Helicobacter pylori–binding nonacid glycosphingolipids in the human stomach

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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 Le[x], Le[a], and H type 2 pentaosylceramides, and the Le[y] 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 Le[b] hexaosylceramide, the H type 1 pentaosylceramide, and the A type 1/ALe[b] heptaosylceramide. Additional *H. pylori*-binding glycosphingolipids recognized by BabA-deficient strains were lactosylceramide, lactotetraosylceramide, the x[2] 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 and 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 Le[b] blood group determinant and related carbohydrate antigens, heparan sulfate, sulfatide, lactosylceramide, neolacto sequences, 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 BabA[2] 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 Le[b] (Fucα2Galβ3(Fucα4)GlcNAcβ-)-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-GlcNAcβ-) and Le[b] 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 Le[b] and related carbohydrate sequences (14). BabA of specialist strains binds only to glycoconjugates with a terminal Fucα2Gal sequence, as the H

[2] The abbreviations used are: BabA, blood group antigen–binding adhesin; ESI, electrospray ionization; LabA, lacdiNAc-binding adhesin; SabA, sialic acid–binding adhesin; Hex, hexose; HexNAc, *N*-acetylhexosamine; Fuc, fucose; Cer, ceramide.

Table 1
Nonacid carbohydrate structures recognized by *H. pylori*

Trivial name	Carbohydrate sequence	BabA binding[a]	Reference
Gangliotetra	Galβ3GalNAcβ4Galβ4Glc	−	57
Le[b]	Fucα2Galβ3 (Fucα4)GlcNAcβ3Galβ4Glc	+	5
H type 1	Fucα2Galβ3GlcNAcβ3Galβ4Glc	+	5
Lactose[b]	Galβ4Glc	−	25
Lactotetra	Galβ3GlcNAcβ3Galβ4Glc	−	17
A type 1 hexa	GalNAcα3 (Fucα2)Galβ3GlcNAcβ3Galβ4Glc	+	16
B type 1 hexa	Galα3 (Fucα2)Galβ3GlcNAcβ3Galβ4Glc	+	16
A type 1 hepta/ALe[b]	GalNAcα3 (Fucα2)Galβ3 (Fucα4)GlcNAcβ3Galβ4Glc	+	14
B type 1 hepta/BLe[b]	Galα3 (Fucα2)Galβ3 (Fucα4)GlcNAcβ3Galβ4Glc	+	14
Neolactotetra	Galβ4GlcNAcβ3Galβ4Glc	−	26
Neolactohexa	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	−	26
Galα3-neolacto	Galα3Galβ4GlcNAcβ3Galβ4Glc	−	26
x_2	GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc	−	26
Globo H	Fucα2Galβ3GalNAcβ3Galα4Galβ4Glc	+	16
Globo A	GalNAcα3 (Fucα2)Galβ3GalNAcβ3Galα4Galβ4Glc	+	16

[a] Recognized by generalist BabA.
[b] Binding to lactosylceramide with phytosphingosine and/or hydroxy fatty acids.

type 1 and Le[b] 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)Galβ3GlcNAcβ- or (Galα3(Fucα2)Galβ3GlcNAcβ-) and ALe[b] or BLe[b] (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ- or Galα3(Fucα2)Galβ3(Fucα4)GlcNAcβ-) 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/Le[b]-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-neolactohexaosylceramide (Neu5Acα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4 Glcβ1Cer) and Neu5Acα3-neolactooctaosylceramide (Neu5Acα3 Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc β1Cer), 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 human 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. 1*A*, *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. 1*A*, *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 Le[b]-binding activity (5). In this assay, the detection limit for binding of the generalist strain J99 to the A type 1/ALe[b] heptaosylceramide is ∼40 ng, whereas the detection limit for binding to the preferred ligand (*i.e.* the Le[b] 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. 1*B*, *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. 1 (*C* and *D*), *lanes 2* and *3*). The two non-BabA strains, on the other hand, bound to compounds migrating in the di- and tetraglycosylceramide regions in the A(Rh+)p stomach sample (Fig. 1 (*C* 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. 1 (*B*–*D*), *lane 1*).

ASBMB

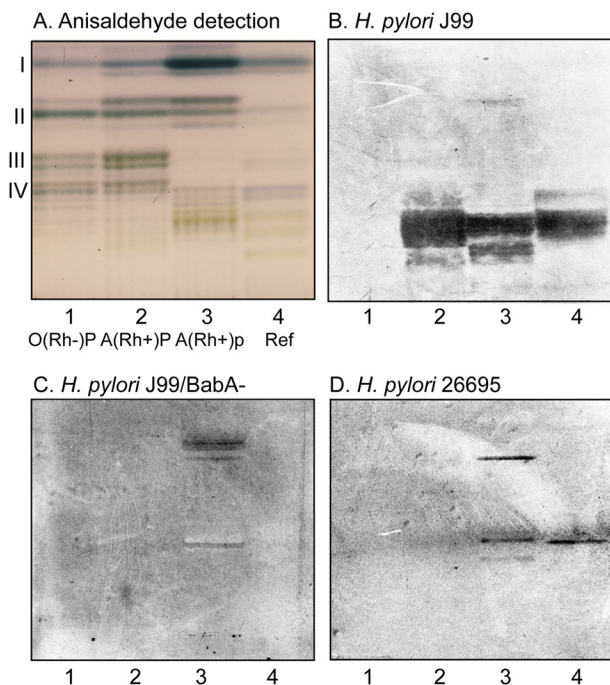*J. Biol. Chem.* (2018) 293(44) 17248 –17266   **17249**

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, 40 μg; *lane 2*, total nonacid glycosphingolipids of human stomach blood group A(Rh+)P, 40 μg; *lane 3*, 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 MS[2] (20). Furthermore, MS[2] spectra of oligosaccharides with a Hex or HexNAc substituted at C-4 have diagnostic cross-ring ^{0,2}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. 2*A*. 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 MS[2], and the oligosaccharides thereby identified are given in the chromatogram.

The annotation procedure is exemplified in Fig. 2 (*B–D*) by the MS[2] 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 MS[2] spectrum of the ion eluting at 15.5 min

(Fig. 2*B*) had an intense ion at *m/z* 364. This fragment ion is obtained by double glycosidic cleavage of the 3-linked branch (C$_2$/Z$_{3\beta}$) and is characteristic for an internal 4-linked GlcNAc substituted with a Fuc at the 3-position (21). Taken together with the C$_2$ ion at *m/z* 528 and the C$_3$ ion at *m/z* 690, a Le^x pentasaccharide (Galβ4(Fucα3)GlcNAcβ3Galβ4Glc) was thus tentatively identified.

A fragment ion at *m/z* 348 was present in the MS[2] spectrum of the ion at *m/z* 852 eluting at 15.9 min (Fig. 2*C*). 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 (C$_2$/Z$_{3\alpha}$). C-type fragment ions at *m/z* 528 (C$_{2\alpha}$) and *m/z* 690 (C$_3$) were present, and taken together, this indicated a Le^a pentasaccharide (Galβ3(Fucα4)GlcNAcβ3-Galβ4Glc).

The MS[2] spectrum of the ion at *m/z* 852 eluting at 20.1 min was distinctly different (Fig. 2*D*) and had a series of C-type fragment ions (C$_2$ at *m/z* 325, C$_3$ at *m/z* 528, and C$_4$ at *m/z* 690), identifying a pentasaccharide with a Fuc-Hex-HexNAc-Hex-Hex sequence. The ^{0,2}A$_3$-H$_2$O fragment ion at *m/z* 409 and the ^{0,2}A$_3$ 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 (Fucα2Galβ4GlcNAcβ3 Galβ4Glc) 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.* neolacto-tetraosylceramide, the Le^x, H type 2, and x$_2$ pentaosylceramides, neolactohexaosylceramide, the Le^y hexaosylceramide, and the H type 2 heptaosylceramide) (see Tables 2 and 3 for glycosphingolipid structures).[3] The presence of a Le^a pentasaccharide and the absence of saccharides with H type 1 and Le^b 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 Iatrobeads columns (Iatron 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.

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[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-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecane).
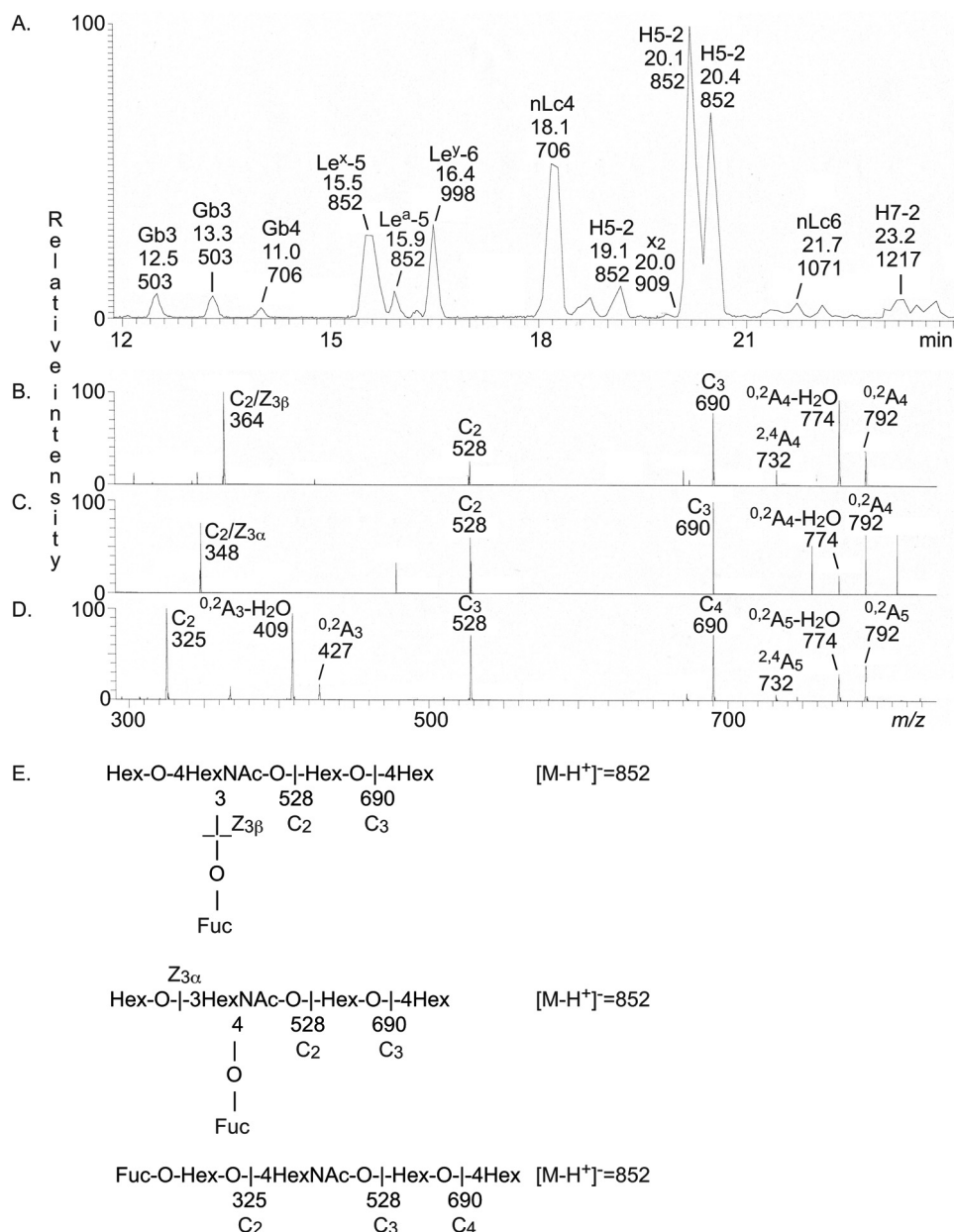
Figure 2. LC-ESI/MS of the oligosaccharides obtained from the total nonacid glycosphingolipid fraction of human blood group O(Rh−)P stomach by hydrolysis with endoglycoceramidase II from *Rhodococcus* spp. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained from human stomach blood group O(Rh−)P. *B*, MS^2 of the ion at *m/z* 852 (retention time 15.5 min). *C*, MS^2 of the ion at *m/z* 852 (retention time 15.9 min). *D*, MS^2 of the ion at *m/z* 852 (retention time 20.1 min). *E*, interpretation formulas showing the deduced oligosaccharide sequences. The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS^2 sequencing. The oligosaccharides identified in the chromatograms were Galα4Galβ4Glc (*Gb3*), GalNAcβ3Galα4Galβ4Glc (*Gb4*), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^x-5*), Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^a-5*), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y-6*), Galβ4GlcNAcβ3Galβ4Glc (*nLc4*); Fucα2Galβ4GlcNAcβ3Galβ4Glc (*H5-2*), GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*x$_2$*), Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*nLc6*), and Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*H7-2*).

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. 3 (*A and B*), *lane 1*), and LC-ESI/MS and subsequent MS^2 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. 3*A*, *lane 2*), whereas fraction p-II contained only compounds migrating as diglycosylceramides (Fig. 3*B*, *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-

Table 2
Oligosaccharides obtained from the total nonacid glycosphingolipid fractions from human stomach by hydrolysis with endoglycoceramidase II from *Rhodococcus* spp. and characterized by LC-ESI/MS and MS^2

Abbreviation	Structure	O(Rh+)P	A(Rh+)P	A(Rh+)p	A(Rh+)P case 3[a]	A(Rh+)P case 4[a]
Gb3	Galα4Galβ4Glc	+	+	−	−	−
Gb4	GalNAcβ3Galα4Galβ4Glc	+	+	−	−	−
Le^b-6[b]	**Fucα2Galβ3 (Fucα4)GlcNAcβ3Galβ4Glc**	−	+	+	+	+
Le^x-5	Galβ4 (Fucα3)GlcNAcβ3Galβ4Glc	+	+	+	+	+
Le^a-5	Galβ3 (Fucα4)GlcNAcβ3Galβ4Glc	+	+	+	+	+
Le^y-6	Fucα2Galβ4 (Fucα3)GlcNAcβ3Galβ4Glc	+	+	+	+	+
H5-1	**Fucα2Galβ3GlcNAcβ3Galβ4Glc**	−	+	−	−	−
Lc4	**Galβ3GlcNAcβ3Galβ4Glc**	−	+	+	−	+
nLc4	**Galβ4GlcNAcβ3Galβ4Glc**	+	+	+	+	+
H5-2	Fucα2Galβ4GlcNAcβ3Galβ4Glc	+	+	+	+	+
x$_2$	**GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc**	+	+	+	+	+
nLc6	**Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc**	+	+	+	−	−
H7-2	Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	+	+	+	−	−
A6-2	GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc	−	+	+	+	+

[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).

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*-acetyllactosamine–binding lectin from *S. tuberosum* also binds to lactosylceramide (Galβ4Glcβ1Cer) 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 (Galα4Galβ4Glcβ1Cer) and globotetraosylceramide (GalNAcβ3 Galα4Galβ4Glcβ1Cer).

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-D-hydroxy fatty acids has been reported (25). According to mass spectrometry, fraction p-II contained a dihexosylceramide with d18:1-h16:0 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-IV:a–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 MS^2. Thereby, a lactotrisaccharide (m/z 544) and a Le^x pentasaccharide (m/z 852) were identified in both fractions (not shown). A globotrisaccharide (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 MS^2 spectrum 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 (C_1 at m/z 220, C_2 at m/z 382, and C_3 at m/z 544), demonstrating a HexNAc-Hex-Hex-Hex sequence. The $^{0,2}A_3$ and $^{0,2}A_3$-H_2O fragment ions at m/z 484 and 466 demonstrated a 4-substituted Hex (20, 21). Thus, a globotetrasaccharide (GalNAcβ3Galα4Galβ4Glc) was identified.

MS^2 of the ion at m/z 706 at the retention time 17.2 min allowed a preliminary identification of a lactotetrasaccharide (Galβ3GlcNAcβ3Galβ4Glc) in both fractions (exemplified in Fig. 5F). This was concluded from the C-type fragment ions (C_2 at m/z 382 and C_3 at m/z 544) identifying a Hex-HexNAc-Hex-Hex sequence. This MS^2 spectrum had a prominent $D_{1−2}$ ion at m/z 202, which is obtained by a C_2-Z_2 double cleavage and diagnostic for a 3-substituted HexNAc (21). The MS^2 spectrum also lacked a $^{0,2}A_2$ fragment ion at m/z 281, further demonstrating that the HexNAc was substituted at the 3-position.

In contrast, the MS^2 spectrum of the ion at m/z 706 at the retention time 17.5 min had a prominent $^{0,2}A_2$ fragment ion at

Table 3
Summary of glycosphingolipids characterized in the subfractions isolated from human stomachs

Fraction	Amount	Migration	Glycosphingolipid	Abbreviation
	mg			
P-I	0.4	Mono	Galβ1Cer	GalCer
			Glcβ1Cer	GlcCer
P-II	1.2	Di + tri	**Galβ4Glcβ1Cer**[a]	**LacCer**
			Galα4Galβ1Cer	Galabia
			Galα4Galβ4Glcβ1Cer	Gb3
P-III	1.3	Tri + tetra	Galα4Galβ4Glcβ1Cer	Gb3
			GlcNAcβ3Galβ4Glcβ1Cer	Lc3
			GalNAcβ3Galα4Galβ4Glcβ1Cer	Gb4
			Galβ3GlcNAcβ3Galβ4Glcβ1Cer	**Lc4**
			Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc4**
			Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^x-5
P-IV	0.7	≥Tetra	GalNAcβ3Galα4Galβ4Glcβ1Cer	Gb4
			Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc4**
			GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**x$_2$**
			Fucα2Galβ3GlcNAcβ3Galβ4Glcβ1Cer	**H5-1**
			Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H5-2
			Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	Le^a-5
			Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^x-5
			Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc6**
			Fucα2Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	**Le^b-6**
			Fucα2Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^y-6
			GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	A6-2
			Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H7-2
			GalNAcα3 (Fucα2)Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	**A7-1**
			GalNAcα3 (Fucα2)Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	A7-2
			GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	A8-2
p-I	1.3	Mono	Galβ1Cer	GalCer
			Glcβ1Cer	GlcCer
p-II	0.7	Di	**Galβ4Glcβ1Cer**	**LacCer**
p-III	0.3	Tetra	GlcNAcβ3Galβ4Glcβ1Cer	Lc3
			Galβ3GlcNAcβ3Galβ4Glcβ1Cer	**Lc4**
			Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc4**
			Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H5-2
			Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^x-5
			GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	A6-2
p-IV[b]	1.2	≥Tetra	**Galβ4GlcNAcβ3Galβ4Glcβ1Cer**	**nLc4**
			GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**x$_2$**
			Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H5–2
			Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	Le^a-5
			Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^x-5
			Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc6**
			Fucα2Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	**Le^b-6**
			Fucα2Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Le^y-6
			GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	A6-2
			GalNAcα3 (Fucα2)Galβ3 (Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	**A7-1**
			GalNAcα3 (Fucα2)Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	A7-2
			Galβ3/4GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Galβ3/4GlcNAcβ3- Le^x-5
			Galβ4 (Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	Le^x-7
			Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H7-2
			Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	**nLc8**
			GalNAcα3 (Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	A8–2
			Fucα2Galβ3/4GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	Fucα2Galβ3/4-GlcNAcβ3-Le^x-5
			Fucα2Galβ4 (Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glclcβ1Cer	Le^y-8
			Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	H9–2
			Fucα2Galβ4 (Fucα3)GlcNAcβ3Galβ4 (Fucα3)GlcNAcβ3Galβ4Glclcβ1Cer	Le^y-9

[a] The compounds in boldface type have been characterized as *H. pylori* binding in previous studies (summarized in Table 1).
[b] The glycosphingolipids listed under fraction p-IV were characterized by LC-ESI/MS and MS^2 of fractions p-IVa–f.

m/z 281 and a $^{0,2}A_2$-H$_2$O 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 C$_2$ ion at m/z 382 and the C$_3$ ion at m/z 544, this demonstrated a neolactotetrasaccharide (Galβ4GlcNAcβ3Galβ4Glc) in both fractions (exemplified in Fig. 5G).

The three MS^2 spectra all had $^{0,2}A_4$ ions at m/z 646 and $^{0,2}A_4$-H$_2$O 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-III.

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 MS^2, and the oligosaccharides thereby identified are given in the chromatogram (Fig. 6) and summarized in Table 3.

By MS^2, four oligosaccharides with terminal blood group A determinants were identified in fraction P-IV. First, MS^2 of the molecular ion at m/z 1055 at retention time 13.1 min (Fig. 6B) gave
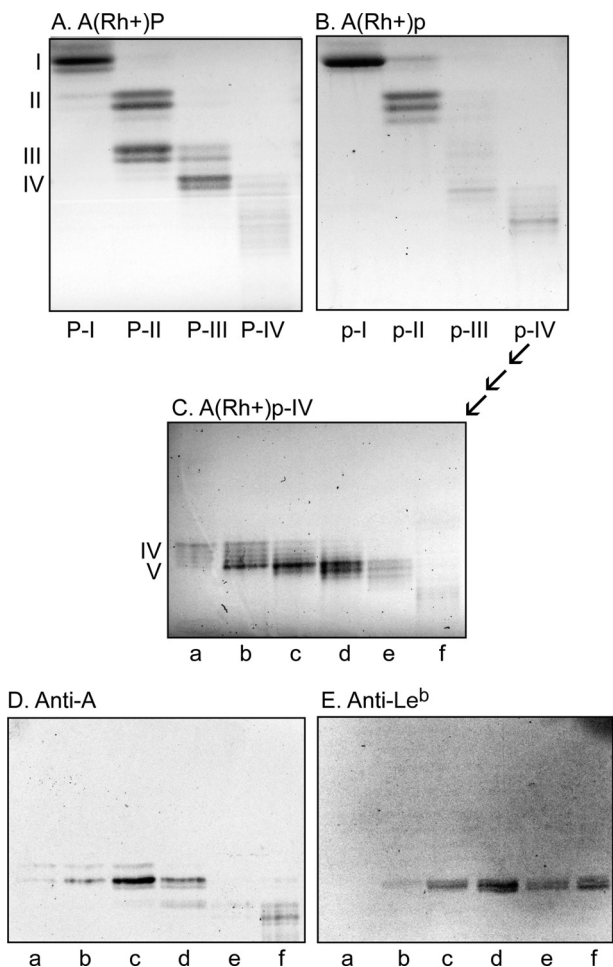
Figure 3. Glycosphingolipid subfractions isolated from human blood group A(Rh+)P and A(Rh+)p stomachs. *A–E*, thin-layer chromatograms after detection with anisaldehyde (*A–C*) and autoradiograms obtained by binding of monoclonal antibodies directed against the blood group A determinant (*D*) and the Le[b] determinant (*E*). The glycosphingolipids (4 μg/lane) were separated on glass or 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. The *Roman numerals* to the *left* of *A* and *C* denote the number of carbohydrates in the bands.
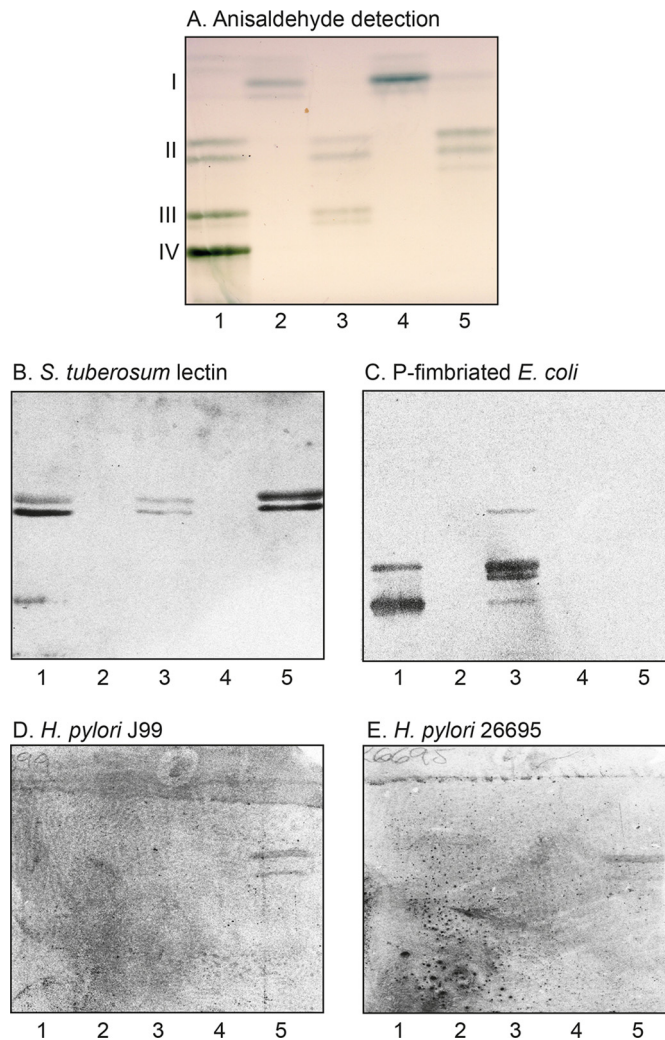

Figure 4. Binding of *H. pylori*, P-fimbriated *E. coli*, and *S. tuberosum* lectin to mono-, di-, and triglycosylceramides of human stomach. *A–E*, thin-layer chromatograms of the mono- to triglycosylceramide fractions isolated from human A(Rh+)P and A(Rh+)p stomachs (*A*) and autoradiograms obtained by binding of *S. tuberosum* lectin (*B*), P-fimbriated *E. coli* strain 291-15 (*C*), *H. pylori* strain J99 (*D*), and *H. pylori* strain 26695 (*E*). The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 65:25:4 (by volume) as the solvent system, and the binding assays were performed as described under "Experimental procedures." Autoradiography was for 12 h. *Lane 1*, reference nonacid glycosphingolipids of human blood group O erythrocytes, 40 μg; *lane 2*, fraction P-I, 4 μg; *lane 3*, fraction P-II, 4 μg; *lane 4*, fraction p-I, 4 μg; *lane 5*, fraction p-II, 4 μg. The *Roman numerals* to the *left* of the chromatogram in *A* denote the number of carbohydrate units in the bands.

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 $^{0,2}A_4$ ion and $^{0,2}A_4$-H_2O ion at *m/z* 630 and 612. Taken together, these spectral features identified a blood group A type 2 hexasaccharide (GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glc).

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-Hex sequence. Both spectra also had a fragment ion at *m/z* 713, which, in analogy with the fragment ion at *m/z* 364 in the MS^2 spectrum of the Le^x pentasaccharide, is obtained by double glycosidic cleavage of the 3-linked branch (C_3/$Z_{3\beta}$) 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/ALe^y heptasaccharide (GalNAcα3 (Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glc).

Third, a blood group A type 1/ALe^b heptasaccharide (GalNAc α3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glc) 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-
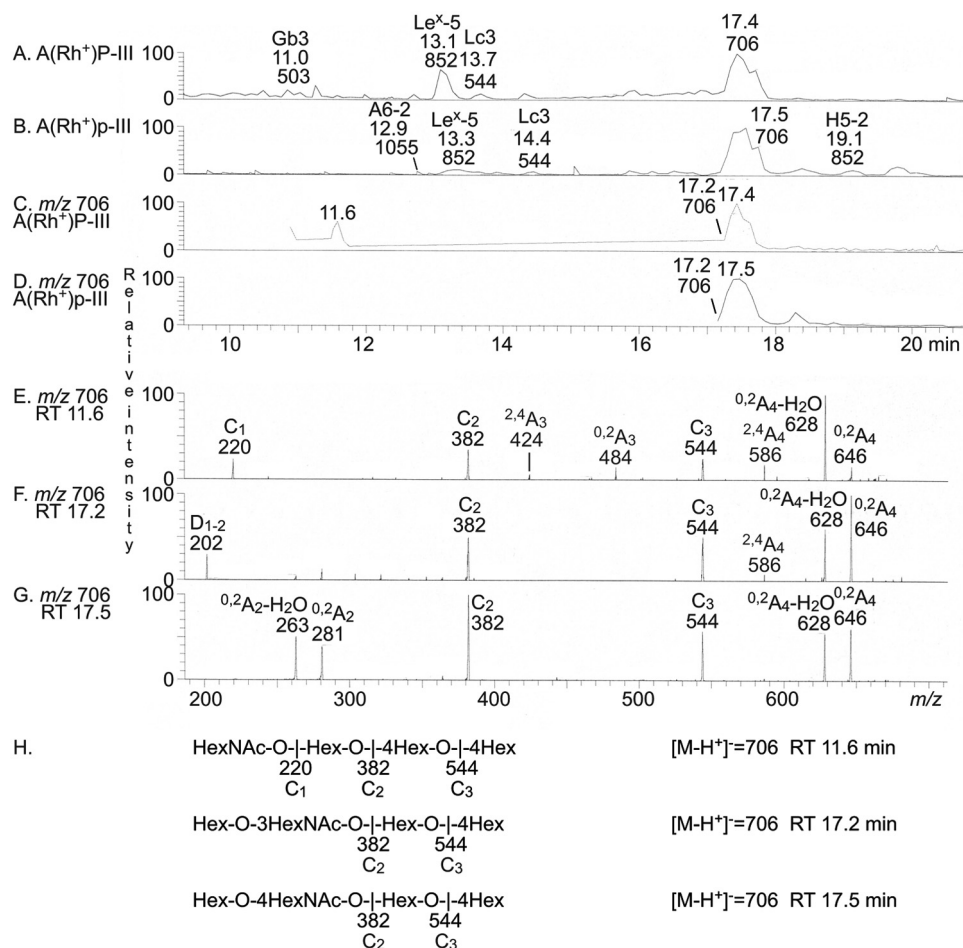
Figure 5. LC-ESI/MS of the oligosaccharides obtained by digestion of the slow-migrating nonacid glycosphingolipids of human blood group A(Rh+)P and A(Rh+)p stomachs with *Rhodococcus* endoglycoceramidase II. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction P-III. *B*, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction p-III. *C*, mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from fraction P-III. *D*, mass chromatogram of m/z 706 from LC-ESI/MS of the oligosaccharides derived from fraction p-III. *E*, MS^2 of the ion at m/z 706 (retention time 11.6 min) from LC-ESI/MS of the oligosaccharides derived from fraction P-III. *F*, MS^2 of the ion at m/z 706 (retention time 17.2 min) from LC-ESI/MS of the oligosaccharides derived from fraction P-III. *G*, MS^2 of the ion at m/z 706 (retention time 17.5 min) from LC-ESI/MS of the oligosaccharides derived from fraction P-III. *H*, interpretation formulas showing the deduced oligosaccharide sequences. The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS^2 sequencing. The oligosaccharides identified in the chromatograms were Galα4Galβ4Glc (*Gb3*), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^x-5*), GlcNAcβ3Galβ4Glc (*Lc3*), GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glc (*A6-2*), and Fucα2Galβ4GlcNAcβ3Galβ4Glc (*H5-2*).

tuted with a Fuc at C-4 and is due to a double glycosidic cleavage of the 3-linked branch at C_3 and $Z_{4\alpha}$ (21).

Finally, the MS^2 spectrum of the molecular ion at m/z 1420 (Fig. 6*E*) had B-type and C-type fragment ion series (C_2 at m/z 528, B_3 at m/z 713, C_3 at m/z 731, C_4 at m/z 893, B_5 at m/z 1078, C_5 at m/z 1096, and C_6 at m/z 1258) demonstrating a HexNAc-(Fuc-)Hex-HexNAc-Hex-HexNAc-Hex-Hex sequence. The $^{0,2}A_4$-H$_2$O ion at m/z 612, along with the $^{0,2}A_5$ ion at m/z 995, showed that both HexNAcs were substituted at C-4. Thereby, a blood group A type 2 octasaccharide (GalNAcα3(Fucα2)-Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc) was identified.

The base peak chromatogram from LC-ESI/MS of the oligosaccharides from fraction p-IV was relatively weak. The largest oligosaccharide identified by MS^2 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. 3*C*). Each fraction was ~0.1 mg.

Fractions p-IV:a–e were all complex oligosaccharide mixtures containing neolactotetra-, Le^x penta-, Le^a penta-, and Le^y hexasaccharides, as found by LC-ESI/MS and MS^2 (data not shown). The Le^b hexasaccharide was present in fractions p-IV: c–e, and the x$_2$ 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-IV-f—LC-ESI/MS of the oligosaccharides obtained from fraction p-IV-f gave a relatively weak base peak chromatogram (Fig. 7*A*). Again, MS^2 of the major molecular ions identified the Le^b and Le^y hexasaccharides; the Le^x, Le^a, 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.
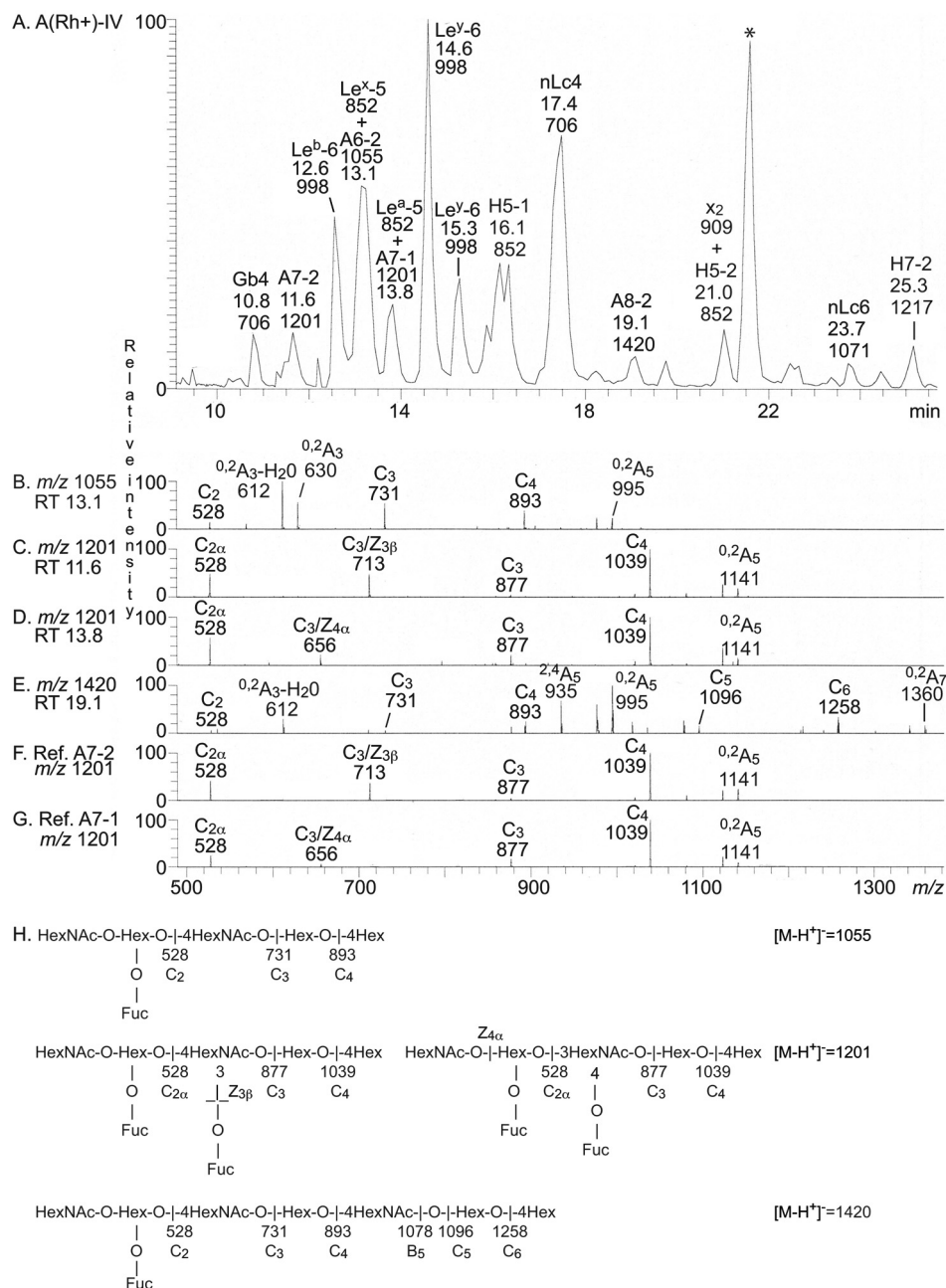
Figure 6. LC-ESI/MS of the oligosaccharides obtained by *Rhodococcus* endoglycoceramidase II digestion of the slow-migrating nonacid glycosphingolipid fraction P-IV from human blood group A(Rh+)P stomach and of reference blood group A heptaosylceramides. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction P-IV. *B*, MS[2] of the ion at *m/z* 1055 (retention time 13.1 min). *C*, MS[2] of the ion at *m/z* 1201 (retention time 11.6 min). *D*, MS[2] of the ion at *m/z* 1201 (retention time 13.8 min). *E*, MS[2] of the ion at *m/z* 1420 (retention time 19.1 min). *F*, MS[2] of the ion at *m/z* 1201 from LC-ESI/MS of the oligosaccharides derived from reference blood group A type 2/ALe^y heptaosylceramide (GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glc). *G*, MS[2] of the ion at *m/z* 1201 from LC-ESI/MS of the oligosaccharides derived from reference blood group A type 1/ALe^b heptaosylceramide (GalNAcα3(Fucα2)Galβ3(Fucα43)GlcNAcβ3Galβ4Glc). *H*, interpretation formulas showing the deduced oligosaccharide sequences. The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS[2] sequencing. The oligosaccharides identified in the chromatograms were GalNAcβ3Galα4Galβ4Glc (*Gb4*), GalNAcα3(Fucα2)Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*A7-2*), Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^b-6*), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^x-5*), GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glc (*A6-2*), Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^a-5*), GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*A7-1*), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y-6*), Fucα2Galβ3GlcNAcβ3Galβ4Glc (*H5-1*), Galβ4GlcNAcβ3Galβ4Glc (*nLc4*), GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*A8-2*), GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*x$_2$*), Fucα2Galβ4GlcNAcβ3Galβ4Glc (*H5-2*), Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*nLc6*), and Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*H7-2*). *, nonoligosaccharide contaminant.

The MS[2] spectrum of the molecular ion at *m/z* 1217 eluting at 15.4 min (Fig. 7*B*) had a series of C-type fragment ions (C$_{2\alpha}$ at *m/z* 382, C$_{3\alpha}$ at *m/z* 544, C$_4$ at *m/z* 893, 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$_{3\beta}$ (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^x determinant. Taken together, this indicated an oligosac-
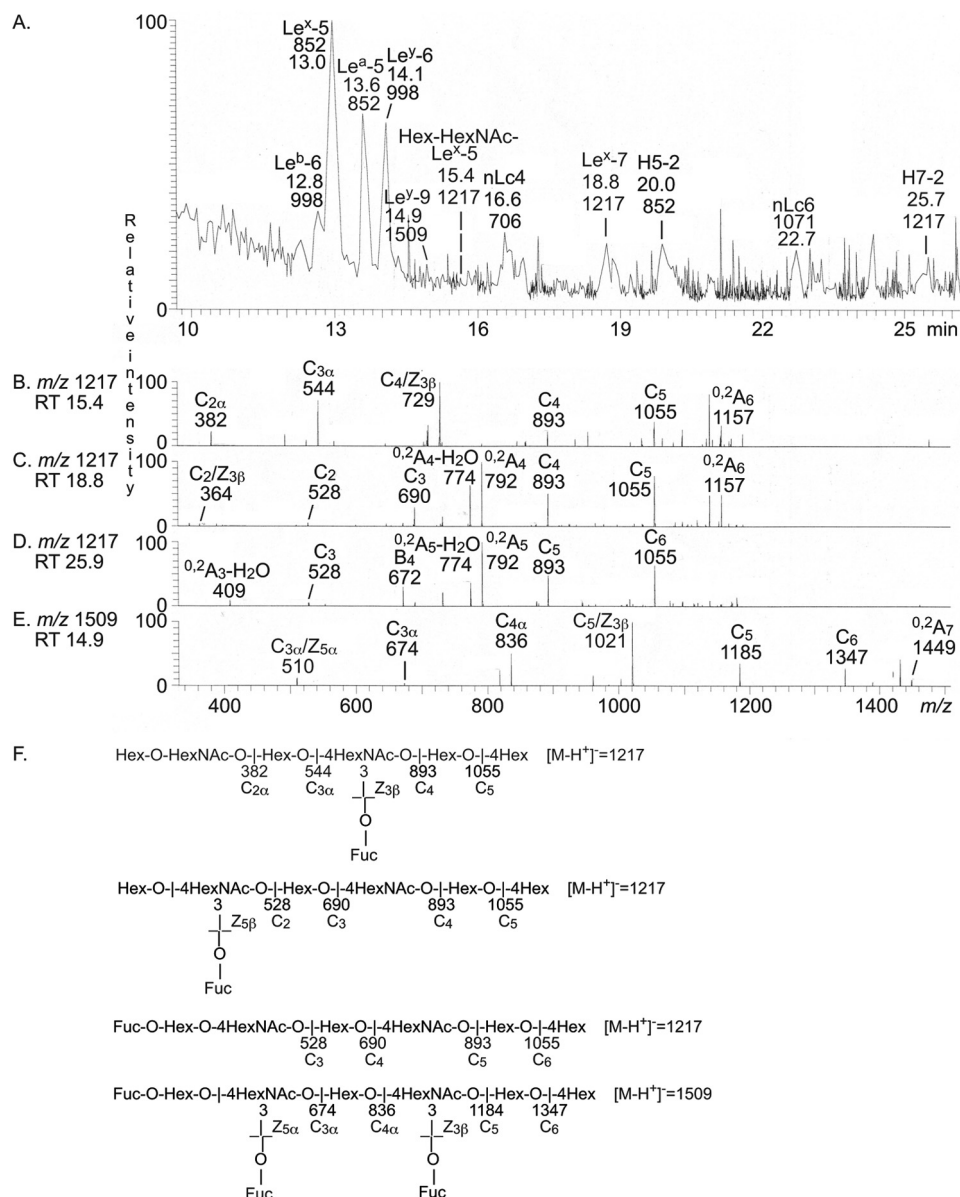
Figure 7. LC-ESI/MS of the oligosaccharides obtained by digestion of the slow-migrating nonacid glycosphingolipid fraction p-IV-f from human blood group A(Rh+)p stomach with *Rhodococcus* endoglycoceramidase II. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides derived from fraction p-IV-f. *B*, MS^2 of the ion at *m/z* 1217 (retention time 15.4 min). *C*, MS^2 of the ion at *m/z* 1217 (retention time 18.8 min). *D*, MS^2 of the ion at *m/z* 1217 (retention time 25.9 min). *E*, MS^2 of the ion at *m/z* 1509 (retention time 14.9 min). *F*, interpretation formulas showing the deduced oligosaccharide sequences. The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS^2 sequencing. The oligosaccharides identified in the chromatograms were Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^b*-6), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^x*-5), Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^a*-5), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y*-6), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y*-9), Galβ3/4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc(*Hex-HexNAc-Le^x-5*), Galβ4GlcNAcβ3Galβ4Glc (*nLc4*), Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*Le^x-7*), Fucα2Galβ4GlcNAcβ3Galβ4Glc (*H5-2*), Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*nLc6*), and Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*H5-2*).

charide with Galβ3/4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3 Galβ4Glc sequence.

MS^2 of the molecular ion at *m/z* 1217, eluting at 18.8 min (Fig. 7C), gave a fragment ion at *m/z* 364, again identifying an internal 4-linked GlcNAc substituted with a Fuc at C-3 (21). C-type fragment ions at *m/z* 528 (C_2), *m/z* 690 (C_3), *m/z* 893 (C_4), and *m/z* 1055 (C_5) were present, as well as $^{0,2}A_4$-H$_2$O fragment ion at *m/z* 774 and a $^{0,2}A_4$ fragment ion at *m/z* 792, demonstrating that the internal HexNAc was substituted at C-4. Taken together, this indicated a Le^x heptasaccharide (Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

The MS^2 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 (C_3 at *m/z* 528, C_4 at *m/z* 690, C_5 at *m/z* 893, and C_6 at *m/z* 1055), which, together with the $^{0,2}A_3$-H$_2$O fragment ion at *m/z* 409, the $^{0,2}A_5$-H$_2$O fragment ion at *m/z* 774, and the $^{0,2}A_5$ fragment ion at *m/z* 792, identified an oligosaccharide with Fuc-Hex-4HexNAc-Hex-4HexNAc-Hex-4Hex sequence (*i.e.* a blood group H type 2 heptasaccharide (Fucα2Galβ4Glc NAcβ3Galβ4GlcNAcβ3Galβ4Glc)).

A molecular ion at *m/z* 1509 corresponds to an oligosaccharide with three Fuc, two HexNAc, and four Hex. Here the MS^2 spec-
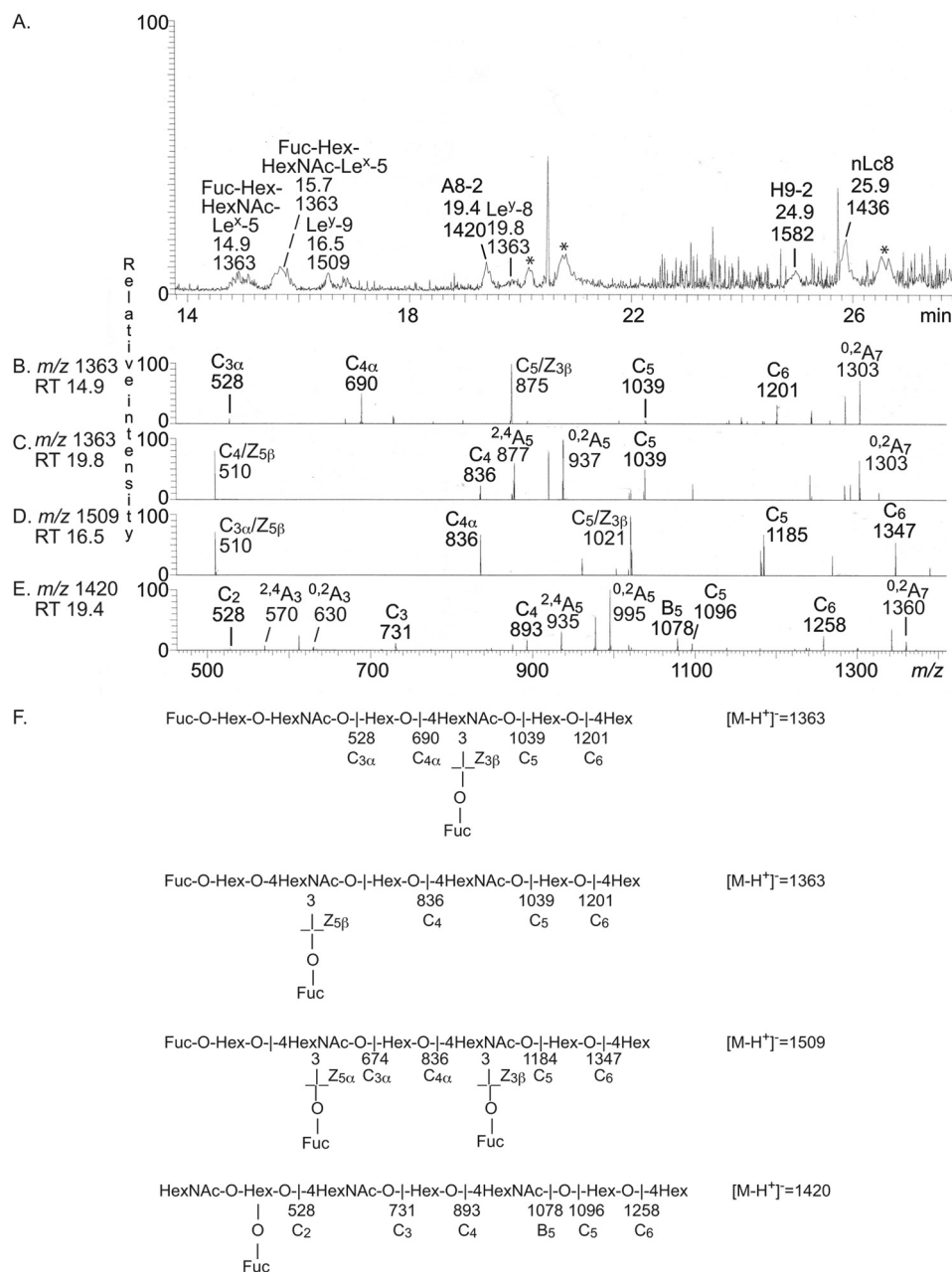
Figure 8. LC-ESI/MS (*m/z* 1300–2000) of the complex oligosaccharides obtained by digestion of the slow-migrating nonacid glycosphingolipid fraction p-IV-f from human blood group A(Rh+)p stomach with *Rhodococcus* endoglycoceramidase II. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides 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 16.5 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 Fucα2Galβ3/4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Fuc-Hex-HexNAc-Le^x-5*), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y-9*), GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*A8-2*), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*Le^y-8*), Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*H9-2*), and Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*nLc8*). *, nonoligosaccharide contaminants.

trum obtained (Fig. 7*E*) had two C/Z$_\beta$ 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$_{3\alpha}$), *m/z* 836 (C$_{4\alpha}$), *m/z* 1185 (C$_5$), and *m/z* 1347 (C$_6$). Collectively, these spectral features allowed a tentative identification of a Le^y nonasaccharide (Fucα2 Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glc).

In a final attempt to characterize the complex glycosphingo-lipids 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. 8*A*). MS² of the ion at *m/z* 1420 identified a blood group A type 2 octasaccharide (GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4 GlcNAcβ3Galβ4Glc) (Fig. 8*E*), and MS² of the ion at *m/z* 1509 identified a Le^y nonasaccharide (Fucα2Galβ4(Fucα3)GlcNAcβ3 Galβ4(Fucα3)GlcNAcβ3Galβ4Glc) (Fig. 8*D*), as above. A neolactooctasaccharide (Galβ4GlcNAcβ3Galβ4GlcNAcβ3

Galβ4GlcNAcβ3Galβ4Glc) was characterized by MS^2 of the ion at *m/z* 1436, and a blood group H nonasaccharide (Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4GlcNAcβ3 Galβ4Glc) was characterized by MS^2 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 MS^2 spectrum of the ion at retention time 14.9 min (Fig. 8*B*) had a series of C-type fragment ions (C$_{3\alpha}$ at *m/z* 528, C$_{4\alpha}$ at *m/z* 690, C$_5$ at *m/z* 1039, and C$_6$ 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 glycosidic cleavage of the 3-linked branch at C$_5$ and Z$_{3\beta}$ (21) (*i.e.* a fragmentation characteristic for an internal 4-linked GlcNAc substituted with a Fuc at 3-position) and thus again demonstrated an internal Le^x determinant. Taken together, these spectral features thus indicated an octasaccharide with Fucα2Galβ3/4GlcNAcβ3 Galβ4(Fucα3)GlcNAcβ3Galβ4Glc sequence.

MS^2 of the ion at *m/z* 1363 eluting at 19.8 min (Fig. 8*C*) gave a C$_4$/Z$_{5\beta}$ 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 (C$_4$) and *m/z* 1039 (C$_5$), and a ^{2,4}A$_5$ ion at *m/z* 877 along with a ^{0,2}A$_5$ ion at *m/z* 937. Taken together, this demonstrated a Le^y octasaccharide (Fucα2Galβ4(Fucα3)-GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc).

Thus, several different complex Le^x and Le^y glycosphingolipids were characterized in fraction p-IV-f. Le^x and Le^y 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 neolactohexaosylceramide, the Le^b hexaosylceramide, 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 stomachs was examined (Fig. 9).

The *H. pylori* strain 26695 bound in the tetraosylceramide region in fractions P-III and p-III (Fig. 9*B*, *lanes 1* and *3*). The structural characterization of fractions P-III and p-III 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. 9*C*, *lanes 2* and *4*). The same binding pattern was obtained with the recombinant truncated adhesin domain of BabA (BabA$_{547}$) (Fig. 9*D*, *lanes 2* and *4*). Ligands for the BabA-mediated binding of the J99 strain to these slow-migrating glycosphingolipids were the H type 1 pentaosylceramide, the Le^b hexaosylceramide, and the A type 1/ALe^b heptaosylceramide identified in fraction P-IV and the Le^b hexaosylceramide and A type 1/ALe^b 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 lec-
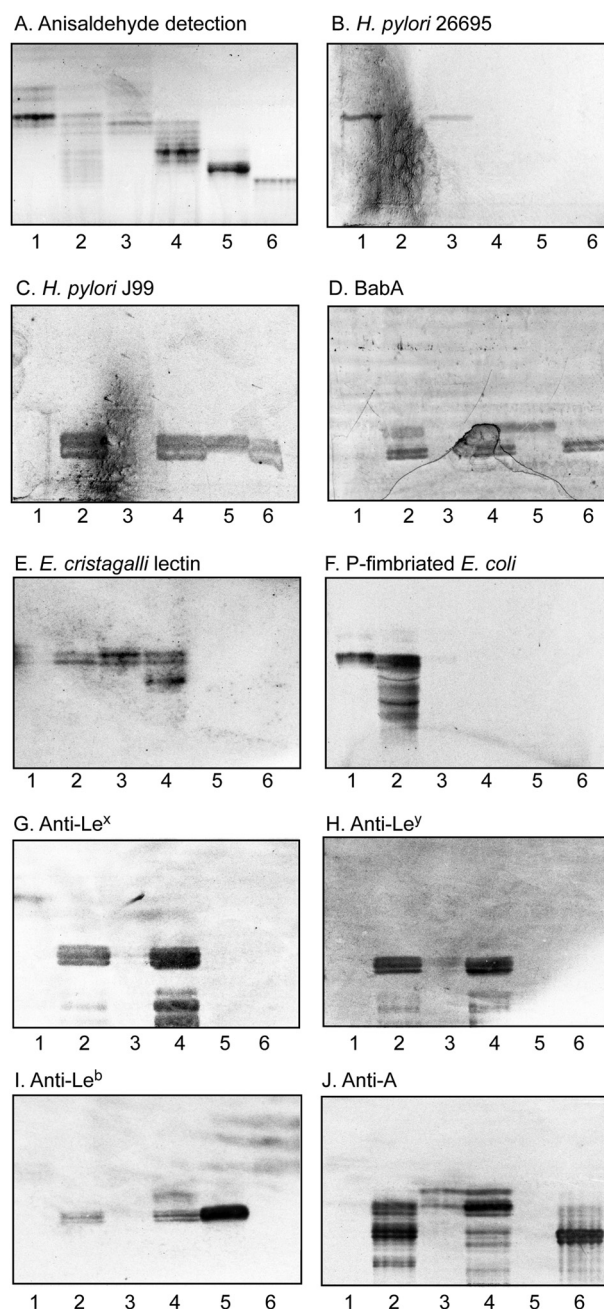

Figure 9. Characterization of the slow-migrating nonacid glycosphingolipids of human blood group A(Rh+)P and A(Rh+)p stomachs by binding of antibodies, lectins, adhesin, and bacteria. *A–J*, thin-layer chromatogram after detection with anisaldehyde (*A*) and autoradiograms obtained by binding of *H. pylori* strain 26695 (*B*), *H. pylori* strain J99 (*C*), BabA$_{547}$ (*D*), *E. cristagalli* lectin (*E*), P-fimbriated *E. coli* strain 291–15 (*F*), and monoclonal antibodies directed against the Le^x determinant (*G*), the Le^y determinant (*H*), the Le^b determinant (*I*), and the blood group A determinant (*J*). 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*, fraction P-III, 4 μg; *lane 2*, fraction P-IV, 4 μg; *lane 3*, fraction p-III, 4 μg; *lane 4*, fraction p-IV, 4 μg; *lane 5*, reference Le^b hexaosylceramide (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 4 μg; *lane 6*, reference ALe^b/A type 1 heptaosylceramide (GalNAcα3(Fucα2)Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer), 4 μg.

tin gave a binding double band in the tetraosylceramide region in fractions P-IV, p-III, and p-IV (Fig. 9*E*, *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. 9*E*, *lane 4*), most likely the blood group H type 2 heptaosylceramide (Fucα2Galβ4 GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer). The binding of Galα4Gal-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. 9*F*, *lanes 1 and 2*) supported the presence of globotriaosylceramide (Galα4Galβ4Glcβ1Cer) and globotetraosylceramide (GalNAcβ3Galα4-Galβ4Glcβ1Cer) in these fractions, whereas the binding of anti-Le^x antibodies in the pentaosylceramide region in fractions P-IV and p-IV (Fig. 9*G*, *lanes 2 and 4*) was in line with the presence of Le^x pentaosylceramide (Galβ4(Fucα3)GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1 Cer). Compounds in the hexaosylceramide region in fraction P-IV and p-IV were recognized by the anti-Le^y and anti-Le^b antibodies (Fig. 9 (*H* and *I*), *lanes 2 and 4*), supporting the presence of Le^y (Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) and Le^b (Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer) hexaosylceramides. The anti-Le^x and anti-Le^y antibodies also bound to slow-migrating glycosphingolipids in fractions P-IV and p-IV, indicating complex Le^x and Le^y 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. 9*J*, *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$^+$]$^{2−}$ ion at *m/z* 868. MS^2 of these ions identified globotriaosylceramide with d18:1-16:0 (*m/z* 1022); globotetraosylceramide and neolactotetra/lactotetraosylceramide with d18:1-16:0 (*m/z* 1225); H, Le^a, or Le^x pentaosylceramide with d18:1-h16:0 (*m/z* 1387); and Le^b or Le^y hexaosylceramide with d18:1-h16:0 (*m/z* 1533), as exemplified in Fig. S2 (*C–E*). Dihexosylceramide with d18:1-h24:0 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 d18:1-16:0. Finally, a blood group A heptaosylceramide with d18:1-h16:0 was characterized by MS^2 of the [M-2H$^+$]$^{2−}$ ion at *m/z* 868 in the nonacid fraction from the A(Rh+)P stomach (Fig. S2*F*).

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 d18:1-16:0 and d18:1-24:1, 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^x

pentaosylceramide and Le^b or Le^y hexaosylceramide, as above, in both cases with d18:1-h16:0.

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. S1*A*) 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^b, Le^y, and blood group A type 2 hexasaccharides 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^y) (*m/z* 1201) and an H type 2 heptasaccharide (*m/z* 1217) in both samples. The molecular ion profile from case 3 (Fig. 10*C*) 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. 10*D*) 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
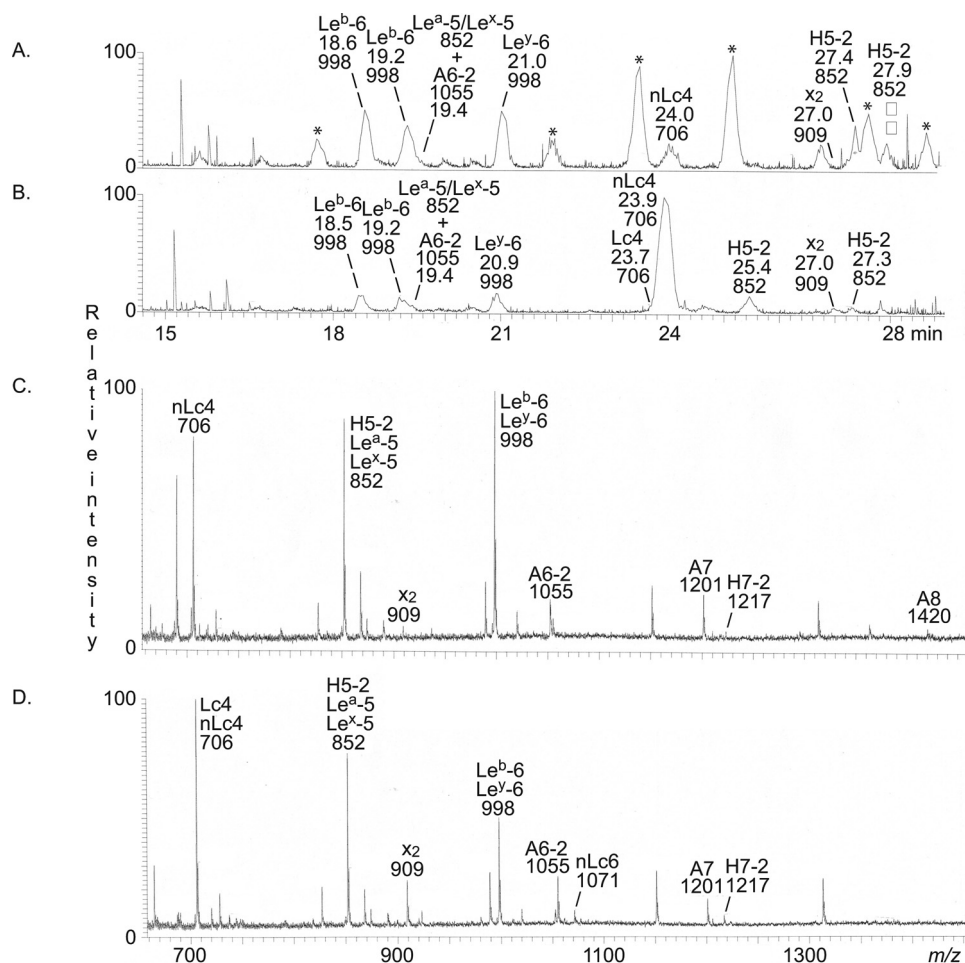
Figure 10. LC-ESI/MS of the oligosaccharides obtained from the total nonacid glycosphingolipid fractions from human gastric mucosa by hydrolysis with endoglycoceramidase II from *Rhodococcus* spp. *A*, base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained from human from human gastric mucosa blood group A(Rh+)P (case 3). Peaks marked with an *asterisk* are noncarbohydrate contaminants. *B*, base peak chromatogram from LC-ESI/MS of the oligosaccharides obtained from human from human gastric mucosa blood group A(Rh+)P (case 4). *C*, molecular ion profile from LC-ESI/MS of from human gastric mucosa blood group A(Rh+)P (case 3). Peaks marked with an *asterisk* are noncarbohydrate contaminants. *D*, molecular ion profile from LC-ESI/MS of from human gastric mucosa blood group A(Rh+)P (case 4). The identification of oligosaccharides was based on their retention times, determined molecular masses, and subsequent MS^2 sequencing. The oligosaccharides identified in the chromatograms were Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^b^-6*), Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^x^-5*), GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glc (*A6-2*), Galβ3(Fucα4)GlcNAcβ3Galβ4Glc (*Le^a^-5*), Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glc (*Le^y^-6*), Galβ4GlcNAcβ3Galβ4Glc (*nLc4*), GalNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*x₂*), Fucα2Galβ4GlcNAcβ3Galβ4Glc (*H5-2*), Fucα2Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*H7-2*), Galβ3GlcNAcβ3Galβ4Glc (*Lc4*), GalNAcα3(Fucα2)Galβ3/4(Fucα4/3)GlcNAcβ3Galβ4Glc (*A7*), Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*nLc6*), and GalNAcα3(Fucα2)Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glc (*A8*).

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* generalist 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- to heptaosylceramides. 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 Le^b^ hexaosylceramide and the A type 1/ALe^b^ heptaosylceramide 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 x₂ 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-

amide, in the A(Rh+)p stomach sample. This is due to lack of the α4-galactosyltransferase converting lactosylceramide to globotriaosylceramide (29). In a previous work, we found that the major dihexosylceramide in the human stomach is galabiaosylceramide (Galα4Galβ1Cer) (18). Here, there was no binding of Galα4Gal recognizing P-fimbriated *E. coli* in the dihexosylceramide region of the stomach sample from the blood group p individual, demonstrating that also galabiaosylceramide 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 immunohistochemistry 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 ALe[b]) are mainly found in the foveolar epithelium, whereas ABH and Le blood group determinants based on type 2 core (*e.g.* Le[y] and ALe[y]) are found both in the foveolar epithelium and in deep gastric glands.

Binding of *H. pylori* to neolactoglycosphingolipids has been characterized, and here the optimal binding sequence is GlcNAcβ3Galβ4GlcNAc, whereas binding to neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer), which lacks the terminal β3-linked GlcNAc, is relatively weak (26). This is in agreement with the nonbinding of *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 *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 human small and large intestine, where the diglycosylceramides, globotriaosylceramide, and globotetraosylceramide are confined to the nonepithelial 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 *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[b] antigens on their epithelial surfaces, because the precursor of the Le[b] sequence (*i.e.* the H type 1 sequence) is not formed due to lack of a functional FUT2 enzyme (39). However, in individuals with little or no Le[b] on the gastric epithelium, an enhanced *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 *H. pylori* SabA-binding gangliosides as Neu5Acα3-

neolactohexaosylceramide (Neu5Acα3Galβ4GlcNAcβ3Galβ4-GlcNAcβ3Galβ4Glc1Cer) and Neu5Acα3-neolactooctaosylceramide (Neu5Acα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4-GlcNAcβ3Galβ4Glcβ1Cer), which thus might function as adhesion factors for SabA-expressing *H. pylori*.

The association between ABO blood groups and *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 *H. pylori* infection, whereas other focus on different *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 *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 *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 *H. pylori* infection rates by secretor status (*i.e.* secretor status and *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 *H. pylori* to the human gastric epithelium. Identified ligands for BabA-mediated binding of *H. pylori* were the Le[b] hexaosylceramide, the H type 1 pentaosylceramide, and the A type 1/ALe[b] heptaosylceramide, and additional characterized *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 *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 *H. pylori* to AGS cells is

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 Le[b] 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 stomachs 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-IVa–e), and six fractions were obtained from fraction p-IV (denoted fractions p-IVa–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 scraped 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 Le[b] determinant, this strain binds to the blood group A or B type 1 and ALe[b] or BLe[b] determinants) (14). The *H. pylori* strain 26695 lacks Le[b]-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 1×10^8 cfu/ml in PBS. The specific activities of the suspensions were ~1 cpm per 100 *H. pylori* organisms.

Recombinant BabA

A truncated BabA domain (BabA$_{547}$), 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-

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J. Biol. Chem. (2018) 293(44) 17248–17266 **17263**

Table 4
Monoclonal antibodies used in chromatogram binding assays

Antibodies	Clone	Manufacturer	Dilution	Isotype
Anti-Lewis[x]	P12	Calbiochem	1:200	IgM
Anti-Lewis[y]	F3	Calbiochem	1:200	IgM
Anti-Lewis[b]	BG-6/T218	Signet/Covance	1:100	IgM
Anti-blood group A	HE-195	Sigma-Aldrich	1:1000	IgM

isobutylmethacrylate (Aldrich). After drying, the chromatograms were soaked in PBS containing 2% BSA (w/v), 0.1% NaN$_3$ (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 ($2–5 \times 10^6$ 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 ^{125}I-labeled monoclonal anti-mouse antibodies (Z0259; DakoCytomation Norden A/S) for detection.

Binding of ^{125}I-labeled *E. cristagalli* lectin (Sigma-Aldrich), ^{125}I-labeled *S. tuberosum* lectin (Sigma-Aldrich), ^{125}I-labeled BabA$_{547}$, and ^{35}S-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 BabA$_{547}$ 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 oligosaccharide-containing upper phase thus obtained was separated from detergent on a Sep-Pak QMA cartridge (Waters). The eluant containing the oligosaccharides was dried under nitrogen and under vacuum.

The glycosphingolipid-derived oligosaccharides were resuspended in 50 μl of water and analyzed by LC-ESI/MS as described (20). The oligosaccharides were separated on a column (100 $\times$ 0.250 mm) packed in-house with 5-μm porous graphite particles (Hypercarb, Thermo-Hypersil, Runcorn, UK). 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 ¹⁄₁₆-inch microvolume-T (0.15-mm bore) (Vici AG International, Schenkon, Switzerland) by a 50 cm $\times$ 50-μm inner diameter fused silica capillary before the injector of the autosampler, allowing ~3–5 μl/min through the column. The oligosaccha-

rides (3 μl) were injected onto the column and eluted with an acetonitrile gradient (A: 10 mM ammonium bicarbonate; B: 10 mM ammonium bicarbonate in 80% acetonitrile). The gradient (0–45% B) was eluted for 46 min, followed by a wash step with 100% B and equilibration of the column for 24 min. A 30 cm $\times$ 50-μm inner diameter fused silica capillary was used as a transfer line to the ion source.

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 MS^2 scans of the three most abundant ions in each scan (2 microscans, maximum 100 ms, target value of 10,000). The threshold for MS^2 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 Glycoworkbench tool (version 2.1) and by comparison of retention times and MS^2 spectra of oligosaccharides from reference glycosphingolipids (20).

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 $\times$ 0.250-mm column, packed in-house with 5-μm polyamine 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 ¹⁄₁₆-inch microvolume-T (0.15-mm bore) (Vici AG International, Schenkon, Switzerland) by a 50 cm $\times$ 50-μm inner diameter fused silica capillary before the injector of the autosampler, allowing ~2–3 μl/min through the column. Samples were eluted with an aqueous gradient (A (100% acetonitrile) to B (10 mM ammonium bicarbonate)). The gradient (0–50% B) was eluted for 40 min, followed by a wash step with 100% B, and equilibration of the column for 20 min. The samples were analyzed in negative ion mode on an LTQ linear quadrupole ion trap mass spectrometer (Thermo Electron, San José, CA), with an IonMax standard ESI source 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 500–1800, two microscans, maximum 100 ms, target value of 30,000) was performed, followed by data-dependent MS^2 scans (two microscans, maximum 100 ms, target value of 10,000) with normalized collision energy of 35%, isolation win-

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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