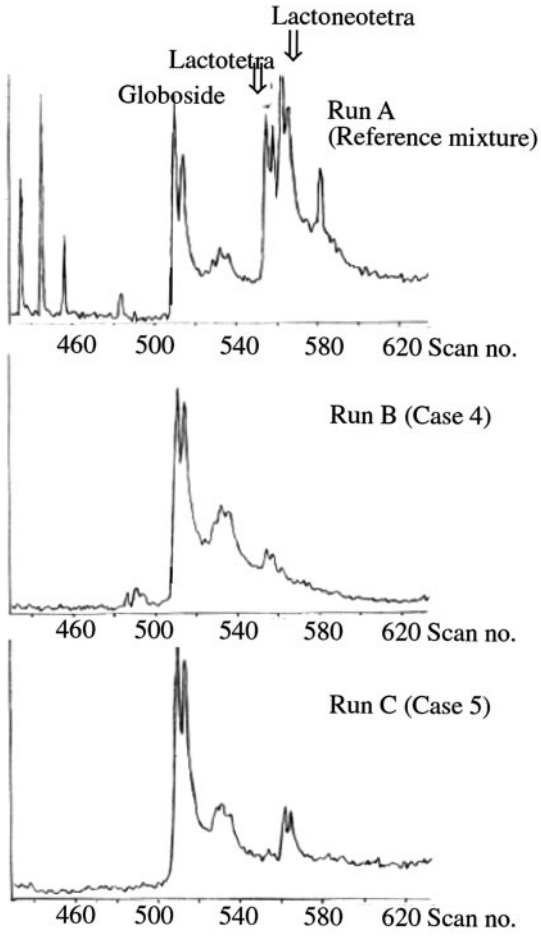

FIG. 9. **Reconstructed ion chromatograms of permethylated oligosaccharides released by ceramide glycanase.** *Run A* was a reference mixture of globoside, lactotetraosylceramide, and lactoneotetraosylceramide, whereas *run B* was the tetraglycosylceramides from the gastric epithelium of case 4 of Table 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.

sylceramide (Fig. 10, *I*) and probably originated from the internal GlcNAc, since no ion at *m/z* 260 was seen.

In conclusion, by gas chromatography and gas chromatography-EI mass spectrometry of permethylated oligosaccharides from the tetraglycosylceramides of cases 4 and 5, the results from proton NMR spectroscopy of these fractions were confirmed. The predominant compound of both fractions was identified as globotetraose, whereas the minor components differed. In the case of the *H. pylori* binding case 4, the minor compound was identified as lactotetraose, whereas the non-binding case 5 had neolactotetraose.

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 1*B*. 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 Le^b binding adhesin (*babA2* mutant strain; Fig. 4*D*) or lacking the Alp protein (strain P1–140; Fig. 4*C*) or lacking the HpaA protein (strain SS1(Δ*hpaA*); not shown).

DISCUSSION

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 tetraglycosylceramide 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 tetrasaccharides 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

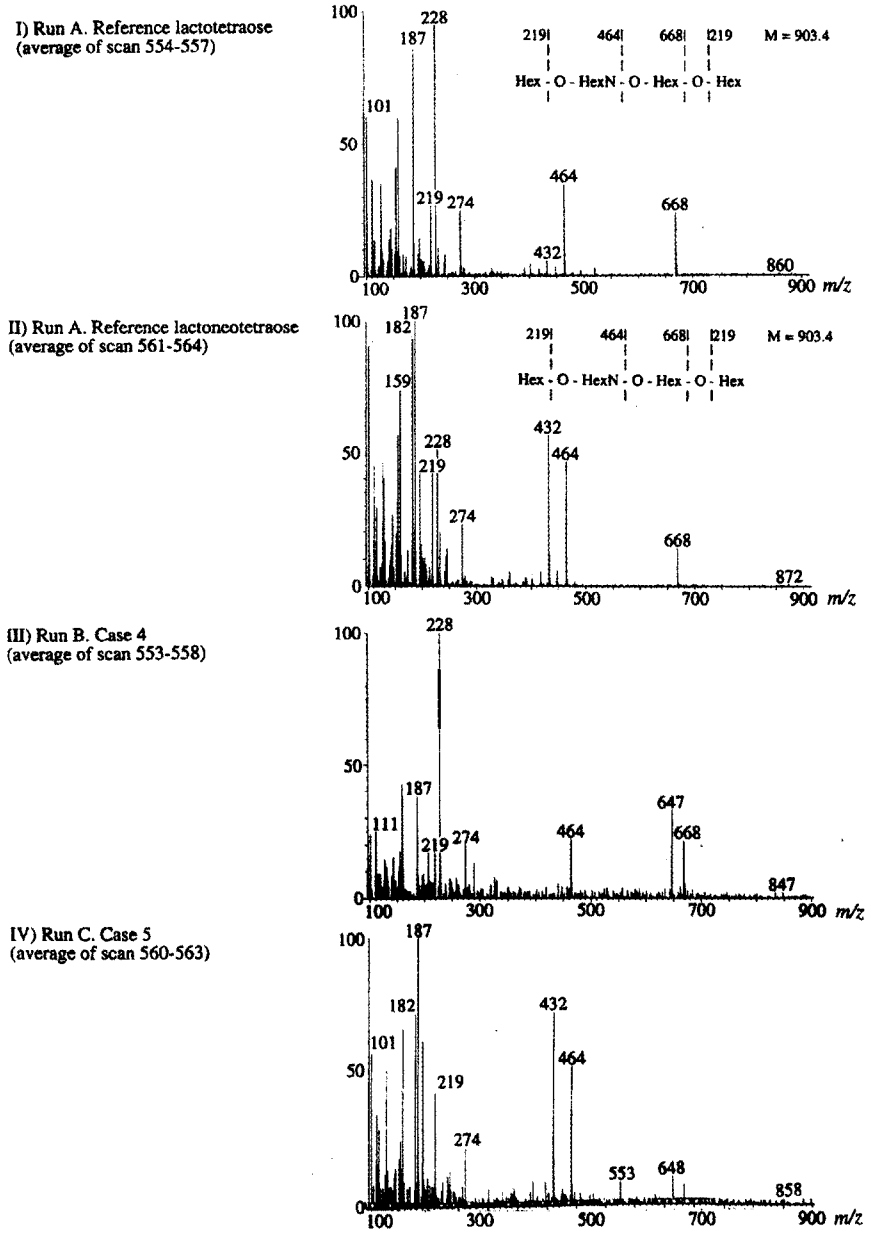

FIG. 10. **Mass spectra obtained by high temperature gas chromatography-EI mass spectrometry of permethylated oligosaccharides released by ceramide glycanase from reference glycosphingolipids (I and II), tetraglycosylceramide fractions from the gastric epithelium of case 4 of Table II (III), and of case 5 of Table II (IV).** For analytical conditions, see "Materials and Methods." The designations *Runs A–C* refer to the partial total ion chromatograms shown in Fig. 10. Interpretation formulae are shown together with the reference spectra.

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+)p 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−)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^c 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 Le^b 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 Le^b determinant (Fucα2Galβ3(Fucα4)GlcNAcβ) 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 Le^b binding activity, as *e.g.* the CCUG 41936 strain (identical to the 26695 strain) and the MO19 strain (14). Furthermore, inactivation of the *bab*A gene coding for the Le^b binding adhesin (14) did not abolish the binding of lactotetraosylceramide. Thus, the binding of *H. pylori* to the Le^b 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 Le^b-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 lactotriaosylceramide (GlcNAcβ3Galβ4Glcβ1Cer) and of lactotetraosylceramide in which the acetamido moiety had been converted to an amine (Galβ3GlcNH$_2\beta$3Galβ4Glcβ1Cer), 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 Galα3 or Fucα2 or substitution of the penultimate GlcNAc by Fucα4 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 Le^b 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 Le^b is inhibited by the free oligosaccharide of the Le^y isostructure (8), the GlcNAcβ3 residue of Le^b is not essential for binding to this compound. Alignment of the minimum energy structures of the terminal tetrasaccharide part of Le^b-6 and Le^y-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 Le^b 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 Le^b 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.

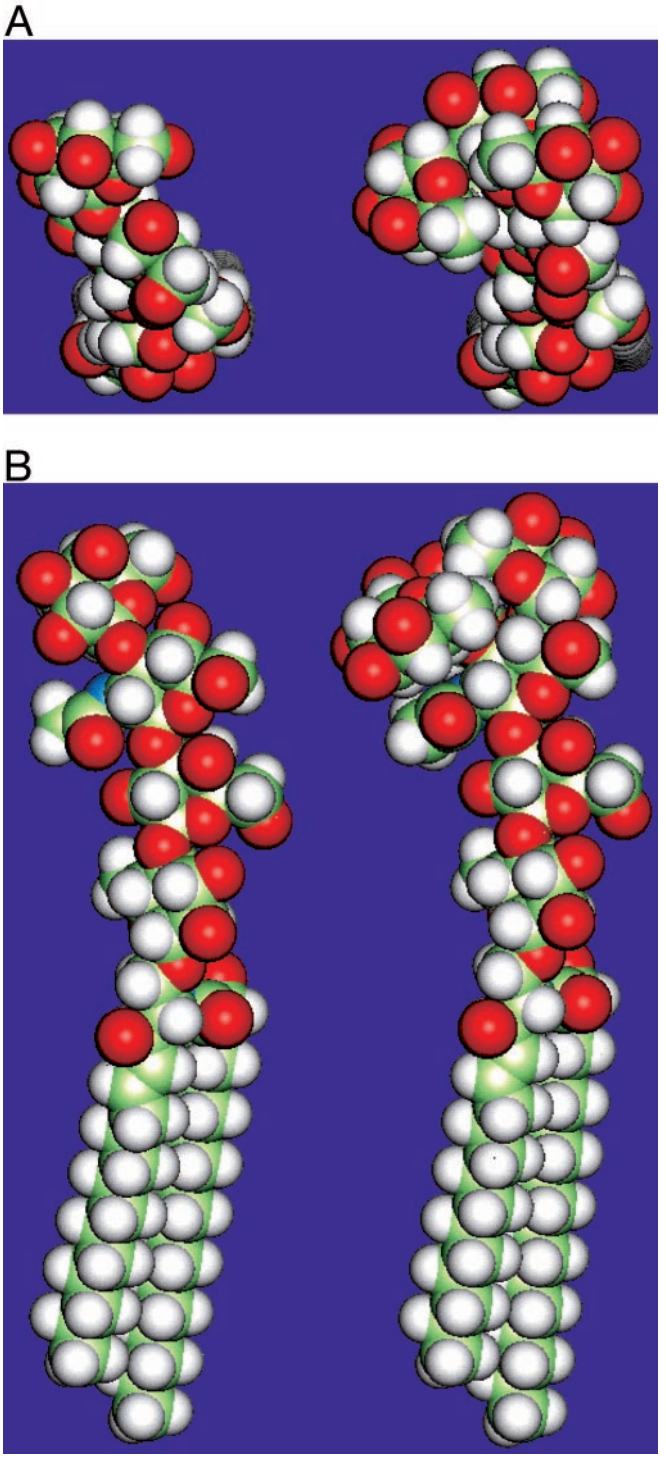

FIG. 11. **Minimum energy conformers of the *H. pylori* binding lactotetraosylceramide (Galβ3GlcNAcβ3Galβ4Glcβ1Cer, *left*) and the Le^b-6 glycosphingolipid (Fucα2Galβ3(Fucα4)GlcNAcβ3-Galβ4Glcβ1Cer, *right*) (*B*).** The top charts (*A*) show the same structures viewed from above. The Glcβ1Cer linkage is shown in an extended conformation. A detailed comparison between these two structures is made in the text.

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REFERENCES

1. Beachey, E. H. (1981) *J. Infect. Dis.* **143,** 325–345
2. Cover, T. L., Berg, D. E., Blaser, M. J., and Mobley, H. L. T. (2001) in *Principals of Bacterial Pathogenesis* (Groisman, E. A., ed) pp. 509–558, Academic Press, Inc., New York
3. Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., Taniyama, K., Sasaki, N., Schlemper, R. J. (2001) *N. Engl. J. Med.* **11,** 784–789
4. Bode, G., Malfertheiner, P., and Ditschuneit, H. (1988) *Scand. J. Gastroenterol.* **23,** Suppl. 142, 25–39
5. Thomsen, L. L., Gavin, J. B., and Tasman-Jones, C. (1990) *Gut* **31,** 1230–1236
6. Falk, P., Roth, K. A., Borén, T., Westblom, T. U., Gordon, J. I., and Normark, S. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2035–2039
7. Lingwood, C. A., Huesca, M., and Kuksis, A. (1992) *Infect. Immun.* **60,** 2470–2474
8. Borén, T., Falk, P., Roth, K. A., Larson, G., and Normark, S. (1993) *Science* **262,** 1892–1895
9. Ascencio, F., Fransson, L.-Å., and Wadström, T. (1993) *J. Med. Microbiol.* **38,** 240–244
10. Saitoh, T., Natomi, H., Zhao, W., Okuzumi, K., Sugano, K., Iwamori, M., and Nagai, Y. (1991) *FEBS Lett.* **282,** 385–387
11. Kamisago, S., Iwamori, M., Tai, T., Mitamura, K., Yazaki, Y., and Sugano, K. (1996) *Infect. Immun.* **64,** 624–628
12. Ångström, J., Teneberg, S., Abul Milh, M., Larsson, T., Leonardsson, I., Olsson, B.-M., Ölwegård Halvarsson, M., Danielsson, D., Näslund, I., Ljungh, Å., Wadström, T., and Karlsson, K.-A. (1998) *Glycobiology* **8,** 297–309
13. Miller-Podraza, H., Abul Milh, M., Bergström, J., and Karlsson, K.-A. (1996) *Glycoconj. J.* **13,** 453–460
14. Ilver, D., Arnqvist, A., Ögren, J., Frick, I. M., Kersulyte, D., Incecik, E. T., Berg, D. E., Covacci, A., Engstrand, L., and Borén, T. (1998) *Science* **279,** 373–377
15. Waldi, D. (1962) in *Dünnschicht-Chromatographie* (Stahl, E., ed.) pp. 496–515, Springer-Verlag, Berlin
16. Hansson, G. C., Karlsson, K.-A., Larson, G., Strömberg, N., and Thurin, J. (1985) *Anal. Biochem.* **146,** 158–163
17. Karlsson, K.-A. (1987) *Methods Enzymol.* **138,** 212–220
18. Samuelsson, B. E., Pimlott, W., and Karlsson K.-A. (1990) *Methods Enzymol.* **193,** 623–646
19. Koerner. T. A. W., Jr., Prestegard, J. H., Demou, P. C., and Yu, R. K. (1983) *Biochemistry* **22,** 2676–2687
20. Yang, H., and Hakomori, S.-i. (1971) *J. Biol. Chem.* **246,** 1192–1200
21. Stellner, K., Saito, H., and Hakomori, S.-i. (1973) *Arch. Biochem. Biophys.* **155,** 464–472
22. Larson, G., Karlsson, H., Hansson, G. C., and Pimlott, W. (1987) *Carbohydr. Res.* **161,** 281–290
23. Breimer, M., Hansson, G. C., Karlsson, K.-A., Larson, G., Leffler, H., Pascher, I., Pimlott, W., and Samuelsson, B. E. (1980) in *Advances in Mass Spectrometry* (Quayle, A., ed.) Vol. 8, pp. 1097–1108, Heyden & Son, London
24. Hansson, G. C., Li, Y.-T., and Karlsson, H. (1989) *Biochemistry* **28,** 6672–6678
25. Hakomori, S.-i. (1983) in *Sphingolipid Biochemistry* (Kanfer, J. N., and Hakomori, S.-i., eds.) Vol. 3, pp. 1–165, Plenum Press, New York
26. Ledeen, R. W., and Yu, R. K. (1978) *Res. Methods Neurochem.* **4,** 371–410
27. Karlsson, H., Karlsson, N., and Hansson, G. C. (1994) *Mol. Biotechnol.* **1,** 165–180
28. Mayo, S. L., Olafson, B. D., and Goddard, W. A., III (1990) *J. Phys. Chem.* **94,** 8897–8909
29. Rappé, A. K., and Goddard, W. A., III (1990) *J. Phys. Chem.* **95,** 3358–3363
30. Karlsson, K.-A. (1976) in *Glycolipid Methodology* (Witting, L. A., ed.) pp. 97–122, American Oil Society, Champaign, Ill
31. Karlsson, K.-A. (1978) *Prog. Chem. Fats Other Lipids* **16,** 207–230
32. Holmes, E. H., and Levery, S. B. (1989) *Arch. Biochem. Biophys.* **274,** 14–25
33. Karlsson, K.-A., and Larson, G. (1979) *J. Biol. Chem.* **254,** 9311–9316
34. Björk, S., Breimer, M. E., Hansson, G. C., Karlsson, K.-A., and Leffler, H. (1987) *J. Biol. Chem.* **262,** 6758–6765
35. Holgersson, J., Jovall, P.-Å., and Breimer, M. E. (1991) *J. Biochem.* (*Tokyo*) **110,** 120–131
36. Breimer, M. E., Cedergren, B., Karlsson, K.-A., Nilsson, K., and Samuelsson, B. E. (1980) *FEBS Lett.* **118,** 209–211
37. Marcus, D. M., Naiki, M., and Kundu, S. K. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3263–3267
38. Gasa, S., Mitsuyama, T., and Makita, A. (1983) *J. Lipid Res.* **24,** 174–182
39. Clausen, H., Levery, S. B., Kannagi, R., and Hakomori, S.-i. (1986) *J. Biol. Chem.* **261,** 1380–1387
40. Natomi, H., Saitoh, T., Sugano, K., Iwamori, M., Fukayama, M., and Nagai, Y. (1993) *Lipids* **28,** 737–742
41. Dohi, T., Ohta, S., Hanai, N., Yamaguchi, K., and Oshima, M. (1990) *J. Biol. Chem.* **265,** 7880–7885
42. Dohi, T., Hanai, N., Yamaguchi, K., and Oshima, M. (1991) *J. Biol. Chem.* **266,** 24038–24043
43. Hattori, H., Uemura, K.-I., and Taketomi, T. (1981) *Biochim. Biophys. Acta* **666,** 361–369
44. Blasco, E., Gutierrez-Hoyos, A., Lojendio, M., Cosme, A., and Arenas, J. I. (1991) *Eur. J. Cancer* **27,** 501–503
45. Henry, S. M., Samuelsson, B. E., and Oriol, R. (1994) *Glycoconj. J.* **11,** 600–607
46. Feldman, R. A. (2001) in *Helicobacter pylori: Molecular and Cellular Biology* (Achtman, M. And Suerbaum, S., eds) pp. 29–51, Horizon Scientific Press, Wymondham, UK
47. Clark, C. A., Wyn Edwards, J., Haddock, D. R. W., Howel-Evans, A. W., McConnell, R. B,., and Sheppard, P. M. (1956) *Br. Med. J.* **2**, 725–731
48. Rotter, J. I. (1983) in *Principles and Practice of Medical Genetics* (Emery, A. E. H., and Riomoin, D. L., eds.) pp. 863–878, Churchill Livingstone, NY
49. Mentis, A., Blackwell, C. C., Weir, D. M., Spiliadis, C., Dailianas, A., and Skandalis, N. (1991) *Epidemiol. Infect.* **106,** 221–229
50. Dickey, W., Collins, J. S. A., Watson, R. P. G., Sloan, J. M., and Porter, K. G. (1993) *Gut* **43,** 351–353
51. Henry, S., Oriol, R., and Samuelsson, B. (1995) *Vox Sang.* **69,** 166–182
52. Huesca, M., Borgia, S., Hoffman, P., and Lingwood, C. A. (1996) *Infect. Immun.* **64,** 2643–2648
53. Quigley, E. M. M., and Turnberg, L. A. (1987) *Gastroenterology* **92,** 1876–1883