Helicobacter pylori has a number of well-characterized carbohydrate-binding adhesins (BabA, Saba, and LabA) that promote adhesion to the gastric mucosa. In contrast, information on the glycoconjugates present in the human stomach remains unavailable. Here, we used MS and binding of carbohydrate-recognizing ligands to characterize the glycosphingolipids of three human stomachs from individuals with different blood group phenotypes (O(Rh−)P, A(Rh+)P, and A(Rh+)p), focusing on compounds recognized by H. pylori. We observed a high degree of structural complexity, and the composition of glycosphingolipids differed among individuals with different blood groups. The type 2 chain was the dominating core chain of the complex glycosphingolipids in the human stomach, in contrast to the complex glycosphingolipids in the human small intestine, which have mainly a type 1 core. H. pylori did not bind to the O(Rh−)P stomach glycosphingolipids, whose major complex glycosphingolipids were neolactotetraosylceramide, the Lea, Le, and H type 2 pentasaccharides, and the Lex hexaosylceramide. Several H. pylori-binding compounds were present among the A(Rh+)P and A(Rh+)p stomach glycosphingolipids. Ligands for BabA-mediated binding of H. pylori were the Leb hexaosylceramide, the H type 1 pentasaccharide, and the A type 1/ALeb heptaosaccharide. Additional H. pylori-binding glycosphingolipids recognized by BabA-deficient strains were lactosylceramide, lactotetraosylceramide, the x, pentaosylceramide, and neolactohexaosylceramide. Our characterization of human gastric receptors required for H. pylori adhesion provides a basis for the development of specific compounds that inhibit the binding of this bacterium to the human gastric mucosa.

Helicobacter pylori colonizes the human gastric mucosa, resulting initially in an acute inflammatory response and damage to epithelial cells progressing to severe gastric diseases ranging from chronic gastritis and peptic ulcers to malignant neoplastic diseases (gastric cancer and mucosa-associated lymphoid tissue lymphoma). Over 50% of the world’s population is infected by H. pylori (1, 2). The infection is acquired during childhood and persists throughout life unless treated with antibiotics.

An essential step in the initiation and establishment of infection is the attachment of a microbe to cell surface receptors on the target tissue. The majority of identified microbial receptors are glycoconjugates (3). For H. pylori, a number of different carbohydrate receptor candidates have been reported (reviewed by Teneberg (4)). Thus, the binding of this bacterium to such diverse compounds as sialic acid-containing glycoconjugates, phosphatidylethanolamine, gangliotetraosylceramide, the Leb blood group determinant and related carbohydrate antigens, heparan sulfate, sulfatide, lactosylceramide, neolactohexaosylceramide, and lactotetraosylceramide has been documented. The nonacid carbohydrate sequences recognized by H. pylori are summarized in Table 1. In contrast to the multitude of candidate H. pylori carbohydrate receptors, there are only three identified carbohydrate-binding adhesins, the blood group antigen–binding BabA² adhesin, the sialic acid–binding SabA adhesin, and the LacdiNAc–binding LabA adhesin (5–7). In addition, HopQ has recently been shown to interact with human carcinoembryonic antigen-related cell adhesion molecules and is thus the first example of a protein-dependent H. pylori adhesin (8, 9). The adherence-associated lipoprotein A and B (AlpA/B) and the outer inflammatory protein A (OipA) are also involved in H. pylori adhesion (10–12).

The Le⁶ (Fucα2Galβ3(Fucα4GlcNAcβ3)-binding BabA was the first identified H. pylori adhesin (5). H. pylori strains expressing BabA together with the vacuolating cytotoxin A and the cytotoxin-associated antigen A (triple-positive strains) are highly associated with severe gastric diseases, such as peptic ulcer and gastric adenocarcinoma (13).

The initial observation that the fucosylated blood group antigens H type 1 (Fucα2Galβ3–GlcNAcβ3) and Le⁶ are recognized by H. pylori BabA was followed by a division of BabA-producing H. pylori strains into specialist and generalist strains, depending on their mode of binding to Le⁶ and related carbohydrate sequences (14). BabA of specialist strains binds only to glycoconjugates with a terminal Fucα2Gal sequence, as the H...
type 1 and Leβ determinants, whereas the generalist BabA tolerates substitution with an αGal or an αGalNAc at the 3-position of the Gal, as in the A or B type 1 (GalNAcα3(Fucα2)Galβ3GlcNCαβ- or (Galα3(Fucα2)Galβ3GlcNCαβ-) and ALeβ or BLeβ (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNCαβ- or Galα3(Fucα2)Galβ3(Fucα4)GlcNCαβ-) determinants. The structural determinants specifying these variant binding modes were recently characterized by X-ray crystallography of the adhesin domain of specialist and generalist BabA, alone and in complex with ABO/Leβ-type oligosaccharides (15).

The structural requirements for carbohydrate recognition by the BabA adhesin of generalist and specialist strains have been redefined (16), demonstrating that BabA recognizes blood group O and A determinants on type 4 core chains in addition to blood group determinants on type 1 core chains.

In contrast to the wealth of information about H. pylori carbohydrate binding, the knowledge about the glycosylation of the human stomach (i.e., the target organ of H. pylori) is limited and mainly consists of immunohistochemistry studies. A few reports with characterization of selected human gastric glycosphingolipids are available (17, 18), but a thorough characterization with the methods available today has not been done. In a recent study, we have characterized the acid glycosphingolipids of human stomach (19) and identified two minor H. pylori SabA-binding gangliosides as Neu5Acα3-neoactoasialo- and neolacto-Neu5Acα3-Galβ4GlcNAcβ3Galβ4GlcNCαβ3Galβ4Glcβ1Cer and Neu5Acα3-neolacto-Neu5Acα3-Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), whereas the other acid human stomach glycosphingolipids characterized (sulfatide and the gangliosides GM3, GD3, GM1, Neu5Acα3-neolactotetraosylceramide, GD1a, and GD1b) were not recognized by the bacteria. The aim of the present study was to characterize the nonacid glycosphingolipids of the human stomach, with particular interest in components with H. pylori-binding activity. Nonacid glycosphingolipids from three stomachs from individuals with blood group O(Rh−)P, A(Rh+)P, and A(Rh+)P were tested for H. pylori binding, and the total nonacid glycosphingolipid fractions and isolated subfractions were characterized with MS and binding of antibodies, lectins, and bacteria.

Results

Binding of H. pylori to total nonacid glycosphingolipids of human stomach

Chemical staining of the human stomach nonacid glycosphingolipids showed that the major compounds in the fractions from the blood group O(Rh−)P and A(Rh+)P individuals migrated as mono- to tetraglycosylceramides (Fig. 1A, lanes 1 and 2), whereas the major glycosphingolipids in the fraction from the blood group A(Rh+)P individual migrated as mono- and diglycosylceramides, and no tri- and tetraglycosylceramides were visualized in this fraction (Fig. 1A, lane 3).

The three nonacid glycosphingolipid fractions from human stomach separated on thin-layer chromatograms were tested for binding of the WT BabA− expressing H. pylori generalist strain J99, the deletion mutant strain lacking BabA (J99/BabA−), and the WT H. pylori strain 26695, which lacks Leβ-binding activity (5). In this assay, the detection limit for binding of the generalist strain J99 to the A type 1/ALeβ heptaosylceramide is ~40 ng, whereas the detection limit for binding to the preferred ligand (i.e. the Leβ hexaosylceramide) is below 40 ng (16).

Probing the stomach glycosphingolipids with the BabA-expressing strain J99 revealed an interaction with glycosphingolipids in the slow-migrating region in the stomach fractions from the blood group A(Rh+)P and A(Rh+)P individuals (Fig. 1B, lanes 2 and 3). The binding was over a rather broad area, suggesting recognition of several compounds. The interaction with these glycosphingolipids was BabA-dependent because binding to these glycosphingolipids was only observed with the J99 strain, and not with the two BabA-negative strains J99/BabA− and 26695 (Fig. 1C and D, lanes 2 and 3). The two non-BabA strains, on the other hand, bound to compounds migrating in the di- and tetracylosylceramide regions in the A(Rh+)P stomach sample (Fig. 1C and D, lane 3) and the 26695 strain also bound weakly to a compound in the penta- to hexaosylceramide region. None of the strains bound to the glycosphingolipids of the blood group O(Rh−)P stomach (Fig. 1B–D, lane 1).

Human gastric H. pylori-binding nonacid glycosphingolipids

Table 1

Nonacid carbohydrate structures recognized by H. pylori

| Carbohydrate sequence | BabA bindinga | Reference |
|-----------------------|--------------|-----------|
| Galβ3GlcNAcβ4Glcβ4Glc | −            | 57        |
| Fuco2Galgβ3(Fucα4)GlcNAcβ3Galβ4Glc | + | 5 |
| Fuco2Galgβ3GlcNAcβ3Galβ4Glc | + | 5 |
| Galβ4Glcβ4Glc | −            | 25        |
| Galβ3GlcNAcβ3Galβ4Glc | −            | 17        |
| GalNAcα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glc | + | 16 |
| Galα3(Fucα2)Galβ3GlcNAcβ3Galβ4Glc | + | 16 |
| Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glc | + | 14 |
| Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glc | + | 14 |
| Galβ4GlcNAcβ3Galβ4Glc | −            | 26        |
| Galβ4Glcα3Galβ4GlcNAcβ3Galβ4Glc | −            | 26 |
| Galβ4Glcα3Galβ4GlcNAcβ3Galβ4Glc | −            | 26 |
| Fuco2Galgβ3GlcNAcβ3Galβ4Glcβ4Glc | + | 16 |
| Fuco2Galgβ3GlcNAcβ3Galβ4Glcβ4Glc | + | 16 |

a Recognized by generalist BabA.

b Binding to lactosylceramide with phytosphingosine and/or hydroxy fatty acids.
Human gastric *H. pylori*-binding nonacid glycosphingolipids

**Figure 1. Binding of *H. pylori* to total nonacid glycosphingolipids of human stomach.** A–D, thin-layer chromatogram detected with anisaldehyde (A) and autoradiograms obtained by binding of *H. pylori* strain J99 (B), *H. pylori* strain J99/BabA– (C), and *H. pylori* strain 26695 (D). 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 assays were performed as described under “Experimental procedures.” Autoradiography was for 12 h. Lane 1, total nonacid glycosphingolipids of human stomach blood group O(Rh–)P; lane 2, total nonacid glycosphingolipids of human stomach blood group A(Rh+)P; lane 3, total nonacid glycosphingolipids of human stomach blood group A(Rh+)P, 40 μg; lane 4, total nonacid glycosphingolipids of human stomach blood group A(Rh+)P, 40 μg; lane 4, reference total nonacid glycosphingolipids of human meconium, 40 μg. The Roman numerals to the left of the chromatogram in A denote the number of carbohydrate unit(s) in the bands.

**Characterization of the total nonacid glycosphingolipids of human blood group O(Rh–)P stomach by LC-ESI/MS**

LC-ESI/MS of oligosaccharides using porous graphitized carbon columns gives resolution of isomeric oligosaccharides, and the carbohydrate sequence can be deduced from series of C-type fragment ions obtained by MS² (20). Furthermore, MS² spectra of oligosaccharides with a Hex or HexNAc substituted at C-4 have diagnostic cross-ring O₂,A-type fragment ions, which allow differentiation of linkage positions.

The base peak chromatogram obtained from LC-ESI/MS of the oligosaccharides obtained by hydrolysis of the nonacid glycosphingolipid fractions from human blood group O(Rh–)P stomach with *Rhodococcus endoglycoceramidase II* is shown in Fig. 2A. Molecular ions corresponding to oligosaccharides ranging from trisaccharides (detected as [M-H⁺]⁻ ions at m/z 503) to heptasaccharides (detected as [M-H⁺]⁻ ions at m/z 1217) were present. All molecular ions were subjected to MS³, and the oligosaccharides thereby identified are given in the chromatogram.

The annotation procedure is exemplified in Fig. 2 (B–D) by the MS³ spectra of the [M-H⁺]⁻ ions at m/z 852 eluting at 15.5, 15.9, and 20.1 min, respectively. A [M-H⁺]⁻ ion at m/z 852 corresponds to an oligosaccharide with one Fuc, one HexNAc, and three Hex. The MS³ spectrum of the ion eluting at 15.5 min (Fig. 2B) had an intense ion at m/z 364. This fragment ion is obtained by double glycosidic cleavage of the 3-linked branch (C₂₁/Z₃⁰) and is characteristic for an internal 4-linked GlcNAc substituted with a Fuc at the 3-position (21). Taken together with the C₂₁ ion at m/z 528 and the C₃₂ ion at m/z 690, a Leα pentasaccharide (Galβ₄(Fucα₃)GlcNAcβ₃Galβ₄Glc) was thus tentatively identified.

A fragment ion at m/z 348 was present in the MS² spectrum of the ion at m/z 852 eluting at 15.9 min (Fig. 2C). This ion is diagnostic for an internal 3-linked GlcNAc substituted with a Fuc at C-4 (21) and is due to a double glycosidic cleavage of the 3-linked branch (C₂₁/Z₃⁰). C-type fragment ions at m/z 528 (C₂₃), and m/z 690 (C₃₂) were present, and taken together, this indicated a Leα pentasaccharide (Galβ₃(Fucα₄)GlcNAcβ₃Galβ₄Glc).

The MS² spectrum of the ion at m/z 852 eluting at 20.1 min was distinctly different (Fig. 2D) and had a series of C-type fragment ions (C₃₂ at m/z 325, C₃₅ at m/z 528, and C₄₃ at m/z 690), identifying a pentasaccharide with a Fuc-Hex-HexNAc-Hex sequence. The δ₂,A₂-H₂O fragment ion at m/z 409 and the δ₂,A₂ fragment ion at m/z 427 are characteristic for 4-substituted HexNAc (i.e. a type 2 carbohydrate chain) (20, 21).

Thus, an H type 2 pentasaccharide (Fucα₂Galβ₄GlcNAcβ₃Galβ₄Glc) was identified.

The oligosaccharides identified by LC-ESI/MS are summarized in Table 2. The majority of the characterized compounds had type 2 (Galβ₄GlcNAc) core chains (i.e. neolactotetraosylceramide, the Leα, H type 2, and x₂ pentaosylceramides, neolactohexaosylceramide, the Leβ hexaosylceramide, and the H type 2 heptaosylceramide) (see Tables 2 and 3 for glycosphingolipid structures). The presence of a Leα pentasaccharide and the absence of saccharides with H type 1 and Leβ determinants suggests that this stomach tissue was derived from a blood group OLe(a+b−) nonsecretor individual (22, 23).

**Separation of the total nonacid glycosphingolipids from human A(Rh+)P and A(Rh+)P stomachs**

LC-ESI/MS of the oligosaccharides from the *H. pylori*-binding nonacid glycosphingolipid fractions from A(Rh+)P and A(Rh+)P stomachs showed that these fractions were complex mixtures (Fig. S1). To enrich the slow-migrating glycosphingolipids, the two stomach fractions (~4 mg each) were separated on latrobeads columns (latron Laboratories). Thereby, four glycosphingolipid-containing fractions were obtained from each fraction (Fig. 3, A and B). These fractions were denoted fractions P-I-IV and P-I-IV, respectively. The amounts obtained for each fraction, and their migration level on thin-layer chromatograms, are summarized in Table 3.

³ The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature (41). It is assumed that Gal, Glc, GlcNAc, GalNAc, NeuAc, and NeuGc are of the D-configuration, that Fuc is of the L-configuration, and that all sugars are 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 trihydroxy. Thus, d18:1 designates sphingosine (1,3-dihydroxy-2-aminoocotadecane) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminoocotadecane).
Characterization of the mono-, di-, and triglycosylceramides

The glycosphingolipids in fractions P-I, p-I, P-II, and p-II were characterized by LC-ESI/MS and by binding of carbohydrate-recognizing ligands. The results are summarized in Table 3.

**LC-ESI/MS of fractions P-I and p-I**—On thin-layer chromatograms, fractions P-I and p-I migrated as monoglycosylceramides (Fig. 3A and B, lane 1), and LC-ESI/MS and subsequent MS² of the native fractions identified monohexosylceramides, with d18:1-16:0 and d18:1-h16:0 as the major species (data not shown).

When analyzed by thin-layer chromatography on borate-impregnated silica gel plates, the major part (>90%) of the compounds in fractions P-I and p-I migrated as galactosylceramides (data not shown).

**LC-ESI/MS of fractions P-II and p-II**—Fraction P-II had compounds migrating as di- and triglycosylceramides (Fig. 3A, lane 2), whereas fraction p-II contained only compounds migrating as diglycosylceramides (Fig. 3B, lane 2). Dihexosylceramide with d18:1-16:0 was the major glycosphingolipid identified by LC-ESI/MS of both fractions, and there were also dihexosylceramide with d18:1-24:0 and dihexosylceramide with d18:1-
h16:0. A trihexosylceramide with d18:1-16:0 was also characterized in fraction P-II (data not shown).

Binding of H. pylori and carbohydrate-recognizing ligands to the mono-, di-, and triglycosylceramides from human stomach—To further characterize the mono-, di-, and triglycosylceramides from human stomach, the binding of Solanum tuberosum lectin and P-fimbriated Escherichia coli to these fractions was examined.

The N-acetylactosamine-binding lectin from S. tuberosum also binds to lactosylceramide (Galβ4Glcβ1 Cer) with sphingosine and nonhydroxy fatty acids (24). Here, a binding to fraction P-II and P-II was obtained (Fig. 4B, lanes 3 and 5), confirming the presence of this variant of lactosylceramide in both fractions.

We have previously reported that the major dihexosylceramide of the human stomach is galabiosylceramide (Galα4Galβ1 Cer) (18). In line with this, the Galα4Gal recognizing P-fimbriated Escherichia coli readily bound in the dihexosylceramide region in the glycosphingolipid fractions from the blood group P individual (Fig. 4C, lane 3), along with binding in the tri- and tetraosylceramide regions, indicating globotriaosylceramide (Galα4Galβ4Glcβ1 Cer) and globotetraosylceramide (Galaβ3 Galaα4Galβ4Glcβ1 Cer).

There was no binding of the P-fimbriated E. coli to the diglycosylceramide fraction from the p individual (Fig. 4C, lane 5). However, binding of both the J99 and the 26695 H. pylori strain in the dihexosylceramide region of the p sample was obtained (Fig. 4 D and E, lane 5). A preferential binding of H. pylori to lactosylceramide having sphingosine/phytosphingosine and 2-α-hydroxy fatty acids has been reported (25). According to mass spectrometry, fraction p-II contained a dihexosylceramide with d18:1-16:0 ceramide, so most likely the H. pylori-binding compound in this fraction is lactosylceramide.

Characterization of the slow-migrating glycosphingolipids from human stomach

The glycosphingolipids in fractions P-III, p-III, P-IV, and p-IVa–f were characterized by LC-ESI/MS and by binding of carbohydrate-recognizing ligands. The results are summarized in Table 3.

LC-ESI/MS of fractions P-III and p-III—TLC showed the presence of glycosphingolipids migrating in the tri- and tetraglycosylceramide region in fraction P-III (Fig. 3A, lane 3), whereas fraction p-III had a main compound that migrated in the tetraglycosylceramide region (Fig. 3B, lane 3).

The base peak chromatograms from LC-ESI/MS of the oligosaccharides obtained from fractions P-III and p-III after Rhodococcus endoglycoceramidase II digestion are shown in Fig. 5 (A and B). There are molecular ions corresponding to oligosaccharides ranging from trisaccharides (detected as [M-H]− ions at m/z 503) to hexasaccharides (detected as [M-H]− ions at m/z 1055). All molecular ions were subjected to MS2. Thereby, a lactotrisaccharide (m/z 544) and a Leα pen-tasaccharide (m/z 852) were identified in both fractions (not shown). A globotetraosylceramide (m/z 503) was characterized in the fraction P-III, whereas in fraction p-III, a blood group A type 2 hexasaccharide (m/z 1055) and a blood group H type 2 pentasaccharide (m/z 852) were present.

To characterize the tetrasaccharide composition of the two fractions, we searched for molecular ions at m/z 706, corresponding to a tetrasaccharide with one HexNAc and three Hex. In fraction P-III, three sets of peaks at m/z 706 were present, eluting at 11.6, 17.2, and 17.4 min, respectively, whereas fraction P-III had two peaks eluting at 17.2 and 17.4 min (Fig. 5, C and D). The MS2 spectra of the ion at m/z 706 at retention time 11.6 min in fraction P-III (Fig. 5E) had a C-type fragment ion series (C1 at m/z 220, C2 at m/z 382, and C3 at m/z 544), demonstrating a hexaHexNAc-Hex-Hex-Hex sequence. The 0.2A3 and 0.2A4-H2O fragment ions at m/z 484 and 466 demonstrated a 4-substituted Hex (20, 21). Thus, a globotetrasaccharide (Galaβ3Galα4Galβ4Glcβ1 Cer) was identified.

MS2 of the ion at m/z 706 at the retention time 17.2 min allowed a preliminary identification of a lactotetrasaccharide (Galβ3GalNAcβ3Galβ4Glc) in both fractions (exemplified in Fig. 5F). This was concluded from the C-type fragment ions (C2 at m/z 382 and C3 at m/z 544) identifying a Hex-HexNAc-Hex-Hex sequence. This MS2 spectrum had a prominent D1–2 ion at m/z 202, which is obtained by a C2–Z2 double cleavage and diagnostic for a 3-substituted HexNAc (21). The MS2 spectrum also lacked a 0.2A3 fragment ion at m/z 281, further demonstrating that the HexNAc was substituted at the 3-position.

In contrast, the MS2 spectrum of the ion at m/z 706 at the retention time 17.5 min had a prominent 0.2A2 fragment ion at m/z 281, further demonstrating that the HexNAc was substituted at the 3-position.

Table 2

| Abbreviation | Structure | O(Rh+)+P | A(Rh+)+P | A(Rh+)+P | A(Rh+)+P case 3 | A(Rh+)+P case 4 |
|--------------|-----------|----------|----------|----------|-----------------|-----------------|
| Galβ3        | Galα4Galβ1 Glc | +         | +         | +         | −               | −               |
| Galβ4        | Galα4Galβ4Glc | +         | +         | +         | −               | −               |
| Leα–β5     | Fuco2Galβ3 (Fuco4) GlcNAcβ3 Galβ4 Glc | −         | +         | +         | +               | −               |
| Leα–β5     | Galβ3 (Fuco3) GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |
| Leα–β6     | Fuco2Galβ3 (Fuco3) GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |
| H5–1        | Fuco2Galβ3 GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |
| Le4        | Galβ3 GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |
| nLe4        | Galβ4 GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |
| H5–2        | Fuco2 Galβ4 GlcNAcβ3 Galβ4 Glc | +         | +         | +         | −               | −               |
| x2        | Galα4 Galβ3 Galβ4 GlcNAcβ3 Galβ4 Glc | +         | +         | +         | −               | −               |
| nLeC4        | Galβ4 GlcNAcβ3 Galβ4 Glc | +         | +         | +         | −               | −               |
| H7–2        | Fuco2 Galβ4 GlcNAcβ3 Galβ4 Glc | +         | +         | +         | −               | −               |
| A6–2        | Galα4 Galβ3 (Fuco2) Galβ4 GlcNAcβ3 Galβ4 Glc | −         | +         | +         | −               | −               |

a Fractions from human gastric mucosa.

b The compounds in boldface type have been characterized as H. pylori binding in previous studies (summarized in Table 1).
A neolactotetrasaccharide (Galβ1Cer) at m/z 281 and a 0.2A₂-H₂O fragment ion at m/z 263, demonstrating a terminal Hex-HexNAc sequence with a 4-substituted HexNAc (i.e. a type 2 chain) (20, 21). In combination with the C₂ ion at m/z 382 and the C₃ ion at m/z 544, this demonstrated a neolactotetrasaccharide (Galβ4GlcNAcβ3Galβ4Glc in both fractions (exemplified in Fig. 5G).

The three MS³ spectra all had 0.2A₂ ions at m/z 646 and 0.2A₃-H₂O ions at m/z 628, which were derived from cross-ring cleavage of the 4-substituted Glc of the lactose unit at the reducing end. Thus, the globotetrasaccharide was present only in fraction P-III, whereas the lacto- and neolactotetrasaccharides were identified in both fractions P-III and P-IV.

**LC-ESI/MS of fractions P-IV and P-IV**—Fraction P-IV and P-IV both contained a number of glycosphingolipids, migrating at the level of tetraglycosylceramide and below (Fig. 3 (A and B), lane 4). LC-ESI/MS of the oligosaccharides obtained from endoglycoceramidase hydrolysis of these fractions showed that both were complex mixtures (exemplified in Fig. 6A). All molecular ions were subjected to MS², and the oligosaccharides thereby identified are given in the chromatogram (Fig. 6) and summarized in Table 3.

By MS², four oligosaccharides with terminal blood group A determinants were identified in fraction P-IV. First, MS² of the molecular ion at m/z 1055 at retention time 13.1 min (Fig. 6B) gave

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**Table 3**

Summary of glycosphingolipids characterized in the subfractions isolated from human stomachs

| Fraction | Amount | Migration | Glycosphingolipid                         | Abbreviation |
|----------|--------|-----------|------------------------------------------|--------------|
| P-I      | 0.4mg  | Mono      | Galβ1Cer                                 | GalCer       |
|          |        |           | Glc1Cer                                  | GlcCer       |
| P-II     | 1.2    | Di + tri  | Galβ4Glcβ1Cer*                           | LacCer       |
|          |        |           | Galα4Galβ1Cer                            | GalβapCer    |
| P-III    | 1.3    | Tri + tetra | Galα4Galβ4Glcβ1Cer                       | Le4          |
|          |        |           | GlcNAcβ3Galβ4Glcβ1Cer                    | Le-5         |
|          |        |           | Galα3GlcNAcβ3Galβ4Glcβ1Cer               | Le-5         |
| P-IV     | 0.7    | ≥Tetra    | Galα3GlcNAcβ3Galβ4Glcβ1Cer               | Le-6         |
|          |        |           | Galα3GlcNAcβ3Galβ4Glcβ1Cer               | Le-6         |
| p-I      | 1.3    | Mono      | Galβ1Cer                                 | GalCer       |
|          |        |           | Glc1Cer                                  | GlcCer       |
| p-II     | 0.7    | Di        | Galβ4Glcβ1Cer                            | LacCer       |
| p-III    | 0.3    | Tetra     | GlcNAcβ3Galβ4Glcβ1Cer                    | Le3          |
|          |        |           | Galβ4GlcNAcβ3Galβ4Glcβ1Cer               | Le4          |
|          |        |           | Galβ4GlcNAcβ3Galβ4Glcβ1Cer               | Le-5         |
| p-IVᵇ    | 1.2    | ≥Tetra    | Galβ4GlcNAcβ3Galβ4Glcβ1Cer               | Le-6         |
|          |        |           | Galβ4GlcNAcβ3Galβ4Glcβ1Cer               | Le-6         |
|          |        |           | Galβ4GlcNAcβ3Galβ4Glcβ1Cer               | Le-6         |

* The compounds in boldface type have been characterized as H. pylori binding in previous studies (summarized in Table 1).

* The glycosphingolipids listed under fraction p-IV were characterized by LC-ESI/MS and MS² of fractions P-IVa–f.

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**Figure 5**

A neolactotetrasaccharide (Galβ4GlcNAcβ3Galβ4Glc) in both fractions (exemplified in Fig. 5G).

**Figure 6**

By MS², four oligosaccharides with terminal blood group A determinants were identified in fraction P-IV. First, MS² of the molecular ion at m/z 1055 at retention time 13.1 min (Fig. 6B) gave
a series of C-type fragment ions (C$_2$ at m/z 528, C$_3$ at m/z 731, and C$_4$ at m/z 893) demonstrating a HexNAc-(Fuc-)Hex-HexNAc-Hex-Hex sequence. A type 2 core chain was identified by the 0,2A4 ion and 0,2A4-H$_2$O ion at m/z 630 and 612. Taken together, these spectral features identified a blood group A type 2 hexasaccharide (GalNAc$_3$(Fuc$_2$)Gal$_4$(Fuc$_3$)GlcNAc$\beta$3Gal$\beta$4Glc).

Second, the MS$^2$ spectrum of the molecular ion at m/z 1201 at retention time 11.6 min (Fig. 6C) was very similar to the MS$^2$ spectrum obtained from the reference blood group A type 2 heptasaccharide (Fig. 6F). Both spectra had a series of C-type fragment ions (C$_2$ at m/z 528, C$_3$ at m/z 877, and C$_4$ at m/z 1039) identifying a HexNAc-(Fuc-)Hex-(Fuc-)HexNAc-Hex sequence. Both spectra also had an ion at m/z 713, which, in analogy with the fragment ion at m/z 364 in the MS$^2$ spectrum of the Le$^b$ pentasaccharide, is obtained by double glycosidic cleavage of the 3-linked branch (C$_3$/Z$_{2pg}$) and thus characteristic for an internal 4-linked GlcNAc substituted with a Fuc at the 3-position (21). Taken together, this identified a blood group A type 2/ALeb heptasaccharide (GalNAc$_3$(Fuc$_2$)Gal$_3$(Fuc$_4$)GlcNAc$\beta$3Gal$\beta$4Glc).

Third, a blood group A type 1/ALeb heptasaccharide (GalNAc$_\alpha$(Fuc$_2$)Gal$_3$(Fuc$_4$)GlcNAc$\beta$3Gal$\beta$4Glc) was identified MS$^2$ of the molecular ion at m/z 1201 at retention time 13.8 min (Fig. 6D). This spectrum had a high similarity with the MS$^2$ spectrum obtained from the reference blood group A type 1 heptasaccharide (Fig. 6G). Again a C-type fragment ion series (C$_2$ at m/z 528, C$_3$ at m/z 877, and C$_4$ at m/z 1039) identifying a HexNAc-(Fuc-)Hex-(Fuc-)HexNAc-Hex-Hex sequence was present in both spectra. In addition, both spectra had an ion at m/z 656, which, in analogy with the fragment ion at m/z 348 obtained by MS$^2$ of the Le$^a$ pentasaccharide, is diagnostic for an internal 3-linked GlcNAc substi-
tuted with a Fuc at C-4 and is due to a double glycosidic cleavage of the 3-linked branch at C₃ and Z₄ (21).

Finally, the MS² spectrum of the molecular ion at m/z 1420 (Fig. 6E) had B-type and C-type fragment ion series (C₂ at m/z 528, B₃ at m/z 713, C₃ at m/z 731, B₄ at m/z 893, B₅ at m/z 1078, C₅ at m/z 1096, and C₆ at m/z 1258) demonstrating a HexNAc-(Fuc-)Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence. The α₂A₅-H₂O ion at m/z 612, along with the α₂A₅ ion at m/z 995, showed that both HexNAcs were substituted at C-4. Thereby, a blood group A type 2 octasaccharide (GalNAc₃(Fuc₃)Gal₃GlcNAc₃Gal₃Glc (Le¹⁻²), GlcNAc₃Galβ4Glc (Le²⁻³), GalNAc₃(Fuc₂)Galβ4GlcNAcβ3Galβ4Glc (A₆⁻²), and Fucα2Galβ4GlcNAcβ3Galβ4Glc (H₅⁻²)).

The base peak chromatogram from LC-ESI/MS of the oligosaccharides from fraction p-IV was relatively weak. The largest oligosaccharide identified by MS² was the A type 2 hexasaccharide, but information about the more complex oligosaccharides was not obtained. Therefore, after the binding assays described below, fraction p-IV was further separated on an Iatrobeads column in a final attempt to identify the complex glycosphingolipids. After pooling, six glycosphingolipid-containing fractions were obtained (denoted fractions p-IVa–f) (Fig. 3C). Each fraction was ~0.1 mg.

Fractions p-IVa–e were all complex oligosaccharide mixtures containing neolactotetra-, Le⁺ penta-, Le⁰ penta-, and Le⁰ hexasaccharides, as found by LC-ESI/MS and MS² (data not shown). The Le⁰ hexasaccharide was present in fractions p-IV: c–e, and the x₂ pentasaccharide and the A type 2 hexasaccharide were present in fractions p-IV: c and d. In addition, the H type 2, the A type 1, and the A type 2 heptasaccharides were present in fractions p-IV: d and e.

LC-ESI/MS of fraction p-IVf—LC-ESI/MS of the oligosaccharides obtained from fraction p-IVf gave a relatively weak base peak chromatogram (Fig. 7A). Again, MS² of the major molecular ions identified the Le⁰ and Le⁰ hexasaccharides; the Le⁰, Le⁰, and H type 2 pentasaccharides; and the neolactotetra- and neolactohexasaccharides. The base peak chromatogram had also three minor molecular ions at m/z 1217 at retention times 15.4, 18.8, and 25.9 min, respectively, and a minor molecular ion at m/z 1509 eluting at 14.9 min.
The MS² spectrum of the molecular ion at \( m/z \) 1217 eluting at 15.4 min (Fig. 7B) had a series of C-type fragment ions (\( C_{2a}, C_{3a} \) at \( m/z \) 544, \( C_4 \) at \( m/z \) 539, and \( C_5 \) at \( m/z \) 1055), which identified an oligosaccharide with Hex-HexNAc-Hex-(Fuc-)HexNAc-Hex-Hex sequence. The ion at \( m/z \) 729 was due to a double glycosidic cleavage of the 3-linked branch at \( C_4 \) and \( Z_{3a} \) (20). This fragmentation is characteristic for an internal 4-linked GlcNAc substituted with a Fuc at the 3-position and thus demonstrated an internal Le⁺ determinant. Taken together, this indicated an oligosaccharide contaminant.
The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS² sequencing. The oligosaccharides identified in the chromatograms were Fuc\(_2\)Gal\(_3\)(Fuc\(_3\))GlcNAc\(_3\)Gal\(_4\) (Leb-6), Gal\(_3\)(Fuc\(_3\))GlcNAc\(_3\)Gal\(_4\) (Lex-5), Gal\(_3\)(Fuc\(_4\))GlcNAc\(_3\)Gal\(_4\) (Leb-9), Gal\(_3\)(Fuc\(_4\))GlcNAc\(_3\)Gal\(_4\) (H5-2), Gal\(_3\)(Fuc\(_5\))GlcNAc\(_3\)Gal\(_4\) (H5-6), Fuc\(_2\)Gal\(_3\)(Fuc\(_3\))GlcNAc\(_3\)Gal\(_4\) (Leb-5), Fuc\(_2\)Gal\(_3\) (Fuc\(_5\))GlcNAc\(_3\)Gal\(_4\) (Lex-7), and Fuc\(_2\)Gal\(_3\)(Fuc\(_5\))GlcNAc\(_3\)Gal\(_4\) (H5-2).

The MS² spectrum obtained of the molecular ion at m/z 1217 eluting at 25.9 min (Fig. 7D) had a series of C-type fragment ions (C₃ at m/z 528, C₄ at m/z 690, C₅ at m/z 893, and C₆ at m/z 1055), which, together with the \(^{18}\)O₂-H₂O fragment ion at m/z 409, the \(^{18}\)O₂-H₂O fragment ion at m/z 774, and the \(^{18}\)O₂-H₂O fragment ion at m/z 792, identified an oligosaccharide with Fuc-Hex-HexNAc-Hex-HexNAc-Hex sequence (i.e. a blood group H type 2 heptasaccharide (Fuc\(_3\)Gal\(_4\)(Fuc\(_3\))GlcNAc\(_3\)Gal\(_4\)Glc\(_4\)).

A molecular ion at m/z 1509 corresponds to an oligosaccharide with three Fuc, two HexNAc, and four Hex. Here the MS² spec-

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**Figure 7.** LC-ESI/MS of the oligosaccharides obtained by digestion of the slow-migrating nonacid glycosphingolipid fraction p-IV-f from human blood group A (Rh\(^-\)) stomach with *Rhodococcus* endoglycoceramidase II. A, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction p-IV-f. B, MS² of the molecular ion at m/z 1217 (retention time 15.4 min). C, MS² of the ion at m/z 1217 (retention time 18.8 min). D, MS² of the ion at m/z 1217 (retention time 25.9 min). E, MS² of the ion at m/z 1509 (retention time 14.9 min). F, interpretation formulas showing the deduced oligosaccharide sequences.
trum obtained (Fig. 7E) had two C/Z ions at m/z 510 and m/z 1021, demonstrating that there were two internal 4-linked GlcNAcs substituted with a Fuc at the 3-position. There was also a series of C-type fragment ions at m/z 674 (C_{30}), m/z 536 (C_{29}), m/z 1185 (C_{18}), and m/z 1347 (C_{17}). Collectively, these spectral features allowed a tentative identification of a Le^{y} nonasaccharide (Fuca2 Gal{3}GlcNAc{3}Gal{4}Glc (Le^{y}-9), GalNAca{3}Gal{4}GlcNAc{3}Gal{4}Glc (Le^{y}-8), Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (Le^{y}-7), Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (H9-2), and Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (nLc8)). * nonoligosaccharide contaminants.

In a final attempt to characterize the complex glycosphingolipids in fraction p-IV-f, this fraction was analyzed at m/z 1300–2000. Thereby, a number of molecular ions were detected at m/z 1363 (retention time 14.9–15.7 min and 19.8 min), m/z 1420, m/z 1436, m/z 1509, and m/z 1582 (Fig. 8A). MS² of the ion at m/z 1509 identified a Le^{y} nonasaccharide (Fuca2 Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (Fig. 8E)), and MS² of the ion at m/z 1582 identified a Le^{y} nonasaccharide (Fuca2 Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc) (Fig. 8E), as above. A neolactooctasaccharide (Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc) (Fig. 8D), as above.

Figure 8. LC-ESI/MS (m/z 1300–2000) of the complex oligosaccharides obtained by digestion of the slow-migrating nonacid glycosphingolipid fraction p-IV-f from human blood group A(Rh+/+p stomach with Rhodococcus endoglycoceramidase II. A, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction p-IV-f. B, MS² of the ion at m/z 1363 (retention time 14.9 min). C, MS² of the ion at m/z 1363 (retention time 19.8 min). D, MS² of the ion at m/z 1509 (retention time 19.8 min). E, MS² of the ion at m/z 1420 (retention time 19.4 min). F, interpretation formulas showing the deduced oligosaccharide sequences. The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS² sequencing. The oligosaccharides identified in the chromatograms were Fuca2Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (Fig. 8E), Fuca2Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (Fig. 8D), and Gal{3}GlcNAc{3}Gal{4}GlcNAc{3}Gal{4}Glc (nLc8). * nonoligosaccharide contaminants.
Galβ4GlcNAcβ3Galβ4Glc was characterized by MS² of the ion at m/z 1436, and a blood group H nonasaccharide (Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc) was characterized by MS² of the ion at m/z 1582 (data not shown).

A molecular ion at m/z 1363 corresponds to an oligosaccharide with two Fuc, two HexNAc, and four Hex. Here, there were two ions at m/z 1363, eluting at retention times 14.9–15.7 min and 19.8 min, respectively. The MS² spectrum of the ion at retention time 14.9 min (Fig. 8B) had a series of C-type fragment ions (Cᵣ at m/z 528, Cₛ at m/z 690, Cₒ at m/z 1039, and Cₒ at m/z 1201) identifying an octasaccharide with Fuc-Hex-HexNAc-Hex-(Fuc-)HexNAc-Hex-Hex sequence. The ion at m/z 875 was obtained by a double glycosic cleavage of the 3-linked branch at Cₒ and Z₂b (1) (i.e., a fragmentation characteristic for an internal 4-linked GlcNAc substituted with a Fuc at 3-position) and thus again demonstrated an internal Leα determinant. Taken together, these spectral features thus indicated an octasaccharide with Fucα2Galβ3/4GlcNAcβ3 Galβ4(Fucα3)GlcNAcβ3Galβ4Glc sequence.

MS² of the ion at m/z 1363 eluting at 19.8 min (Fig. 8C) gave a Cₒ/Z₂b ion at m/z 510 demonstrating an internal 4-linked GlcNAc substituted with a Fuc at the 3-position, C-type fragment ions at m/z 836 (Cᵣ), and m/z 1039 (Cₛ), and a 2,4Aᵣ ion at m/z 877 along with a 0,2Aᵣ ion at m/z 937. Taken together, this demonstrated a Leα octasaccharide (Fucα2Galβ4(Fucα3)-GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

Thus, several different complex Leα and Leβ glycosphingolipids were characterized in fraction P-IV. Leα and Leβ do not, however, belong to the reported spectrum of H. pylori-binding glycans. The only potential H. pylori-binding ligands present in this fraction were neolactotetra- and neolactohexaoysylceramide, the Leβ hexaolsylceramide, and the A type 1 heptaosylceramide.

Binding of H. pylori and carbohydrate recognizing ligands to the slow-migrating glycosphingolipids from human stomach—To substantiate the data from MS, the binding of a number of antibodies, lectins, BabA adhesin, and bacteria to the slow-migrating glycosphingolipid subfractions from human stomach homogenates was examined (Fig. 9).

The H. pylori strain 26695 bound in the tetraosylceramide region in fractions P-III and P-IV (Fig. 9B, lanes 1 and 2). The structural characterization of fractions P-III and P-IV demonstrated the presence of lactotetraosylceramide and neolactotetraosylceramide in these fraction, both of which have previously been shown to be recognized by H. pylori (17, 26).

H. pylori generalist strain J99, on the other hand, bound to slow-migrating compounds in fractions P-IV and P-IV (Fig. 9C, lanes 2 and 4). The same binding pattern was obtained with the recombinant truncated adhesin domain of BabA (BabA547) (Fig. 9D, lanes 2 and 4). Ligands for the BabA-mediated binding of the J99 strain to these slow-migrating glycosphingolipids were the H type 1 pentaoysylceramide, the Leβ hexaolsylceramide, and the A type 1/ALeβ heptaosylceramide identified in fraction P-IV and the Leβ hexaolsylceramide and A type 1/ALeβ heptaosylceramide in fraction P-IV.

The Erythrina cristagalli lectin binds to glycoconjugates with terminal Galβ4GlcNAc and Fucα2Galβ4GlcNAc (27). Probing of the slow-migrating glycosphingolipid fractions with this lectin gave a binding double band in the tetraosylceramide region in fractions P-IV, p-III, and p-IV (Fig. 9E, lanes 2–4), confirming the presence of a Galβ4GlcNAc-terminated tetraosyl-
ceramide (i.e. neolactotetraosylceramide (Galβ4GlcNAcβ3 Galβ4Glcβ1Cer)). A more slow-migrating compound in fraction p-IV was also recognized (Fig. 9E, lane 4), most likely the blood group H type 2 heptaoxasaccharide (Fucα2Galβ4 GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). The binding of Galex4Gal-binding P-fimbriated E. coli in the tri- and tetraosylceramide region of the nonacid fractions of the stomach of the A(Rh + )P individual (Fig. 9F, lanes 1 and 2) supported the presence of globotriaosylceramide (Galα4Galβ4Glcβ1Cer) and globotetraosylceramide (Galα4Galβ4Glcα1Galβ4Glcβ1Cer) in these fractions, whereas the binding of anti-Le\(^b\) antibodies in the pentaosylceramide region in fractions P-IV and p-IV (Fig. 9G, lanes 2 and 4) was in line with the presence of Le\(^b\) pentaosylceramide (Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). Compounds in the hexaosylceramide region in fraction P-IV and p-IV were recognized by the anti-Le\(^b\) and anti-Le\(^a\) antibodies (Fig. 9 (H and I), lanes 2 and 4), supporting the presence of Le\(^b\) (Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) and Le\(^b\) (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer) hexaosylceramides. The anti-Le\(^a\) and anti-Le\(^b\) antibodies also bound to slow-migrating glycosphingolipids in fractions P-IV and p-IV, indicating complex Le\(^a\) and Le\(^b\) glycosphingolipids. Finally, the anti-A antibodies bound in the hexa-, hepta-, and octaosylceramide regions in the slow-migrating fractions from the blood group A(Rh + )P and A(Rh + )p stomachs (Fig. 9J, lanes 2 and 4).

**LC-ESI/MS of native glycosphingolipids from human stomach**

To characterize the ceramide composition, the native total nonacid glycosphingolipid fractions from human stomach were also analyzed by LC-ESI/MS. The base peak chromatograms from LC-ESI/MS of the blood group O(Rh−)P stomach and the blood group A(Rh + )P stomach (Fig. S2, A and B) were very similar, and both had [M-H\(^−\)]\(^−\) ions at \(m/z\) 1022, \(m/z\) 1225, \(m/z\) 1387, and \(m/z\) 1533, and the blood group A(Rh + )P sample also had a [M-2H\(^−\)]\(^−\) ion at \(m/z\) 868. MS\(^2\) of these ions identified globotriaosylceramide with \(m/z\) 18:1-16:0 (only \(m/z\) 1022); globotetraosylceramide and neolactotetra/lactotetraosylceramide with \(m/z\) 18:1-16:0 (only \(m/z\) 1225); H, Le\(^a\), or Le\(^b\) pentaosylceramide with \(m/z\) 18:1-16:0 (only \(m/z\) 1387); and Le\(^b\) hexaosylceramide with \(m/z\) 18:1-16:0 (only \(m/z\) 1533), as exemplified in Fig. S2 (C–E). Dihexosylceramide with \(m/z\) 18:1-16:0 was demonstrated by MS\(^2\) of the ([M-H\(^−\)]\(^−\) ion at \(m/z\) 988 in the sample from the O(Rh−)P stomach, whereas MS\(^2\) of the ([M-H\(^−\)]\(^−\) ion at \(m/z\) 860 in the sample from the A(Rh + )P stomach identified a dihexosylceramide with \(m/z\) 18:1-16:0. Finally, a blood group A heptaoxasaccharide with \(m/z\) 18:1-16:0 was characterized by MS\(^2\) of the [M-2H\(^−\)]\(^−\) ion at \(m/z\) 868 in the nonacid fraction from the A(Rh + )P stomach (Fig. S2F).

The base peak chromatograms from LC-ESI/MS of the blood group A(Rh + )P stomach (data not shown) was dominated by [M-H\(^−\)]\(^−\) ions at \(m/z\) 860 and \(m/z\) 970, which upon MS\(^2\) identified dihexosylceramide with \(m/z\) 18:1-16:0 and \(m/z\) 18:1-24:1, respectively. No ions corresponding to globotria- or globotetraosylceramide were present. There was a minor ion at \(m/z\) 1225, and here MS\(^2\) gave a Hex-HexNAc-Hex-Hex carbohydrate sequence, as in neolactotetra- or lactotetraosylceramide. Minor ions at \(m/z\) 1387 and \(m/z\) 1533 identified H, Le\(^a\), or Le\(^b\) pentaosylceramide and Le\(^b\) or Le\(^a\) hexaosylceramide, as above, in both cases with \(m/z\) 18:1-16:0.

Thus, the di-, tri-, and tetraosylceramides of human stomach had mainly sphingosine with nonhydroxy 16:0 fatty acid, whereas the predominant ceramide species among the pentaosylceramides and larger glycosphingolipids was sphingosine with hydroxy 16:0 fatty acid.

**LC-ESI/MS of nonacid glycosphingolipids from human gastric mucosa**

Because the glycosphingolipids from human stomach were isolated from whole stomach tissue and potentially could be derived from the submucosal tissue, we also analyzed the nonacid glycosphingolipids from human gastric mucosal scrapings from two blood group A(Rh + )P individuals. LC-ESI/MS of the oligosaccharides derived from the nonacid glycosphingolipids of human gastric mucosa showed also in these cases a predominance of compounds based on type 2 core chains (Fig. 10). The base peak chromatograms of the two samples (Fig. 10, A and B) were very similar to the base peak chromatogram of the sample from A(Rh + )P stomach (Fig. S1A) but lacked ions corresponding to globotri-, globotetra-, Le\(^a\), and H type 1 oligosaccharides. The oligosaccharides identified in the nonacid glycosphingolipid fractions from human gastric mucosa are summarized in Table 2. Thus, the Le\(^a\), Le\(^b\), and blood group A type 2 hexaosyl- and tetraosylceramides were the largest oligosaccharides characterized by MS\(^2\) in both samples. However, the molecular ion profiles (Fig. 10, C and D) indicated the presence of a blood group A heptasaccharide (ALe\(^b\) and/or ALe\(^a\)) (\(m/z\) 1201) and an H type 2 heptasaccharide (\(m/z\) 1217) in both samples. The molecular ion profile from case 3 (Fig. 10C) also had an ion at \(m/z\) 1420, indicating the presence of a blood group A octasaccharide, and the ion at \(m/z\) 1071 in the molecular ion profile from case 4 (Fig. 10D) indicated a neolactohexasaccharide.

**Discussion**

A high proportion of the world's population is infected by *H. pylori*, mainly in developing countries, where the infection occurs in up to 80% of the middle-aged adults (1, 2). The majority of infected individuals develop an acute gastritis, which evolves into a chronic active gastritis. Most cases remain asymptomatic, and only a subset of infected individuals develop severe gastric diseases, as peptic ulcers or gastric cancer. The virulence factors expressed by the infecting *H. pylori* strain is one known determinant factor of the outcome of the infection.

Differences in the host genetic background, such as variability in host immune responses (see Mărginean et al. (28) and references therein) and differential expression of receptors in the gastric mucosa, are also involved in the final outcome of the infection. In this study, the nonacid glycosphingolipids of three human stomachs were characterized, demonstrating differences in the composition of glycosphingolipids, and also differences in *H. pylori*-binding compounds, between individuals.

No binding of *H. pylori* to the blood group O(Rh−)P stomach glycosphingolipids was obtained. LC-ESI/MS of the oligosaccharides obtained from the glycosphingolipids of this stomach demonstrated the presence of a Le\(^a\) pentasaccharide, whereas oligosaccharides with H type 1 and Le\(^b\) determinants were absent. This
suggests that this stomach was derived from a blood group OLe(a+b−) nonsecretor individual (22, 23). Due to low amounts, this fraction was not further characterized.

Glycosphingolipids of the A(Rh+)P and A(Rh+)p stomachs were recognized by both the BabA-expressing H. pylori generation strain J99 and the two strains without BabA (the J99/BabA− mutant strain and the 26695 strain), but with different binding patterns. The J99 strain and the recombinant truncated adhesin domain of BabA bound to compounds migrating as penta- or hexaosylceramides. Binding to these glycosphingolipids was not obtained with the BabA-negative strains, further demonstrating that this interaction was BabA-dependent. The candidate ligands for BabA-mediated binding identified by LC-ESI/MS and antibody binding were the Leb hexaosylceramide and the A type 1/ALe hexaosylceramide in both stomach samples and also the H type 1 pentaosylceramide in the A(Rh+)P stomach.

The BabA-negative strains J99/BabA− and 26695, on the other hand, both bound to compounds migrating as di- and tetraosylceramides in the A(Rh+)P stomach sample, and the 26695 strain also bound in the penta- to hexaosylceramide region. The binding of H. pylori in the dihexosylceramide region of the A(Rh+)p sample is most likely due to recognition of lactosylceramide (25). The tetraosylceramides of the A(Rh+)P stomach were characterized as lactotetraosylceramide and neolactotetraosylceramide, both of which are recognized by H. pylori (17, 26). The penta- or hexaosylceramide recognized by the BabA-negative H. pylori 26695 strain is likely to be the x3 pentaosylceramide or neolactohexaosylceramide (26), which both were characterized in the A(Rh+)P stomach fraction.

The most obvious difference between the A(Rh+)P and A(Rh+)p stomachs was the absence of globoseries glycosphingolipids, such as globotriaosylceramide and globotetraosylcer-
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amide, in the A(Rh+)p stomach sample. This is due to lack of the \(\alpha\)-galactosyltransferase converting lactosylceramide to globotriaosylceramide (29). In a previous work, we found that the major dihexosylceramide in the human stomach is galabioasylceramide (Galα4Galβ1Cer) (18). Here, there was no binding of Ga4Gal recognizing P-fimbriated \textit{E. coli} in the dihexosylceramide region of the stomach sample from the blood group \(P\) individual, demonstrating that also galabiosyl ceramide is absent in this case.

An unexpected finding was the predominance of nonacid glycosphingolipids with type 2 core chains in the human stomach. This is in contrast to the glycosphingolipids of human small intestine, which mainly have type 1 core (30, 31). Interestingly, several immunochemistry studies have demonstrated a differential distribution of ABH and Le blood group determinants with type 1 and type 2 core chains in the normal human gastric mucosa (32–36). Determinants with type 1 core (e.g., Le\(^b\) and ALeb) are mainly found in the foveolar epithelium, whereas ABH and Le blood group determinants are found on type 2 core (e.g., Le\(^a\) and ALeb) found both in the foveolar epithelium and in deep gastric glands.

Binding of \textit{H. pylori} to neolactoglycosphingolipids has been characterized, and here the optimal binding sequence is GlcNACβ3Galβ4GlcNACα, whereas binding to neolactotetraosylceramide (Galβ4GlcNACβ3Galβ4Glcβ1Cer), which lacks the terminal β3-linked GlcNAC, is relatively weak (26). This is in agreement with the nonbinding of \textit{H. pylori} to several fractions containing neolacto compounds (neolactotetraosylceramide, neolactohexaosylceramide, and the \(x_2\) pentaosylceramide) characterized in this study (e.g., the nonacid glycosphingolipid fraction of the blood group O(Rh-)P stomach).

Several of the potential \textit{H. pylori} receptors (lactotetraosylceramide, the \(x_2\) pentaosylceramide, and the Le\(^b\) hexaosylceramide) were also characterized in the human gastric mucosal scrapings, although also these samples had a predominance of type 2 core glycosphingolipids. However, globotriaosylceramide and globotetraosylceramide, found in the analyses of glycosphingolipids from whole human stomach, were not present in these samples. An analogous situation has been reported for the small intestine, where the diglycosylceramides, globotriaosylceramide, and globotetraosylceramide are confined to the nonpiliated tissue, whereas monoglycosylceramides and glycosphingolipids with five or more carbohydrate residues are present in the epithelial cells (30, 31, 37, 38).

There was no binding of \textit{H. pylori} to the nonacid glycosphingolipids from the blood group O(Rh-+)p stomach that was deduced to be derived from a blood group OLe\((a+b-)\) nonsecretor individual. Nonsecretor individuals have low amounts or no Le\(^a\) antigens on their epithelial surfaces, because the precursor of the Le\(^a\) sequence (i.e., the H type 1 sequence) is not formed due to a functional FUT2 enzyme (39). However, in individuals with little or no Le\(^b\) on the gastric epithelium, an enhanced \textit{H. pylori} colonization density is obtained by interactions between the SabA adhesin and sialylated gastric glycoconjugates (40). In a recent study, we have characterized the acid glycosphingolipids of the human stomach (19) and identified two minor \textit{H. pylori} SabA-binding gangliosides as Neu5Aca3-neolactohexaosylceramide (Neu5Aca3Galβ4GlcNACβ3Galβ4GlcNACαβ3Galβ4Glcβ1Cer) and Neu5Aca3-neolactoctaosylceramide (Neu5Aca3Galβ4GlcNACβ3Galβ4GlcNACαβ3Galβ4GlcNACαβ3Galβ4Glcβ1Cer), which thus might function as adhesion factors for SabA-expressing \textit{H. pylori}.

The association between ABO blood groups and \textit{H. pylori} infection has been examined in several studies (reviewed by Cooling (22) and Brandão de Mattos and de Mattos (42)). The results are, however, often discordant because some studies focus on \textit{H. pylori} infection, whereas other focus on different \textit{H. pylori}-related diseases (e.g., peptic ulcers or gastric cancer). One example is the nine studies that tested the association between ABO blood group and \textit{H. pylori} infection in healthy subjects; two of them found a statistically significant association between the infection and blood group A status. However, in other studies, no association was detected. An increased risk for atrophic gastritis in individuals with blood group A has also been reported, but this was not confirmed in other studies on gastritis or gastric cancer patients.

Several studies have also examined the impact of Lewis and Secretor status on \textit{H. pylori} infection or gastric diseases (reviewed by Cooling 22), also with contradictory results. In early studies, an increased incidence of peptic ulcers among nonsecretor individuals was found. However, later it was demonstrated that although nonsecretors are significantly more likely to have gastric disease, there is no difference in \textit{H. pylori} infection rates by secretor status (i.e., secretor status and \textit{H. pylori} infection are independent risk factors for gastroduodenal diseases).

In summary, the characterization of the nonacid glycosphingolipids of the three human stomachs demonstrated a high degree of structural complexity. Although type 2 chain was the dominating core chain of the complex glycosphingolipids of human stomach, there were several glycosphingolipids identified that are known to have roles in mediating binding of \textit{H. pylori} to the human gastric epithelium. Identified ligands for BabA-mediated binding of \textit{H. pylori} were the Le\(^b\) hexaosylceramide, the H type 1 pentaosylceramide, and the A type 1/ALeb heptaosylceramide, and additional characterized \textit{H. pylori}-binding compounds recognized by strains lacking BabA were lactosylceramide, lactotetraosylceramide, the \(x_2\) pentaosylceramide, and neolactohexaosylceramide.

The chromatogram binding assay is a powerful tool for defining ligands for carbohydrate recognizing proteins. However, the thin-layer plate is very far removed from the dynamic lipid bilayer in the biological cell membranes. In this bilayer, there are ordered and tightly packed microdomains, which can be viewed as floating lipid rafts composed of sphingolipids and cholesterol with sequestered glycosylphosphatidylinositol-linked proteins (43). Several microbes and microbial toxins selectively bind to lipid rafts due to the presence of their specific receptors, such as glycosphingolipids, in these domains, thereby gaining access to their host cells. Interaction with lipid rafts in gastric epithelial cells is important for intoxication by \textit{H. pylori} vacuolating cytotoxin A (44, 45), and also type IV secretion system-dependent translocation of cytotoxin-associated antigen A is associated with lipid rafts (46). It has been demonstrated that the initial binding of \textit{H. pylori} to AGS cells is
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independent of cellular rafts, whereas internalization in AGS cells requires the presence of sufficient cellular cholesterol on rafts (46). However, these studies were done using the H. pylori 26695 strain, which lacks Leb and sialic acid–binding capacities (6). Therefore, it would be of interest to investigate whether human gastric cell binding of BabA- or SabA-expressing H. pylori strains is raft-related and whether the different H. pylori–binding glycosphingolipids are present in lipid rafts.

Experimental procedures

Glycosphingolipid preparations

The studies were performed in accordance with the Declaration of Helsinki. The isolation of total acid and total nonacid glycosphingolipids from human stomach was done during the 1970s by the method described by Karlsson (47). The stomachs were collected in 1974 at Sahlgrenska University Hospital (Göteborg, Sweden) (before the hospital had an ethics committee). The blood group A(Rh +)P individual was an 84-year-old patient who died of heart failure due to a myocardial abscess, whereas the blood group O(Rh −)P individual was an 80-year-old patient who died of cerebral trauma. The tissues were dissected out at autopsy and frozen at −70 °C. The tissues were lyophilized and thereafter extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and 1:9 by volume, respectively). The extracts were subjected to mild alkaline hydrolysis and dialysis, followed by separation on a silicic acid column. Acid and nonacid glycosphingolipid fractions were obtained by chromatography on a DEAE-cellulose column. To separate the nonacid glycosphingolipids from alkali-stable phospholipids, the nonacid fractions were acetylated and separated on a second silicic acid column, followed by deacetylation and dialysis. Final purifications were done by chromatographies on DEAE-cellulose and silicic acid columns.

The isolation of glycosphingolipids from the stomach of a blood group A(Rh +)P individual was described previously (48). The tissue was in this case obtained after surgery for peptic ulcer disease. The glycosphingolipids were dissolved in chloroform/methanol (2:1 by volume) and kept at −20 °C.

After initial tests for H. pylori binding and characterization by LC-ESI/MS (see below), the total nonacid glycosphingolipid fractions from the blood group A(Rh +)P and A(Rh +)P stomachs were separated on Iatrobeads (Iatrobeads 6RS-8060; Iatron Laboratories, Tokyo) columns eluted with increasing amounts of methanol in chloroform. The partly purified subfractions (denoted fractions P-I-IV and p-I-IV) obtained were further characterized by LC-ESI/MS and binding of antibodies, lectins, and bacteria. Finally, fractions P-IV and p-IV were again separated on Iatrobeads columns, and after pooling, five glycosphingolipid–containing fractions were obtained from fraction P-IV (denoted fractions P-IIVa–e) and six fractions were obtained from fraction p-IV (denoted fractions p-IIVa–f). Each fraction was ∼0.1 mg.

The nonacid glycosphingolipids from human gastric mucosa were from a previous study (cases 3 and 4, both blood group A(Rh +)P) (17). The materials used were stomach tissue (10 × 10-cm pieces) 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 scrapped off and kept at −70 °C. The material was lyophilized, and acid and nonacid glycosphingolipids were isolated as described (17).

H. pylori strains, culture conditions, and labeling

The H. pylori strain J99 and the construction of the J99/BabA− mutant babA::cam were described by Ilver et al. (5). The H. pylori strain 26695 was described previously (49). The J99 strain has a generalist BabA (i.e. in addition to the Leb determinant, this strain binds to the blood group A or B type 1 and ALeb or BLeb determinants) (14). The H. pylori strain 26695 lacks Leb-binding activity (5).

For chromatogram binding experiments, the bacteria were grown in a microaerophilic atmosphere at 37 °C for 48 h on Brucella medium (Fisher) containing 10% fetal calf serum (Fisher) inactivated at 56 °C and BBL IsoVitaleX Enrichment (Fisher). The mutant strain J99/BabA− was cultured on the same medium supplemented with chloramphenicol (20 μg/ml). Bacteria were radiolabeled by the addition of 50 μCi of [35S]methionine (PerkinElmer Life Sciences) diluted in 0.5 ml of PBS, pH 7.3, to the culture plates. After incubation for 12–72 h at 37 °C under microaerophilic conditions, the bacteria were harvested, centrifuged three times, and thereafter suspended to 1 × 10⁸ cfu/ml in PBS. The specific activities of the suspensions were ∼1 cpm per 100 H. pylori organisms.

Recombinant BabA

A truncated BabA domain (BabA547), lacking the C-terminal β-barrel part, was expressed with a periplasmic leader sequence in E. coli and purified as described (50).

Reference glycosphingolipids

Total acid and nonacid glycosphingolipid fractions were isolated as described (47). Individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns and by HPLC and identified by mass spectrometry (20, 51) and 1H NMR spectroscopy (52).

TLC

TLC was done on aluminum- or glass-backed silica gel 60 high-performance TLC plates (Merck). Glycosphingolipid mixtures (40 μg) or pure glycosphingolipids (4 μg) were applied to the plates and eluted with chloroform/methanol/water (60:35:8 by volume) or chloroform/methanol/water (65:25:4 by volume) as the solvent system. Chemical detection was done with anisaldehyde (53).

Borate-impregnated plates (54) were prepared by spraying glass-backed silica gel 60 high-performance TLC plates with 1% (w/v) aqueous sodium tetraborate, followed by activation for 30 min at 120 °C. Chromatography on the borate-impregnated plates was done using chloroform/methanol/water (100:30:4 by volume) as the solvent system.

Chromatogram binding assays

Binding of 35S-labeled H. pylori to glycosphingolipids on thin-layer chromatograms was done as reported previously (17). Dried chromatograms were dipped for 1 min in diethyl ether/n-hexane (1:5 by volume) containing 0.5% (w/v) poly-
isobutylmethacrylate (Aldrich). After drying, the chromatograms were soaked in PBS containing 2% BSA (w/v), 0.1% NaN₃ (w/v), and 0.1% Tween 20 (by volume) for 2 h at room temperature. The chromatograms were subsequently covered with radiolabeled bacteria diluted in PBS (2–5 × 10⁶ cpm/ml). Incubation was done for 2 h at room temperature, followed by repeated washings with PBS. The chromatograms were thereafter exposed to XAR-5 X-ray films (Eastman Kodak Co.) for 12 h.

The mouse monoclonal antibodies tested for binding to the nonacid glycosphingolipids in the chromatogram binding assay are given in Table 4. Binding of antibodies to glycosphingolipids separated on thin-layer chromatograms was performed as described (55), using 125I-labeled monoclonal anti-mouse antibodies (Z02S95; DakoCytomation Norden A/S) for detection.

Binding of 125I-labeled E. cristagalli lectin (Sigma-Aldrich), 125I-labeled S. tuberosum lectin (Sigma-Aldrich), 125I-labeled BabA547, and 35S-labeled P-fimbriated E. coli strain 291-15 to glycosphingolipids on thin-layer chromatograms was done as described (18, 24, 27). The binding assays with BabA₅₄⁷ were done using Carbo-Free blocking solution (Vector Laboratories, Burlingame, CA) for blocking of the chromatograms.

### Endoglycoceramidase digestion and LC-ESI/MS

Endoglycoceramidase II from Rhodococcus spp. (Takara Bio Europe S.A., Gennevilliers, France) was used for hydrolysis of the nonacid glycosphingolipids. The glycosphingolipids (50 mg) were resuspended in 100 ml of 0.05 M sodium acetate buffer, pH 5.0, containing 120 mg of sodium cholate and sonicated briefly. Thereafter, 1 milliunit of enzyme was added, and the mixture was incubated at 37 °C for 48 h. The reaction was stopped by the addition of chloroform/methanol/water to the final proportions 8:4:3 (by volume). The oligosaccharides were separated on thin-layer chromatograms as described (55), using 125I-labeled monoclonal anti-mouse antibodies (Z02S95; DakoCytomation Norden A/S) for detection.

The binding assays with BabA₅₄⁷ were done using Carbo-Free blocking solution (Vector Laboratories, Burlingame, CA) for blocking of the chromatograms.

### LC-ESI/MS of native glycosphingolipids

The native nonacid glycosphingolipid fractions were analyzed by LC-ESI/MS as described (56). Aliquots of the glycosphingolipid fractions were dissolved in methanol/acetonitrile (75:25 by volume) and separated on a 100 × 0.250-mm column, packed in-house with 5-μm polystyrene II particles (YMC Europe GmbH, Dinslaken, Germany). An autosampler, HTC-PAL (CTC Analytics AG, Zwingen, Switzerland), equipped with a Cheminert valve (0.25-mm bore) and a 2-μl loop, was used for sample injection. An Agilent 1100 binary pump (Agilent Technologies, Palo Alto, CA) delivered a flow of 250 μl/min, which was split down in a 1/4-inch microvolume T (0.15-mm bore) (Vici AG International, Schenkon, Switzerland) by a 50 cm × 50-μm inner diameter fused silica capillary before the injector of the autosampler, allowing ~2–3 μl/min through the column. The LC-ESI/MS analysis was performed in the positive mode using an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA). The LTQ quadrupole was operated using an ESI source with a voltage of 2.2 kV. The LTQ capillary voltage was 30 V, and the tube lens voltage was 50 V. The LTQ mass spectrometer was equipped with a stainless steel needle kept at ~3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270 °C, and the capillary voltage was −50 kV. Full scan (m/z 200–2000, two microscans, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent MS² scans of the three most abundant ions in each scan (2 microscans, maximum 100 ms, target value of 10,000). The threshold for MS² was set to 500 counts. Normalized collision energy was 35%, and an isolation window of 3 units, an activation q = 0.25, and an activation time of 30 ms were used. Selected fractions were also analyzed at m/z 1300–2000. Data acquisition and processing were conducted with Xcalibur software (version 2.0.7).

### Table 4

| Antibodies | Clone | Manufacturer | Dilution | Isotype |
|------------|-------|--------------|----------|---------|
| Anti-Lewis⁺ | P12   | Calbiochem   | 1:200    | IgM     |
| Anti-Lewis⁺ | F3    | Calbiochem   | 1:200    | IgM     |
| Anti-Lewis⁻ | BG-6/T218 | Signet/Covance | 1:100 | IgM     |
| Anti-blood group A | HE-195 | Sigma-Aldrich | 1:1000 | IgM     |

The oligosaccharides were analyzed in negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA). The IonMax standard ESI source on the LTQ mass spectrometer was equipped with a stainless steel needle kept at ~3.5 kV. Compressed air was used as nebulizer gas. The heated capillary was kept at 270 °C, and the capillary voltage was −50 kV. Full scan (m/z 380–2000, two microscans, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent MS² scans of the three most abundant ions in each scan (2 microscans, maximum 100 ms, target value of 10,000). The threshold for MS² was set to 500 counts. Normalized collision energy was 35%, and an isolation window of 3 units, an activation q = 0.25, and an activation time of 30 ms were used. Selected fractions were also analyzed at m/z 1300–2000. Data acquisition and processing were conducted with Xcalibur software (version 2.0.7).

Manual assignment of glycan sequences was done on the basis of knowledge of mammalian biosynthetic pathways, with the assistance of the Glycoworbench tool (version 2.1) and by comparison of retention times and MS² spectra of oligosaccharides from reference glycosphingolipids (20).
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dow of 2.5 units, activation \( q = 0.25 \), and activation time 30 ms). The threshold for MS\(^2\) was set to 500 counts.

Data acquisition and processing were conducted with Xcalibur software (version 2.0.7). Manual assignment of glycosphingolipid sequences was done with the assistance of the Glycoworkbench tool (version 2.1), and by comparison of retention times and MS\(^2\) spectra of reference glycosphingolipids.

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