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Glycosphingolipid Binding Specificities of *Neisseria meningitidis* and *Haemophilus influenzae*: Detection, Isolation, and Characterization of a Binding-Active Glycosphingolipid from Human Oropharyngeal Epithelium[1]

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The glycosphingolipid binding specificities of *Haemophilus influenzae* and *Neisseria meningitidis* were investigated as to the binding of radiolabeled bacteria to glycosphingolipids on thin-layer chromatograms. Thereby, similar binding profiles, for the binding of the two bacteria to lactosylceramide, isoglobotriaosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, lactotetraosylceramide, neolactotetraosylceramide, and sialyl-neolactohexaosylceramide, were obtained. On a closer view the binding preferences of the bacteria could be differentiated into three groups. The first specificity is recognition of lactosylceramide. The second specificity is binding to gangliotriaosylceramide and gangliotetraosylceramide, since conversion of the acetamido group of the *N*-acetylgalactosamine of gangliotriaosylceramide and gangliotetraosylceramide to an amine prevented the binding of the bacteria, and thus the binding to these two glycosphingolipids represents a separate specificity from lactosylceramide recognition. Preincubation of *H. influenzae* with neolactotetraose inhibited the binding to neolactotetraosylceramide, while the binding to lactosylceramide, gangliotetraosylceramide, or lactotetraosylceramide was unaffected. Thus, the third binding specificity is represented by neolactotetraosylceramide, and involves recognition of other neolacto series glycosphingolipids with linear *N*-acetyllactosamine chains, such as sialyl-neolactohexaosylceramide. The relevance of the detected binding specificities for adhesion to target cells was addressed as to the binding of the bacteria to glycosphingolipids from human granulocytes, epithelial cells of human nasopharyngeal tonsils and human plexus choroideus. Binding-active neolactotetraosylceramide was thereby detected in human granulocytes and the oropharyngeal epithelium.

Key words: bacterial adhesion, glycosphingolipid receptor, *Haemophilus influenzae*, *Neisseria meningitidis*, oropharyngeal epithelium.

Acute bacterial meningitis is a worldwide health problem with severe morbidity, significant mortality, and a high incidence of residual sequelae in survivors (*1-4*). Two of the most important causative agents for this disease are the capsulated bacteria *Neisseria meningitidis* (serogroups A, B, and C) and *Haemophilus influenzae* type b. The port of entry for both bacteria is the epithelial cells of the upper respiratory tract. After hematogenous dissemination they eventually cause inflammation of the pia and the arachnoid, which is manifested as purulent meningitis. *N. meningitidis* (serogroups A, B, and C) and *H. influenzae* type b also cause other invasive infections, such as septicemia, and *H.*

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[3] Abbreviations: CFU, colony forming units; EI, electron ionization; FAB, fast atom bombardment; Hex, hexose; HexN, *N*-acetylhexosamine; LPS, lipopolysaccharide; PBS, phosphate-buffered saline. 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 denotes sphingosine (1,3-dihydroxy-2-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecene). The glycosphingolipid nomenclature follows the recommendations by the IUPAC-IUB Commission on Biochemical Nomenclature [CBN for Lipids: *Eur. J. Biochem.* (1977) **79**, 11-21; *J. Biol. Chem.* (1982) **257**, 3347-3351; *J. Biol. Chem.* (1987) **262**, 13-18; and *Eur. J. Biochem.* (1997) **243**, 9]. It is assumed that Gal, Glc, GlcNAc, GalNAc, NeuAc and NeuGc are of the D-configuration, Fuc of the L-configuration, and all sugars present in the pyranose form.

influenzae type b is the major pathogen involved in acute epiglottitis. Non-typable *H. influenzae* rarely causes invasive diseases, but is a common etiologic agent of respiratory tract infections, such as otitis, sinusitis, and pneumonia.

Both *H. influenzae* and *N. meningitidis* are capable of inducing an oxidative burst reaction in human granulocytes, which is enhanced by opsonising antibodies and complement proteins (*5*).

The selectivity for certain hosts, tissues, and cells, found for both commensal and pathogenic bacteria, is partly due to the expression of bacterial adhesins with different receptor-binding properties. The distribution of the eukaryotic cell receptors to which the bacterial adhesins bind is a determinant of which site the bacterium will successfully colonize (*6*). A large number of microbial carbohydrate receptors have been identified (*7-9*). Among the most well-known examples are the mannose receptors of type 1 fimbriae of enterobacteriae (*10*), and the Galα4Gal receptors of P-fimbriated *Escherichia coli* (*11-13*). Different detailed carbohydrate binding specificities have been described for type 1 fimbriae and P-fimbriae (*10, 14*).

H. influenzae and *N. meningitidis* have the same target cells. However, while no information on carbohydrate recognition by *N. meningitidis* is available, several different glycosphingolipid binding specificities of *H. influenzae* have been reported. Thus, these bacteria bind to gangliotriaosylceramide and gangliotetraosylceramide (*15, 16*). The fimbriae-mediated adhesion of *H. influenzae* to human erythrocytes and oropharyngeal epithelial cells is inhibited by gangliosides, such as $GM3^3$, GM2, GM1, and GD1a (*17*). In addition, the binding of *H. influenzae* to minor unidentified gangliosides of HEp-2 cells has been reported (*18*). Recently, it was described that heat shock treatment induces the binding of *H. influenzae* to sulfatide (*19*).

The aim of the present study was twofold. The first intention was to compare the glycosphingolipid binding properties of *H. influenzae* and *N. meningitidis*, and the second aim was to investigate potential binding-active glycoconjugates in the target tissues of the bacteria. On the binding of radiolabeled *H. influenzae* and *N. meningitidis* to a large number of glycosphingolipids on thin-layer chromatograms it was found that both bacteria bound to lactosylceramide, isoglobotriaosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, lactotetraosylceramide, neolactotetraosylceramide, and sialylneolactohexaosylceramide. By means of binding assays utilizing chemically modified glycosphingolipids, and by inhibition experiments, the binding-active compounds were classified into three groups, which should correspond to three separate adhesins.

Glycosphingolipids isolated from epithelial cells of human oropharynx and human choroideal plexus, two models of the natural target tissues of the bacteria, were examined for the presence of binding-active glycosphingolipids. A binding-active non-acid tetraglycosylceramide was isolated from human oropharyngeal epithelium by affinity chromatography on immobilized *Erythrina cristagalli* lectin, and characterized as neolactotetraosylceramide.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Labeling—During this study four *N. meningitidis* strains were used in parallel. The strains were isolated from clinical specimens and were serogrouped by the co-agglutination technique (*20*). There was one strain each of *N. meningitidis* serogroups A [non-typable (nt), non-subtypable (nst), 884], B (serotype 15, nst, 9-076/86), and C (nt, nst, gbg 3271) isolated from blood or cerebrospinal fluid of patients with septicemia and/or meningitis, and one strain of *N. meningitidis* serogroup 29E (nt, nst, 9-070/86) isolated from a nasopharyngeal specimen of a healthy carrier.

The four *H. influenzae* strains used were obtained from clinical specimens. They were serotyped by agglutination with hyperimmune serum from rabbits immunized with freshly cultivated bacteria (*21*). Two strains of *H. influenzae* serotype b (457-92 and 055-92) isolated from cerebrospinal fluid of patients with meningitis, and two strains of non-typable *H. influenzae* (75-00555 and 3-09962) isolated from nasopharyngeal specimens were used.

Hemagglutinating activity was examined by mixing a 3% (v/v) suspension of human blood group A erythrocytes in phosphate-buffered saline (PBS), pH 7.3, with a 1/2 or 1/4 volume of a stock solution containing 1×10^{10} bacteria in PBS. Human erythrocytes were agglutinated by all four *H. influenzae* strains, while the four *N. meningitidis* strains were non-hemagglutinating.

The *N. meningitidis* strains were cultured on agar plates containing non-selective gonococcal medium (*22*) and the *H. influenzae* strains on hematinagar. The plates were supplemented with 50 μCi ^{35}S-methionine (Amersham, UK) in 0.5 ml PBS and incubated under 4-6% CO_2 at 37°C overnight. The bacteria were then scraped off the plates and washed three times with PBS. For binding assays the bacteria were suspended in PBS to approximately 1×10^8 CFU/ml. The specific activities of the suspensions were approximately 1 cpm per 100 bacterial cells.

Preparation of Outer Membrane Proteins—Outer membrane proteins of *N. meningitidis* serogroups B and 29E, *H. influenzae* serotype b (457-92), and non-typable *H. influenzae* (75-00555) were prepared as described by Tam *et al.* (*23*), with minor modifications. Briefly, the bacteria were harvested and suspended in 30 ml cold (+4°C) 0.17 M ethanolamine solution containing 29 mM EDTA. The suspension was homogenized using a Turrax Homogenizer (Mod. X1020, Intern. Laborat. App. GmbH 7801, Dottingen, Germany) at 30,000 rpm/min for 30 min at 40°C. The bacterial debris was pelleted by centrifugation two times for 10 min at $12,000 \times g$, and once for 25 min at $30,000 \times g$, and the resultant pellet was discarded. Finally, the outer membrane proteins in the supernatant were pelleted by centrifugation for 1 h at $143,000 \times g$. LPS was separated from the proteins by homogenization in 30 ml 3% (w/v) sodium deoxycholate solution, followed by centrifugation for 4 h at $150,000 \times g$. The protein pellet was suspended in PBS, and then the protein concentration was determined by spectrophotometry of the Ponceau-S stained proteins (*24*).

From each extract an aliquot of approximately 100 μg protein was taken, and labeled with ^{125}I by the Iodogen method (*25*) to a specific activity of $2\text{-}5 \times 10^3$ cpm/μg.

Thin-Layer Chromatography—Thin-layer chromatography was performed on glass- or aluminum-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany), using chloroform/methanol/water (60:35:8, by volume) as the solvent system. Chemical detection was accomplished with

anisaldehyde (*26*).

Chromatogram Binding Assay—The chromatogram binding assay was performed as described previously (*27*). Briefly, chromatograms with separated mixtures of glycosphingolipids (20-40 μg/lane) or pure glycosphingolipids (1-4 μg/lane) were dipped in 0.3-0.5% (w/v) polyisobutylmethacrylate (Plexigum P28, Röhm, GmbH, Darmstadt, Germany) in diethylether/*n*-hexane (1:5, by volume) for 1 min, and then air-dried. Blocking of non-specific binding sites was performed by immersing the plates in PBS containing either 2% bovine serum albumin (w/v; Solution 1), 2% bovine serum albumin (w/v), and 0.1% Tween 20 (w/v; Solution 2), or 2% bovine serum albumin (w/v) and 0.2% deoxycholic acid (w/v; Solution 3), for 2 h at room temperature. Thereafter, suspensions of radiolabeled bacteria (diluted in PBS to 1×10^8 CFU/ml and $1\text{-}5\times10^6$ cpm/ml) or ^{125}I-labeled bacterial surface proteins [diluted in PBS containing 0.1% Tween 20 (w/v) to approximately 2×10^6 cpm/ml] were gently sprinkled over the chromatograms, followed by incubation for 2 h at room temperature. After washing six times with PBS and drying, the thin-layer plates were autoradiographed for 12-48 h using XAR-5 X-ray films (Eastman Kodak, Rochester, NY).

Binding of ^{125}I-labeled lectin from *Erythrina cristagalli* to glycosphingolipids on thin-layer plates was performed as described (*28*).

Reference Glycosphingolipids—Total acid and non-acid glycosphingolipid fractions, from the sources given in Table I, were isolated as described previously (*29*). The pure glycosphingolipids used in the binding studies were isolated by repeated chromatography of native glycosphingolipids or acetylated derivatives (*30*) on silicic acid columns (Iatrobeads 6RS-8060, Iatron Laboratories, Tokyo) or by HPLC on silicic acid columns. The isolated glycosphingolipids were characterized by mass spectrometry (*31*), proton NMR spectroscopy (*32-35*), and degradation studies (*36, 37*).

De-N-Acylation of Glycosphingolipids—The hexosamine residues in selected glycosphingolipids were de-*N*-acylated by treatment with anhydrous hydrazine, as described (*38*). The glycosphingolipids (200 μg) were dissolved in 300 μl of freshly distilled anhydrous hydrazine (Pierce, Rockford, IL) by sonication for 30 s, and then the reaction was allowed to proceed for 72 h at 76°C. The hydrazine was subsequently removed using N_2(g) at 40°C, followed by two cycles of redissolution in toluene and evaporation. Finally, the sample was desalted on an C18 Extract-Clean column (Alltech Assoc., Deerfield, IL). The identity of the reaction products was verified by negative ion FAB mass spectrometry and proton NMR spectroscopy.

Inhibition Studies—The inhibitory activities of lactose (J.T. Baker Chem., Phillipsburg, NJ), lactotetraose and neolactotetraose (Glycorex, Lund, Sweden) were determined by preincubating ^{35}S-labeled *H. influenzae* with each substance (1 mg/ml in PBS) for 1 h at room temperature. The suspensions were thereafter utilized in the chromatogram binding assay.

Isolation of Glycosphingolipids from Human Granulocytes, Plexus Choroideus, and Oropharyngeal Epithelium—Total acid and non-acid glycosphingolipids were isolated from human granulocytes as described (*39*). Lactosylceramide and neolactosylceramide were isolated from 110 mg of total non-acid granulocyte glycosphingolipids by HPLC on a 2.12×25 cm column of silica (Kromasil 5 Silica, 5 μm particles; Phenomenex, Torrance, CA). The column was equilibrated in chloroform/methanol/water (80:20:1, by volume; solvent A) and eluted (4 ml/min) with a linear gradient of chloroform/methanol/water (40:40:12, by volume; solvent B) in solvent A. Aliquots of each 4 ml fraction were analyzed by thin-layer chromatography and anisaldehyde staining. After pooling, 34.4 mg of pure lactosylceramide and 1.3 mg of pure neolactotetraosylceramide were obtained.

Human plexus choroideus were collected at autopsy from patients without intracranial neoplastic disease. After dissection the material was carefully washed with 0.9% NaCl (w/v), and the histopathological features of the preparation were verified by light microscopy. The tissues from three individuals were pooled and lyophilized, giving a dry weight of 1.15 g.

Normal oropharyngeal epithelium surrounding the palatine tonsils was dissected after tonsillectomy for upper airway obstruction. The epithelial cell preparations were analyzed by light microscopy to ensure that they did not contain subepithelial components. The preparations from seven individuals were pooled, and after lyophilization a dry weight of 1.24 g was obtained.

Total acid and non-acid glycosphingolipid fractions were isolated as described (*29*). Briefly, the lyophilized material was extracted in two steps in a Soxhlet apparatus with chloroform and methanol (2:1 and 1:9, by volume, respectively). The extract was subjected to mild alkaline methanolysis and dialysis, followed by separation on a silicic acid column. Acid and non-acid glycolipids were separated by chromatography on a DEAE-cellulose column. In order to separate the non-acid glycolipids from alkali-stable phospholipids, this fraction was acetylated and then separated on a second silicic acid column, followed by deacetylation and dialysis. After final purification on DEAE-cellulose and silicic acid columns, 21.4 mg acid glycosphingolipids (18.6 mg/g dry weight) and 4.3 mg non-acid glycosphingolipids (3.7 mg/g dry weight) were obtained from the pooled plexus choroideus material. The pooled epithelial cell preparations from human oropharyngeal epithelium yielded 10.6 mg acid glycosphingolipids (8.5 mg/g dry weight) and 6.7 mg non-acid glycosphingolipids (5.4 mg/g dry weight).

The non-acid glycosphingolipid fraction from human oropharyngeal epithelium (5 mg) was subsequently separated on a silicic acid column, eluted stepwise with increasing concentrations of methanol in chloroform. The fractions obtained were examined for *E. cristagalli*-binding activity, using the chromatogram binding assay, and the binding-active fractions were pooled, giving 1.1 mg.

Affinity Chromatography of Glycosphingolipids Using Immobilized Erythrina cristagalli Lectin—The pooled glycosphingolipid fraction containing the *E. cristagalli*-binding compound from human oropharyngeal epithelium was separated on a column of *E. cristagalli*-agarose (Vector Laboratories, Burlingame, CA). The column (2 ml) was equilibrated in PBS. The glycosphingolipid sample was loaded on the column in 1 ml PBS, and then left overnight at room temperature. The column was first eluted with $2\times$ 100 ml PBS, and thereafter eluted with 100 ml PBS containing 0.2 M lactose. The three fractions were subjected to Folch partitioning (*40*), and the lower phases obtained were

subsequently dialyzed against distilled water.

Mass Spectrometry—For EI mass spectrometry portions of the isolated glycosphingolipid fractions were permethylated (*41*), or permethylated and reduced with $LiAlH_4$ (*42*). The derivatized samples were analyzed with a JEOL SX-102A mass spectrometer (JEOL, Tokyo) using the in beam technique (*43*). The analyses of both derivatives were performed with an electron energy of 70 eV, a trap current of 300 μA and an acceleration voltage of 10 kV. The temperature was raised from 150 to 410°C, with increases of 10 or 15°C/min.

Negative ion FAB mass spectra of native glycosphingolipids were obtained with a JEOL SX-102A mass spectrometer. The spectra were produced by 8 kV Xe atom bombardment, using triethanolamine (Fluka AG, Buchs, Switzerland) as the matrix.

RESULTS

Chromatogram Binding Assay—During the initial binding studies, mixtures of glycosphingolipids isolated from various species and organs were utilized, in order to expose the bacteria to a large number of potentially binding-active carbohydrate structures. Figure 1 shows the binding of ^{35}S-labeled *H. influenzae* and *N. meningitidis* to glycosphingolipid mixtures. Although most compounds were not recognized by the bacteria, selective binding to some glycosphingolipids was detected. The binding patterns obtained with *H. influenzae* (Fig. 1B) and *N. meningitidis* (Fig. 1C) were very similar to the binding patterns obtained with bacteria classified as "lactosylceramide-binding" (*8*), with binding to the di- and triglycosylceramide regions in

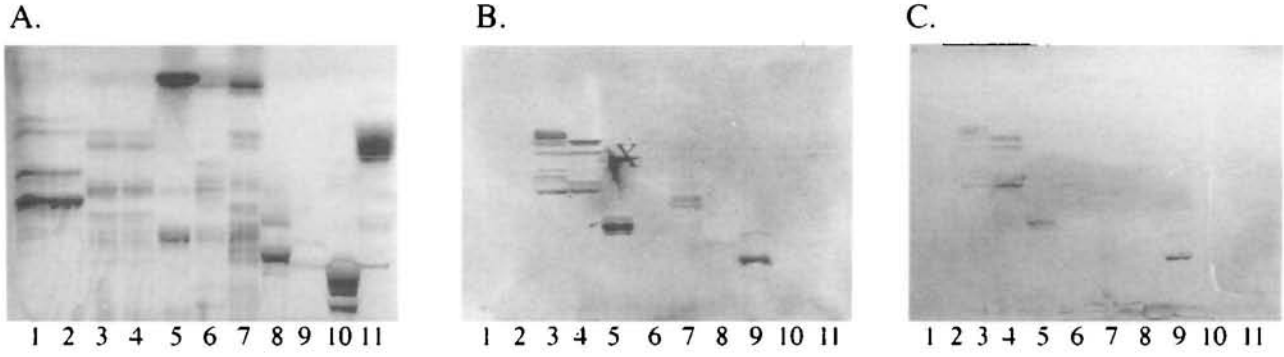

Fig. 1. **Binding of ^{35}S-labeled *Haemophilus influenzae* and *Neisseria meningitidis* to mixtures of glycosphingolipids on thin-layer chromatograms.** The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as the solvent system. The chromatogram in (A) was visualized with anisaldehyde. Duplicate chromatograms were incubated with radiolabeled *H. influenzae* (B) and *N. meningitidis* (C), followed by autoradiography for 12-48 h, as described under "MATERIALS AND METHODS." The lanes contained: (1) non-acid glycosphingolipids of human erythrocytes, blood group AB, 40 μg; (2) non-acid glycosphingolipids of human erythrocytes, blood group O, 40 μg; (3) non-acid glycosphingolipids of dog intestine, 40 μg; (4) non-acid glycosphingolipids of guinea pig intestine, 40 μg; (5) non-acid glycosphingolipids of mouse feces, 40 μg; (6) non-acid glycosphingolipids of epithelial cells of rat intestine, 40 μg; (7) non-acid glycosphingolipids of human meconium, 40 μg; (8) acid glycosphingolipids of human erythrocytes, 40 μg; (9) acid glycosphingolipids of rabbit thymus, 40 μg; (10) calf brain gangliosides, 40 μg; (11) acid glycosphingolipids of human meconium, 40 μg. The spot denoted by X is due to damage to the silica gel.

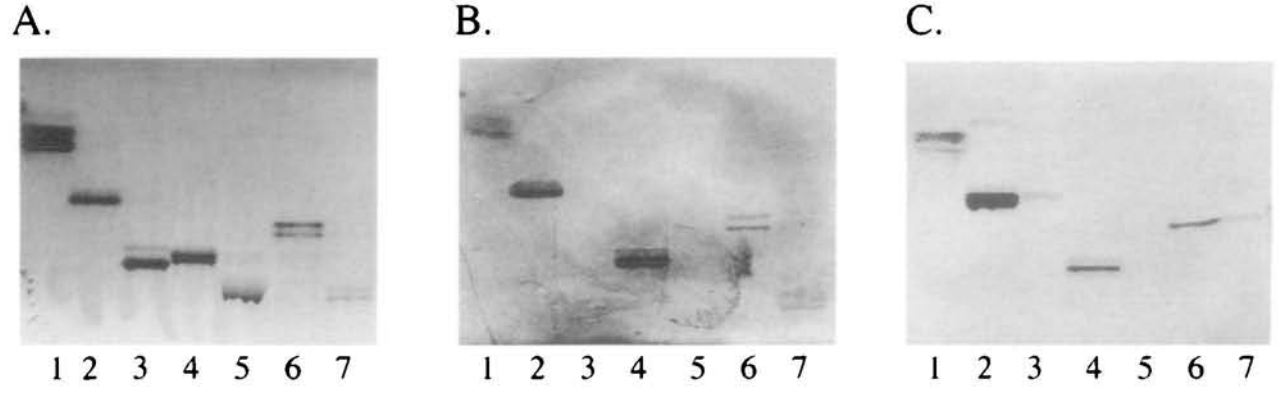

Fig. 2. **Chromatogram binding experiment showing the binding of *Haemophilus influenzae* and *Neisseria meningitidis* to pure glycosphingolipids.** The glycosphingolipids were chromatographed on aluminum-backed silica gel plates and visualized with anisaldehyde (A). Duplicate chromatograms were incubated with radiolabeled *H. influenzae* (B) and *N. meningitidis* (C), followed by autoradiography for 12-48 h, as described under "MATERIALS AND METHODS." The solvent system used was chloroform/methanol/water 60:35:8 (by volume). The lanes contained: (1) Galβ4Glcβ1Cer (lactosylceramide) with phytosphingosine and hydroxy 16:0-24:0 fatty acids isolated from dog intestine, 4 μg; (2) GalNAcβ4Galβ4Glcβ1Cer (gangliotriaosylceramide) from guinea pig erythrocytes, 4 μg; (3) $GalNH_2\beta$4Galβ4Glcβ1Cer, approximately 4 μg; (4) Galβ3GalNAcβ4Galβ4Glcβ1Cer (gangliotetraosylceramide) from mouse feces, 4 μg; (5) Galβ3$GalNH_2\beta$4Galβ4Glcβ1Cer, approximately 4 μg; (6) Galβ4GlcNAcβ3Galβ4Glcβ1Cer (neolactotetraosylceramide) from human granulocytes, 4 μg; (7) Galβ4$GlcNH_2\beta$3Galβ4Glcβ1Cer, approximately 4 μg.

the non-acid glycosphingolipid fractions of dog intestine (lane 3) and guinea pig intestine (lane 4), a slow-migrating compound in the non-acid glycosphingolipid fraction of mouse feces (lane 5), and the tetraglycosylceramide region in the non-acid glycosphingolipid fraction of human meconium (lane 7). In addition, binding to a slow-migrating compound in the acid fraction of rabbit thymus (lane 9) was detected.

To further define the binding characteristics of the two bacteria a number of pure glycosphingolipids were examined by means of the chromatogram binding assay, as exemplified in Fig. 2. The results are summarized in Table I. Thus, both *H. influenzae* and *N. meningitidis* bound to lactosylceramide. The binding to lactosylceramide was only observed when this glycosphingolipid had a ceramide with sphingosine or phytosphingosine and hydroxy fatty acids (Nos. 5 and 6 in Table I, Fig. 2, lane 1, and present in lanes 3 and 4 in Fig. 1), whereas lactosylceramide with sphin-

TABLE I. **Binding of ^{35}S-labeled *Haemophilus influenzae* and *Neisseria meningitidis* to glycosphingolipids on thin-layer chromatograms.**

No. Trivial name	Structure	*H. influenzae*	*N. meningitidis*	Source
Simple compounds				
1. Cerebroside	Galβ1Cer	−	−	Various
2. Cerebroside	Glcβ1Cer	−	−	Various
3. Sulfatide (d18:1-16:0 and 24:0)[b]	SO_3-Galβ1Cer	−	−	Human meconium
4. LacCer (d18:1-16:0-24:0)	Galβ4Glcβ1Cer	−	−	Human granulocytes
5. LacCer (d18:1-h22:0)	Galβ4Glcβ1Cer	+	+	Dog intestine
6. LacCer (t18:0-h16:0-h24:0)	Galβ4Glcβ1Cer	+	+	Rabbit small intestine
7. Isoglobotri (d18:1-h16:0)	Galα3Galβ4Glcβ1Cer	+	+	Dog intestine
8. Globotri (d18:1-16:0 and 24:0)	Galα4Galβ4Glcβ1Cer	−	−	Human erythrocytes
9. Lactotri (d18:1-16:0 and 24:1)	GlcNAcβ3Galβ4Glcβ1Cer	−	−	Malignant melanoma
Ganglioseries				
10. GgO3 (d18:1-16:0 and 24:0)	GalNAcβ4Galβ4Glcβ1Cer	+	+	Guinea pig erythrocytes
11.	$GalNH_2$β4Galβ4Glcβ1Cer	−	−	Guinea pig erythrocytes[c]
12. GgO4 (t18:0-h16:0 and h24:0)	Galβ3GalNAcβ4Galβ4Glcβ1Cer	+	+	Mouse feces
13.	Galβ3$GalNH_2$β4Galβ4Glcβ1Cer	−	−	Mouse feces[c]
14. Fuc-GgO4 (d18:0-h16:0)	Fucα2Galβ3GalNAcβ4Galβ4Glcβ1Cer	−	−	Mouse small intestine
Neolactoseries				
15. Neolactotetra (d18:1-16:0 and 24:1)	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	Human granulocytes
16.	Galβ4$GlcNH_2$β3Galβ4Glcβ1Cer	+	−	Human granulocytes[c]
17.	H5-2 Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human erythrocytes
18. Le^x-5	Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	Dog intestine
19. P1	Galα4Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human erythrocytes
20. B5	Galα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Rabbit erythrocytes
21. x_2	GalNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	Human erythrocytes
22. Le^y-6	Fucα2Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer	−	−	Dog intestine
23. B6-2	Galα3(Fucα2)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human erythrocytes
24. Neolactohexa (d18:1-16:0-24:0)	Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	−	−	Bovine buttermilk
25.	GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	Rabbit thymus[d]
26. Neolactohexa (d18:1-16:0)	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	Rabbit thymus[e]
Lactoseries				
27. Lactotetra	Galβ3GlcNAcβ3Galβ4Glcβ1Cer	+	+	Human meconium
28.	Galβ3$GlcNH_2$β3Galβ4Glcβ1Cer	−	−	Human meconium[c]
29. Le^a-5	Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human meconium
30. Le^b-6	Fucα2Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human meconium
Globoseries				
31. Globotetra (d18:1-16:0 and 24:0)	GalNAcβ3Galα4Galβ4Glcβ1Cer	−	−	Human erythrocytes
32. Forssman (d18:1-16:0 and 224:0)	GalNAcα3GalNAcβ3Galα4Galβ4Glcβ1Cer	−	−	Dog intestine
Gangliosides				
33. GM3	NeuAcα3Galβ4Glcβ1Cer	−	−	Human brain
34. GM1 (d18:1-18:0 and d18:1-20:0)	Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	−	−	Human brain
35. GD1a	NeuAcα3Galβ3GalNAcβ4(NeuAcα3)Galβ4Glcβ1Cer	−	−	Human brain
36. NeuAcα3SPG	NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human erythrocytes
37. NeuAcα6SPG	NeuAcα6Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human meconium
38. NeuGcα3SPG	NeuGcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	−	−	Rabbit thymus
39. NeuAcα3-Le^a	NeuAcα3Galβ3(Fucα4)GlcNAcβ3Galβ4Glcβ1Cer	−	−	Human bile bladder tumor
40. NeuGcα3neolactohexa (d18:1-16:0)	NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4-Glcβ1Cer	+	+	Rabbit thymus

TABLE I. Continued.

No. Trivial name	Structure	*H. influenzae*	*N. meningitidis*	Source
41. NeuAcα3neolactohexa	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	Human placenta
42.	Galβ4GlcNAcβ6(NeuAcα6Galβ4GlcNAcβ3)Galβ4Glcβ1Cer	–	–	Bovine buttermilk
43.	NeuAcα3Galβ4GlcNAcβ6(NeuAcα3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–	Human placenta
44.	Galα3Galβ4GlcNAcβ6(NeuAcα3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–	Bovine erythrocytes
45.	Fucα2Galβ4GlcNAcβ6(NeuAcα3Galβ4GlcNAcβ3)Galβ4GlcNAcβ3Galβ4Glcβ1Cer	–	–	Human erythrocytes

[a]Binding is defined as follows: + denotes significant darkening on the autoradiogram when 4 μg was applied to the thin-layer plate, while – denotes no binding. [b]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 denotes sphingosine (1,3-dihydroxy-2-aminooctadecene) and t18:0 phytosphingosine (1,3,4-trihydroxy-2-aminooctadecene). [c]Glycosphingolipids Nos. 11, 13, 16, and 28 were prepared from Nos. 10, 12, 15, and 27, respectively, by treatment with anhydrous hydrazine, as described under "MATERIALS AND METHODS." [d]Glycosphingolipid No. 25 was prepared from No. 40 by mild acid hydrolysis and subsequent treatment with β-galactosidase. [e]Glycosphingolipid No. 26 was prepared from No. 40 by mild acid hydrolysis.

gosine and non-hydroxy fatty acids (No. 4 in Table I, and present in lanes 1 and 2 in Fig. 1) was consistently non-binding.

Further glycosphingolipids recognized by both bacteria were isoglobotriaosylceramide (No. 7 in Table I, and present in lanes 3 and 4 in Fig. 1), gangliotriaosylceramide (No. 10 in Table I, and Fig. 2, lane 2), gangliotetraosylceramide (No. 12 in Table I, and Fig. 2, lane 4, and present in lane 5 in Fig. 1), neolactotetraosylceramide (No. 15 in Table I, and Fig. 2, lane 6), and lactotetraosylceramide (No. 27 in Table I). No dependence on the ceramide structure for the binding of these tri- and tetraglycosylceramides to occur was found. The binding of *H. influenzae* and *N. meningitidis* to lactotetraosylceramide was, however, only observed when Tween 20 or deoxycholic acid was present in the coating buffer, suggesting that the presence of a detergent was necessary for optimal presentation of the binding epitope in this case.

The only gangliosides recognized by the bacteria were NeuGcα3-neolactohexaosylceramide from rabbit thymus (No. 40 in Table I, and present in lane 9 in Fig. 1) and NeuAcα3-neolactohexaosylceramide from human placenta (No. 41), while no consistent binding to the other gangliosides examined (Nos. 33–39 and 42–45 in Table I, and present in lanes 8–11 in Fig. 1) was observed. However, the hexa- and pentaglycosylceramides obtained on hydrolysis of NeuGcα3-neolactohexaosylceramide (Nos. 26 and 25 in Table I) were also bound by both bacteria, demonstrating that the terminal sialic acid and the penultimate galactose are not necessary for the binding process. The occasional binding of *N. meningitidis* and *H. influenzae* to the x_2 glycosphingolipid (No. 21) was also observed, in agreement with the proposed conformational similarity between the terminal trisaccharides of the x_2 glycosphingolipid and GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (*44*).

Other substitutions of the binding-active compounds were, however, not tolerated. Thus, an α-fucose at the 2-position of the terminal galactose of gangliotetraosylceramide (No. 14 in Table I) or neolactotetraosylceramide (No. 17) abolished the binding. The binding to neolactotetraosylceramide was also abrogated by an α-galactose at the 3- or 4-position of the terminal galactose (Nos. 19 and 20), and by an α-fucose at the 3-position of the *N*-acetylglucosamine (No. 18). Branching of neolactotetraosylceramide with a β-linked Galβ4GlcNAcβ at the 6-position of the internal galactose (No. 24) also abolished the binding of the bacteria.

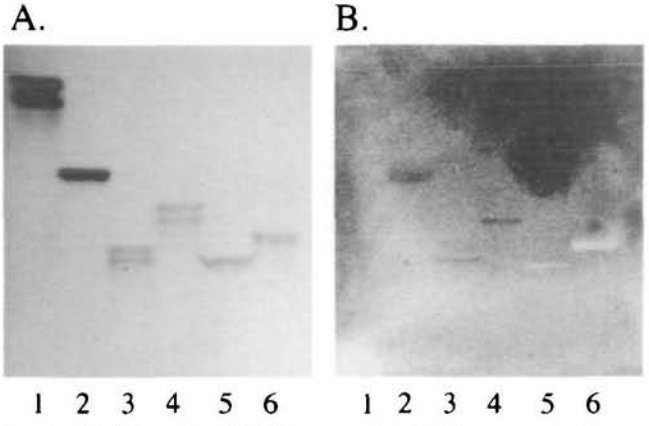

Fig. 3. **Binding of a ^{125}I-labeled extract of outer membrane proteins from *Neisseria meningitidis* to glycosphingolipids on thin-layer chromatograms.** The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as the solvent system. The chromatogram in (A) was visualized with anisaldehyde. A duplicate chromatogram was incubated with ^{125}I-labeled outer membrane proteins from *N. meningitidis* (B), followed by autoradiography for 12 h. The lanes contained: (1) Galβ4Glcβ1Cer (lactosylceramide) with sphingosine and non-hydroxy 16:0 and 24:1 fatty acids from human granulocytes, 4 μg; (2) GalNAcβ4Galβ4Glcβ1Cer (gangliotriaosylceramide) from guinea pig erythrocytes, 4 μg; (3) Galβ3GalNAcβ4Galβ4Glcβ1Cer (gangliotetraosylceramide) from mouse feces, 4 μg; (4) Galβ4GlcNAcβ3Galβ4Glcβ1Cer (neolactotetraosylceramide) from human granulocytes, 4 μg; (5) NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer (NeuAcα3-neolactotetraosylceramide) from human granulocytes, 4 μg; (6) Fucα2Galβ4GlcNAcβ3Galβ4Glcβ1Cer (H5 type 2 pentaglycosylceramide) from human erythrocytes, 4 μg.

Binding to lactosylceramide with sphingosine or phytosphingosine and hydroxy fatty acids (Nos. 5 and 6 in Table I), gangliotriaosylceramide (No. 10), gangliotetraosylceramide (No. 12), neolactotetraosylceramide (No. 15), and lactotetraosylceramide (No. 27) was also observed using ^{125}I-labeled extracts of bacterial outer membrane proteins,

from both *N. meningitidis* (examplified in Fig. 3B) and *H. influenzae* (data not shown). Again, no binding to lactosylceramide with sphingosine and non-hydroxy fatty acids (Fig. 3B, lane 1) was observed.

The same binding patterns were obtained with the four strains of *N. meningitidis, i.e.* there were no differences between the pathogenic strains of serogroups A, B, and C, and non-pathogenic *N. meningitidis* 29E. Also the four strains of *H. influenzae* exhibited identical glycosphingolipid binding patterns, without consistent differences between *H. influenzae* type b and non-typeable *H. influenzae* strains.

To assess the role of the acetamido groups of the *N*-acetylhexosamines of the binding-active compounds, the acetamido groups were converted to amines by treatment with anhydrous hydrazine. After de-*N*-acylation of the *N*-acetylgalactosamine of gangliotriaosylceramide (No. 11 in Table I, and Fig. 2, lane 3) and gangliotetraosylceramide (No. 13 in Table I, and Fig. 2, lane 5), no binding of the bacteria occurred, which reflects the importance of *N*-acetylgalactosamine for the interaction. De-*N*-acylation of the *N*-acetylglucosamine of neolactotetraosylceramide (No. 16 in Table I) decreased the binding of *H. influenzae* (Fig. 2B, lane 7), and abolished the binding of *N. meningitidis* (Fig. 2C, lane 7). No binding of the bacteria to de-*N*-acylated lactotetraosylceramide (No. 28 in Table I) was observed (data not shown).

Inhibition Studies—Radiolabeled *H. influenzae* was preincubated with lactose, lactotetraose or neolactotetraose (1 mg/ml) before assaying the binding to lactosylceramide, lactotetraosylceramide, gangliotetraosylceramide, and neolactotetraosylceramide on thin-layer chromatograms. Preincubation with lactose or lactotetraose did not affect the binding (Fig. 4, B and C). However, with preincubation with neolactotetraose the binding of the bacteria to neolactotetraosylceramide (Fig. 4D) was abolished, while the binding to lactosylceramide, lactotetraosylceramide, and gangliotetraosylceramide was unaffected. It should however be noted that this inhibition was only observed in two of five experiments, indicating the low efficacy of the inhibitory substance, possibly due to the monovalent presentation.

Glycosphingolipids from Target Cells—The non-acid glycosphingolipid fractions isolated from human granulocytes, oropharyngeal epithelium and plexus choroideus are shown in Fig. 5A, while the corresponding acid fractions are

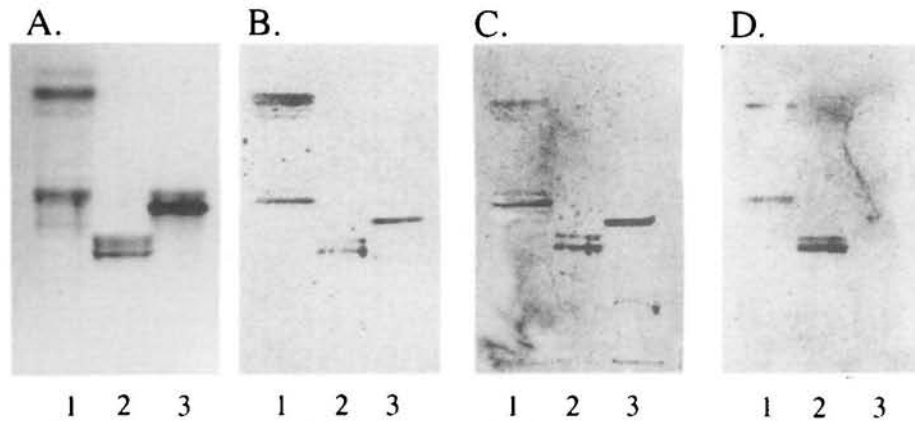

Fig. 4. **Effect of incubation of *Haemophilus influenzae* with oligosaccharides.** Radiolabeled *H. influenzae* was incubated with lactose, lactotetraose or neolactotetraose (1 mg/ml) in PBS for 1 h at room temperature. Thereafter the suspensions were utilized for the chromatogram binding assay. (A) Thin-layer chromatogram stained with anisaldehyde, (B) binding of *H. influenzae* preincubated with lactose, (C) binding of *H. influenzae* preincubated with lactotetraose, (D) binding of *H. influenzae* preincubated with neolactotetraose. The lanes contained: (1) Galβ4Glcβ1Cer (lactosylceramide) from dog intestine, 2 μg, and Galβ3GlcNAcβ-3Galβ4Glcβ1Cer (lactotetraosylceramide) from human meconium, 2 μg; (2) Galβ3GalNAcβ4Galβ4Glcβ1Cer (gangliotetraosylceramide) from mouse feces, 2 μg; (3) Galβ4GlcNAcβ3Galβ4Glcβ1Cer (neolactotetraosylceramide) from human erythrocytes, 2 μg. Chloroform/methanol/water 60:35:8 (by volume) was used as the solvent system. Autoradiography was performed for 12 h.

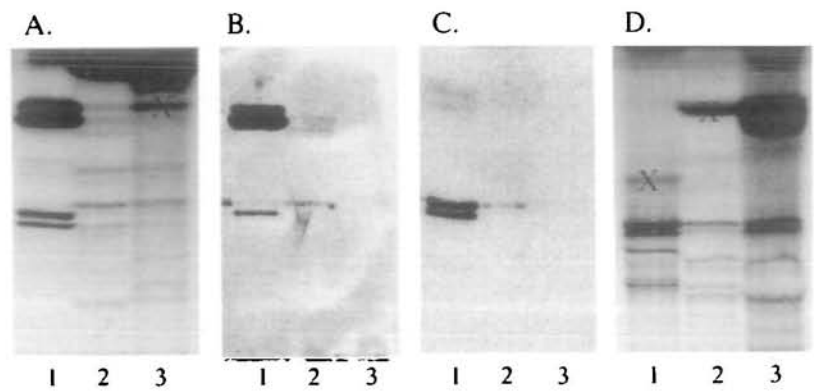

Fig. 5. **Chromatogram binding experiment showing the binding of radiolabeled *Neisseria meningitidis* and *Erythrina cristagalli* lectin to non-acid glycosphingolipids of human granulocytes, human oropharyngeal epithelium, and human plexus choroideus.** Total non-acid glycosphingolipids of human granulocytes, human oropharyngeal epithelium, and human plexus choroideus are shown in (A), while the results of binding of radiolabeled *N. meningitidis* and *E. cristagalli* lectin to the non-acid glycosphingolipid fractions are shown in (B) and (C), respectively. Total acid glycosphingolipids of human granulocytes, human oropharyngeal epithelium, and human plexus choroideus are shown in (D). The lanes contained: (1) human granulocyte glycosphingolipids, 40 μg/lane; (2) human oropharyngeal epithelium glycosphingolipids, 40 μg/lane; (3) human plexus choroideus glycosphingolipids, 40 μg/lane. The thin-layer plates were chromatographed with chloroform/methanol/water 60:35:8 (by volume), and the chromatograms in (A) and (D) were stained with anisaldehyde. The chromatograms in (B) and (C) were incubated with radiolabeled bacteria or lectin, followed by autoradiography for 12 h, as detailed under "MATERIALS AND METHODS." The bands denoted by X are non-glycosphingolipid contaminants.

shown in Fig. 5D. Thus, the non-acid fraction of human granulocytes was dominated by a double band migrating in the diglycosylceramide region, and also gave double bands migrating in the mono- and tetraglycosylceramide regions. The non-acid glycosphingolipid fraction of human oropharyngeal epithelial cells exhibited bands migrating as mono-, di-, tri-, and tetraglycosylceramides. The non-acid fraction of human choroideal plexus was dominated by a compound migrating in the monoglycosylceramide region, and also gave a faint band migrating in the tetraglycosylceramide region.

The acid fraction of human granulocytes (Fig. 5D) gave a number of bands migrating as GM3 and below. Two compounds migrating as GM3 and GD3/NeuAcα3-neolactotetraosylceramide were observed for the acid fraction of human oropharyngeal epithelium, while the acid fraction of human plexus choroideus was dominated by a compound migrating as sulfatide, and also gave a minor band migrating as GM3 and GD3/NeuAcα3-neolactotetraosylceramide.

The binding of *H. influenzae* and *N. meningitidis* to these glycosphingolipid preparations was investigated next. No consistent binding to the acid glycosphingolipids of human granulocytes, oropharyngeal epithelium, or choroideal plexus was observed (data not shown). Both *H. influenzae* (not shown) and *N. meningitidis* (Fig. 5B, lane 1) bound to the tetraglycosylceramide region of the non-acid fraction of human granulocytes. A binding-active tetraglycosylceramide was also detected in the non-acid fraction of human oropharyngeal epithelium (Fig. 5B, lane 2), while no binding to the non-acid fraction of plexus choroideus was detected. Parallel binding of the Galβ4GlcNAcβ-recognizing lectin of *E. cristagalli* in the tetraglycosylceramide region in the non-acid fractions of human granulocytes and human oropharyngeal epithelium was also observed (Fig. 5C).

The binding-active compound from the epithelial cells of human oropharynx was isolated by silicic acid chromatography and affinity chromatography using immobilized *E. cristagalli* lectin. The tetraglycosylceramide in the fraction eluted with 0.2 M lactose from the *E. cristagalli* column exhibited *H. influenzae*-binding activity (Fig. 6B, lane 4).

Mass Spectrometry of Glycosphingolipids of Human Oropharyngeal Epithelium—EI mass spectrometric analyses of the non-acid glycosphingolipid fraction of the epithelial cells of human oropharynx were performed using permethylated, and permethylated and $LiAlH_4$-reduced derivatives. The EI mass spectra of the permethylated and reduced total non-acid glycosphingolipid fraction of human oropharyngeal epithelium were dominated by series of immonium ions (F fragments), giving information about the carbohydrate structure and the fatty acid composition. The spectra obtained in the lower temperature range (approximately 220–250°C) were dominated by a series of immonium ions of monoglycosylceramide with h16:0-h24:0 fatty acids, at m/z 546-658. Terminal Hex was indicated by ions at m/z 219 and 187 (219−32). The spectra recorded at 240–280°C contained immonium ions of dihexaosylceramide with 16:0, h16:0, 22:0, 24:1, 24:0, and h24:0 fatty acids, at m/z 720, 750, 804, 830, 832, and 862, respectively. The ions at m/z 219 and 423 indicated a terminal Hex-Hex sequence. A series of immonium ions at m/z 924-1036, indicating trihexaosylceramide with 16:0 to 24:0, were found in the spectra recorded at 260–290°C.

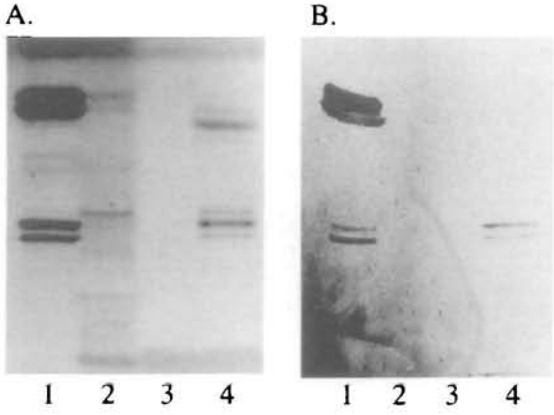

Fig. 6. **Binding of ^{35}S-labeled *Haemophilus influenzae* to non-acid glycosphingolipids from human oropharyngeal epithelium separated by affinity chromatography on *Erythrina cristagalli*-agarose.** The glycosphingolipids were separated on aluminum-backed silica gel plates, using chloroform/methanol/water 60:35:8 (by volume) as the solvent system, and visualized with anisaldehyde (A). Duplicate chromatograms were incubated with radiolabeled *H. influenzae* (B), followed by autoradiography for 12 h, as described under "MATERIALS AND METHODS." The lanes contained: (1) reference non-acid glycosphingolipids of human granulocytes, 40 μg; (2) and (3) fractions eluted from the *E. cristagalli* column with PBS; (4) fraction eluted from the *E. cristagalli* column with PBS containing 0.2 M lactose. Autoradiography was performed for 12 h.

Immonium ions of tetraglycosylceramide with 16:0, 22:0, 24:0, and h24:1 fatty acids, at m/z 1,155, 1,239, 1,267, and 1,295, were dominant in the spectra recorded at 280–310°C. In the EI mass spectra of permethylated, and permethylated and reduced glycosphingolipids with the Hexβ4HexN sequence, a diagnostic ion is found at m/z 182 (*45, 46*). However, this ion was not seen in the tetraglycosylceramide spectra of the permethylated, or permethylated and reduced non-acid fraction of human oropharyngeal epithelium. Instead, a terminal HexN was indicated by the ions at m/z 260 and 228 (260−32) in the spectrum of the permethylated derivative, and a carbohydrate sequence ion at m/z 464 suggested that the major tetraglycosylceramide had a HexN-Hex-Hex-Hex sequence.

In recordings in the higher temperature range (280–350°C) two weak series of immonium ions were found. One series was at m/z 1,442, 1,471, and 1,499, indicating a glycosphingolipid with three Hex and two HexN, and with 20:0, 22:0, and 24:0 fatty acids. The other series, at m/z 1,647, 1,675, and 1,703, suggested the presence of a glycosphingolipid with four Hex and two HexN, and with 20:0, 22:0, and 24:0 fatty acids. However, no conclusive sequence-derived fragments were obtained.

The successive evaporation of monohexosylceramide (m/z 628), dihexaosylceramide (m/z 832), trihexaosylceramide (m/z 1,036), tetraglycosylceramide (m/z 1,267), pentaglycosylceramide (m/z 1,499), and hexaglycosylceramide (m/z 1,703) is illustrated in Fig. 7.

Ions confirming the concluded Hex-Cer, Hex-Hex-Cer, Hex-Hex-Hex-Cer, and HexN-Hex-Hex-Hex-Cer structures were found in the EI mass spectra of the permethylated non-acid glycosphingolipid fraction of human oropharyngeal epithelium (data not shown). The dominant ceramide

ions were seen at m/z 548-660 (d18:1-16:0-24:0), while the ions at m/z 692 and 722 indicated t18:0-24:0 and t18:0-h24:0, respectively.

Thus, by EI mass spectrometry of the total non-acid glycosphingolipid fraction of human oropharyngeal epithelium the sequences of four compounds, *i.e.* Hex-Cer, Hex-Hex-Cer, Hex-Hex-Hex-Cer, and HexN-Hex-Hex-Hex-Cer, were determined. Trace amounts of glycosphingolipids with a $(HexN)_2$-$(Hex)_3$-Cer or $(HexN)_2$-$(Hex)_4$-Cer composition were also found. Sphingosine with non-hydroxy fatty acids was the major ceramide species, but phytosphingosine and hydroxy fatty acids were also present.

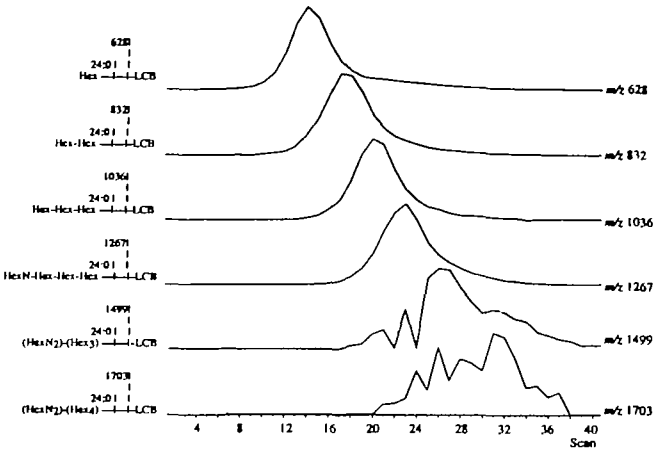

Fig. 7. **Selected reconstructed mass chromatograms obtained on EI mass spectrometry of the permethylated and reduced non-acid glycosphingolipid fraction of human oropharyngeal epithelium.** The curves reproduced correspond to the relative abundance of the immonium ions (indicated in the formulae) as a function of the evaporation temperature. LCB, long chain base. The sample (8 μg) was evaporated with increases of 10°C/min, starting at 150°C. Spectra (mass range, 100-3,200) were recorded every 40 s.

The acid fraction of human oropharyngeal epithelium was investigated by negative ion FAB mass spectrometry. The spectrum thus obtained (not shown) contained a series of molecular ions of NeuAc-GM3 with d18:1-16:0, d18:1-h16:0, d18:1-20:0, d18:1-22:0, d18:1-24:0, and d18:1-24:1 at m/z 1,152, 1,168, 1,208, 1,236, 1,264, and 1,262, respectively. The dominant ceramide species were d18:1-16:0 and d18:1-24:1.

Mass Spectrometry of Glycosphingolipids of Human Plexus Choroideus—EI mass spectra of the permethylated total non-acid fraction of human choroideal plexus are shown in Fig. 8. Simplified formulae for interpretation are shown above the spectra, representing the species with d18:1-24:0. The mass spectrometric analysis showed the presence of two compounds, one evaporating from the probe at approximately 270°C and the other at approximately 310°C. In the lower temperature range molecular ions and fragment ions specific for monohexosylceramides were observed (Fig. 8A). The ions at m/z 894 and 924 were the molecular ions of monohexosylceramide with d18:1-24:0 and d18:1-h24:0, respectively. Immonium ions of monohexosylceramide with 24:0 and h24:0 were seen at m/z 642 and 672. The only carbohydrate sequence ions were at m/z 219 and 187 (219−32), demonstrating a terminal Hex. The ion at m/z 292 was a rearrangement ion containing the carbohydrate and part of the fatty acid. Ceramide ions for d18:1-24:1 and d18:1-h24:0 were seen at m/z 658 and 690, respectively.

The compound evaporating at 310°C (Fig. 8B) was a glycosphingolipid with one HexN and three Hex, containing

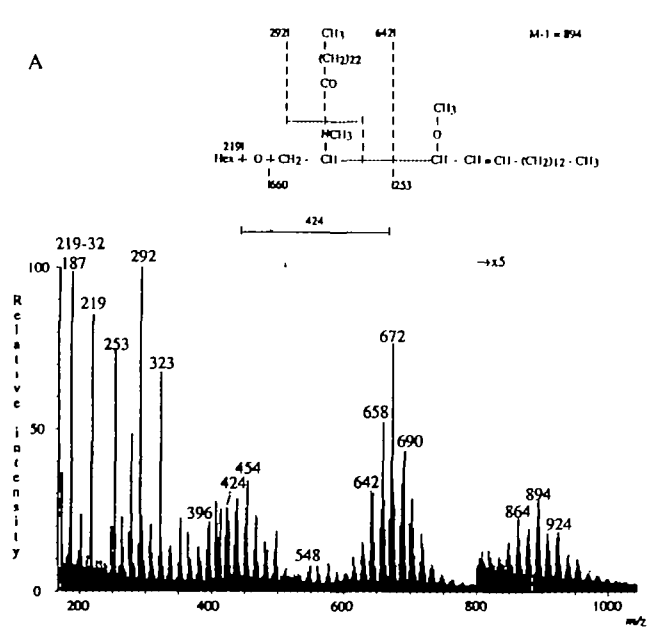

Fig. 8. **EI mass spectrum of the permethylated total non-acid glycosphingolipid fraction isolated from human plexus choroideus.** Above the spectrum simplified formulae for interpretation are shown, representing the species with sphingosine and non-hydroxy 24:0 fatty acid. The analytical conditions were: sample amount, 4 μg; electron energy, 70 eV; trap current, 500 μA; acceleration voltage, 8 kV. Starting at 150°C, the temperature was increased by 15°C/min. The spectrum in (A) was recorded at 270°C, and the spectrum in (B) at 310°C.

non-hydroxy 16:0 to 24:0 fatty acids, as demonstrated by the series of immonium ions at m/z 1,183 to 1,295. The ion at m/z 364 indicated a sphingosine long-chain base, and a series of ceramide ions of d18:1-16:0 to 24:0 were seen at m/z 548 to m/z 660. Carbohydrate sequence ions were found at m/z 219 and 187 (terminal Hex), and at m/z 464 (Hex-HexN). Fragment ions due to the loss of carbohydrate units from the molecular ions were also observed, as explained below the formula.

Thus, the analysis of the permethylated total non-acid fraction of human choroideal plexus by EI mass spectrometry demonstrated the presence of two glycosphingolipids. One was monohexosylceramide with mainly d18:1-24:0/24:1 and d18:1-h24:0. The other compound was a tetraglycosylceramide with the Hex-HexN-Hex-Hex sequence and d18:1-16:0-24:0. Interestingly, no significant ion at m/z 182 (see above) was seen in the spectrum of the latter compound, indicating that this glycosphingolipid did not have the Hexβ4HexN sequence.

Ions indicating four different compounds were present in the spectrum obtained on negative ion FAB mass spectrometry of the acid glycosphingolipid fraction of human plexus choroideus (data not shown). The dominant compound was sulfated monohexosylceramide with d18:1-16:0, d18:1-18:0, d18:1-24:0, d18:1-h24:1, and d18:1-24:0, as evidenced by the series of molecular ions at m/z 778, 806, 890, 904, and 906. The major ceramide species were d18:1-h24:1 and d18:1-24:0. A small ion at m/z 1,052 was also seen, indicating the presence of sulfated dihexaosylceramide with d18:1-24:0. In addition, a series of molecular ions at m/z 1,152, 1,168, 1,208, 1,236, 1,264, and 1,280 indicated NeuAc-GM3 with d18:1-16:0, d18:1-20:0, d18:1-22:0, d18:1-24:0, and d18:1-h24:0. Molecular ions corresponding to NeuAc-GD3 with t18:0-22:0 and t18:0-24:0 were found at m/z 1,544 and 1,572.

Mass Spectrometry of the Permethylated H. influenzae-Binding Fraction from the E. cristagalli Column—EI mass spectrometry of the permethylated fraction from the *E. cristagalli*-column, containing the *H. influenzae*-binding glycosphingolipid, showed the presence of two glycosphingolipids (Fig. 9). The spectrum recorded at 270°C (Fig. 9A) contained a series of molecular ions of dihexosylceramide at m/z 986 to 1,160, demonstrating a mixed population of ceramides with both sphingosine and phytosphingosine, and non-hydroxy and hydroxy 16:0 to 24:0 fatty acids. A corresponding series of ceramide ions was found at m/z 548 to 722. Immonium ions of dihexaosylceramide with 16:0, h16:0, h22:0, and h24:0 were found at m/z 734, 764, 848 and 876, respectively. Terminal Hex was indicated by the ions at m/z 219 and 187 (219−32), and the Hex-Hex sequence was verified by the rearrangement ion at m/z 496, containing the whole carbohydrate chain and a portion of the fatty acid (see interpretation formula above the spectrum). From the relative intensities of the ceramide ions and the immonium ions it was concluded that the dominant ceramide species was sphingosine with non-hydroxy 16:0 fatty acid.

In the spectrum recorded at a higher temperature (Fig. 9B) carbohydrate sequence ions corresponding to the Hex-HexN-Hex sequence were seen at m/z 219 and 187 (219−32), m/z 464 and 432 (464−32), and m/z 668. The ion at m/z 182 indicated a type 2 carbohydrate chain, Hexβ4-HexN (see above). The ions at m/z 548-722 were due to the ceramide part, and demonstrated a mixture of sphingosine and phytosphingosine long-chain bases, combined with both hydroxy and non-hydroxy 16:0-24:0 fatty acids. Fragments containing the whole carbohydrate chain and the fatty acid appeared at m/z 1,183 (non-hydroxy 16:0) and m/z 1,325 (hydroxy 24:0). The presence of phytosphingosine combined with hydroxy 24:0 fatty acid was indicated

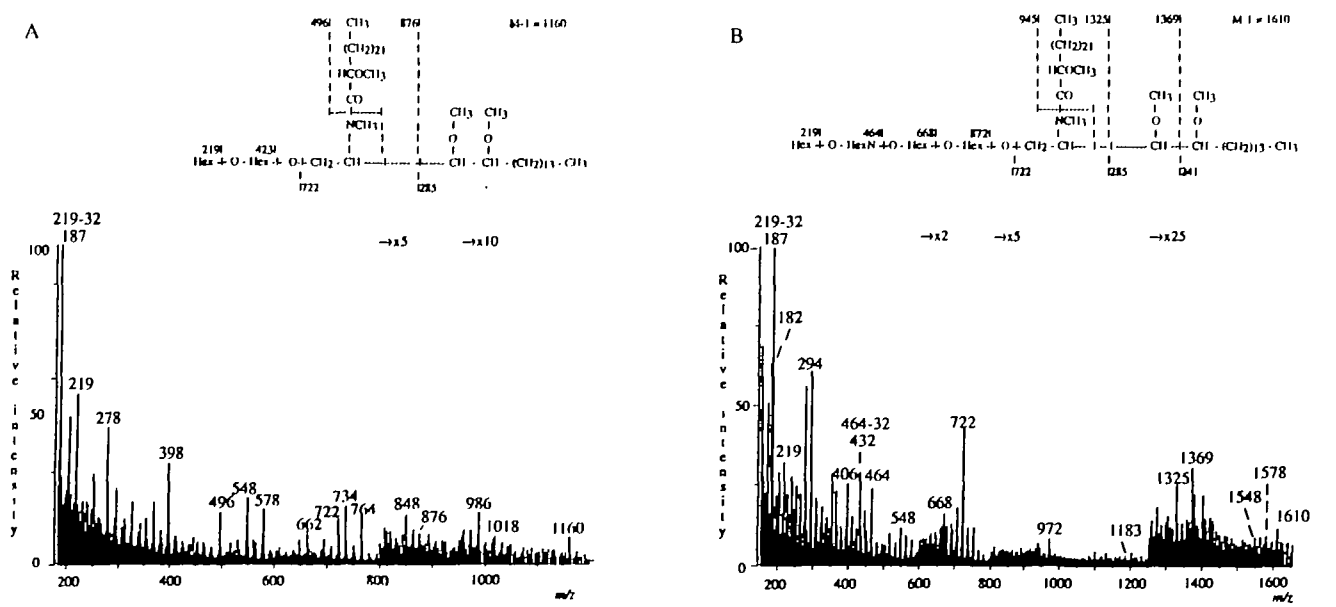

Fig. 9. **EI mass spectrometry of the permethylated fraction with *Haemophilus influenzae* binding-activity, isolated from human oropharyngeal epithelium by affinity chromatography on *Erythrina cristagalli* agarose.** Above the spectra formulae for interpretation are shown, representing the species with phytosphingosine and hydroxy 24:0 fatty acid. The analytical conditions were: sample amount, 2 μg; electron energy, 70 eV; trap current, 300 μA; acceleration voltage, 10 kV. Starting at 150°C, the temperature was increased by 15°C/min. The spectrum in (A) was recorded at 270°C, and the spectrum in (B) at 360°C.

by the ion at m/z 1,369 (1610−241). Molecular ions were seen at m/z 1,582 (t18:0-h22:0) and m/z 1,610 (t18:0-h24:0).

Thus, glycosphingolipids with the Hex-Hex-Cer and Hex-HexN-Hex-Hex-Cer sequences were identified on mass spectrometry of the *H. influenzae*-binding fraction. The latter compound had a type 2 linkage (Hexβ4HexN) between the terminal Hex and the penultimate HexN.

DISCUSSION

Three Binding Specificities—The Opa adhesins of *N. meningitidis* bind to members of the carcinoembryonic antigen receptor family (*47, 48*), and it was recently demonstrated that meningococcal Opc adhesin binds to heparan sulphate (*49*). However, binding of *N. meningitidis* to glycosphingolipids has not been reported previously. The present comparison of the glycosphingolipid binding properties of *H. influenzae* and *N. meningitidis* showed that these bacteria bound to glycosphingolipids in a similar manner, *i.e.* both bacteria recognized lactosylceramide, isoglobotriaosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, lactotetraosylceramide, neolactotetraosylceramide, and NeuGcα3-neolactohexaosylceramide. De-*N*-acylation of the *N*-acetylgalactosamine of gangliotriaosylceramide and gangliotetraosylceramide, and the *N*-acetylglucosamine of neolactotetraosylceramide and lactotetraosylceramide abolished or diminished the binding of both bacteria, indicating that the *N*-acetylhexosamines are involved in the recognition of these compounds.

Binding to lactosylceramide, with concomitant binding to isoglobotriaosylceramide, gangliotriaosylceramide, and gangliotetraosylceramide, has previously been reported for several, bacteria, *i.e.* both pathogens and members of the indigenous flora (*8*). Lactosylceramide-binding has also been demonstrated for some pathogenic fungi such as *Cryptococcus neoformans* and *Candida albicans* (*50*).

Binding to gangliotetraosylceramide *per se* has been reported for a number of human pathogens with different target tissues, such as lung pathogens (*e.g. Pseudomonas aeruginosa* and *H. influenzae*; Ref. *15*), enterotoxigenic *E. coli* with colonization factor antigens (*51*), and the human gastroduodenal pathogen, *Helicobacter pylori* (*52*). Also several human enteric viruses, *e.g.* rotavirus, have been shown to bind to gangliotetraosylceramide (*53*).

There are conflicting suggestions concerning which part of the gangliotriaosylceramide/gangliotetraosylceramide structure is recognized by the adhesins of the lactosylceramide-binding bacteria. One alternative is that there is one adhesin that primarily recognizes the lactose saccharide, and that extensions specifying the ganglio-series (*i.e.* GalNAcβ4 and Galβ3GalNAcβ4) are tolerated for sterical reasons, while other extensions make the lactose epitope inaccessible (*8*). Another suggestion is that the binding to lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represents two separate binding specificities (*54*).

The results of binding of *H. influenzae* and *N. meningitidis* to the de-*N*-acylated glycosphingolipids are most compatible with the interpretation that lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide represent two separate binding specificities for these bacteria. Similar results were recently reported for *H. pylori* (*38*). However, the binding of *H. influenzae* and *N. meningitidis* to lactosylceramide on thin-layer chromatograms is frequently accompanied by binding to gangliotriaosylceramide and gangliotetraosylceramide. This suggests that the factors controlling the expression of the lactosylceramide-binding adhesins are similar or identical to those which regulate the expression of the gangliotriaosylceramide/gangliotetraosylceramide-binding adhesins.

Our results indicating that the binding to lactosylceramide and gangliotriaosylceramide/gangliotetraosylceramide is due to two different adhesins agree with those of Baker *et al.* (*54*), who demonstrated that for *P. aeruginosa* the binding to these two glycosphingolipids represents two separate binding specificities. This finding was confirmed by the demonstration that *P. aeruginosa* PAK and PAO pili are responsible for the binding to gangliotetraosylceramide (*55*), and that the minimum sequence required for binding of these pili is GalNAcβ4Gal (*56*).

The binding of *H. influenzae* to neolactotetraosylceramide was abrogated when the bacteria were preincubated with neolactotetraose. However, the binding to lactosylceramide, gangliotetraosylceramide, or lactotetraosylceramide was unaffected by this treatment. Recognition of neolactotetraosylceramide is thus a specificity separate from the recognition of lactosylceramide or gangliotriaosylceramide/gangliotetraosylceramide. Most substitutions of the *N*-acetyllactosamine moiety abolished the binding. However, substitution with β3-linked *N*-acetylglucosamine at the terminal galactose of neolactotetraosylceramide, as in linear chains with repetitive *N*-acetyllactosamine moieties, was tolerated with retained binding activity.

In summary, three separate binding specificities were identified for *H. influenzae* and *N. meningitidis*. The first specificity is lactosylceramide recognition, where the minimum binding epitope is Galβ4Glc, and a ceramide with phytosphingosine and/or hydroxy fatty acids is necessary for correct presentation of the binding epitope. The second specificity is binding to gangliotriaosylceramide and gangliotetraosylceramide, where the minimum element recognized is GalNAcβ4Gal. Recognition of neolacto series glycosphingolipids is the third binding specificity, the minimum binding epitope in this case being Galβ4GlcNAcβ.

Presence of Binding-Active Glycosphingolipids in Human Tissues—While some of the *H. influenzae*- and *N. meningitidis*-binding glycosphingolipids are found in a variety of human cells, some have hitherto not been identified in human tissues. The most prominent example of the former group is lactosylceramide, which is an almost ubiquitous glycosphingolipid expressed in a large variety of cells (*57*). However, the lactosylceramide of most cell types, apart from the epithelial cells of the gastrointestinal tract, has mainly non-hydroxy ceramide, which is not recognized by *H. influenzae* or *N. meningitidis*.

Neolactotetraosylceramide is also found in several human tissues, such as erythrocytes (*57*), granulocytes (*58*), and semen (*59*), and the terminal Galβ4GlcNAcβ sequence of neolactotetraosylceramide is a common core structure in the carbohydrate chains of glycoproteins (*60*). Sialyl-neolactohexaosylceramide with terminal *N*-acetylneuraminic acid has been identified in human erythrocytes (*57*), semen (*59*), granulocytes (*61*), and placenta (*62*).

Lactotetraosylceramide, on the other hand, has only been chemically identified in human meconium (*63*), and in the small intestine of an individual previously resected *ad modum* Billroth II (*64*).

Isoglobotriaosylceramide is present in *e.g.* dog intestine (*65*), but has hitherto not been found in human tissues.

Gangliotriaosylceramide or gangliotetraosylceramide has not been chemically identified in peripheral human tissue. However, on the binding of monoclonal antibodies (*15, 66*), the presence of gangliotetraosylceramide in human lung tissue and umbilical vein endothelial cells was indicated, although the precise chemical structures of the binding-active compounds were not clearly established.

Glycosphingolipids of Target Tissues—The glycosphingolipid composition of human granulocytes has been thoroughly characterized (*58, 61, 67*). The major non-acid glycosphingolipid is lactosylceramide, with sphingosine and non-hydroxy 16:0 and 24:1 fatty acids, which constitutes 75% of the total non-acid fraction (*58*). In addition, this fraction contains lactotriaosylceramide, neolactotetraosylceramide, neolactohexaosylceramide, and a series of glycosphingolipids carrying the Le^x determinant. When the non-acid fraction of human granulocytes was examined for *H. influenzae*- and *N. meningitidis*-binding activity, preferential binding of both bacteria in the tetraglycosylceramide region, *i.e.* to neolactotetraosylceramide, was observed.

In some binding assays binding of the bacteria in the diglycosylceramide region of the non-acid fraction of human granulocytes, *i.e.* to lactosylceramide with sphingosine and non-hydroxy fatty acids (*58*), was also detected (Figs. 5 and 6). This was judged to represent non-specific binding, due to the large quantity of lactosylceramide (approximately 30 μg) present in the sample. No binding was noted when lactosylceramide purified from human granulocytes was used in the chromatogram binding assay. Furthermore, the lactosylceramide of human granulocytes is predominantly derived from intracellular compartments (*68*), and thus is not accessible for bacterial adherence under physiological conditions.

The glycosphingolipid compositions of the other target tissues of *H. influenzae* and *N. meningitidis, i.e.* the human respiratory epithelium and leptomeninges, have not been investigated previously. We selected the epithelium surrounding palatine tonsils as a model of respiratory epithelium, and since enough material for glycosphingolipid preparation from the pia and arachnoid is difficult to obtain, plexus choroideus was chosen as a model of the leptomeninges. Acid and non-acid glycosphingolipids were isolated from these tissues, and screened for reactivity with *H. influenzae* and *N. meningitidis*. No binding to acid or non-acid glycosphingolipids of plexus choroideus, or acid glycosphingolipids of the oropharyngeal epithelium was observed. However, since the glycosphingolipid compositions of these tissues have not been reported before, and the tissues in question are the target tissues for a number of microbial pathogens, the acid and non-acid glycosphingolipid fractions of the oropharyngeal epithelium and plexus choroideus were characterized by mass spectrometry. The results are summarized in Fig. 10.

In the non-acid glycosphingolipid fraction of human oropharyngeal epithelium a binding-active tetraglycosylceramide was detected, and this compound was isolated by affinity chromatography on immobilized *E. cristagalli* lectin. In hemagglutination and hemagglutination-inhibition studies the carbohydrate binding specificity of this lectin has been defined as Galβ4GlcNAc > Galβ4Glc $\gg$ Gal (*69*). On thin-layer chromatograms the *E. cristagalli* lectin binds to glycosphingolipids with terminal Galβ4GlcNAβ- and Fucα2Galβ4GlcNAβ-sequences, but not to lactosylceramide (*28*). However, the fraction eluted with 0.2 M lactose in PBS from the column with immobilized *E. cristagalli* lectin contained both diglycosylceramides (presumably lactosylceramide) and tetraglycosylceramides (presumably neolactotetraosylceramide), illustrating that different binding patterns might be obtained using different assay systems (*70*). The presence of a glycosphingolipid with the hexose-*N*-acetylhexosamine-hexose-hexose sequence, and with a type 2 chain (hexoseβ4-*N*-acetylhexosamine), in the binding-active fraction was demonstrated by mass spectrometry. Taken together with the lectin binding data this strongly suggests that binding-active neolactotetraosylceramide is expressed by human oropharyngeal cells, although as a minor compound, and may thus

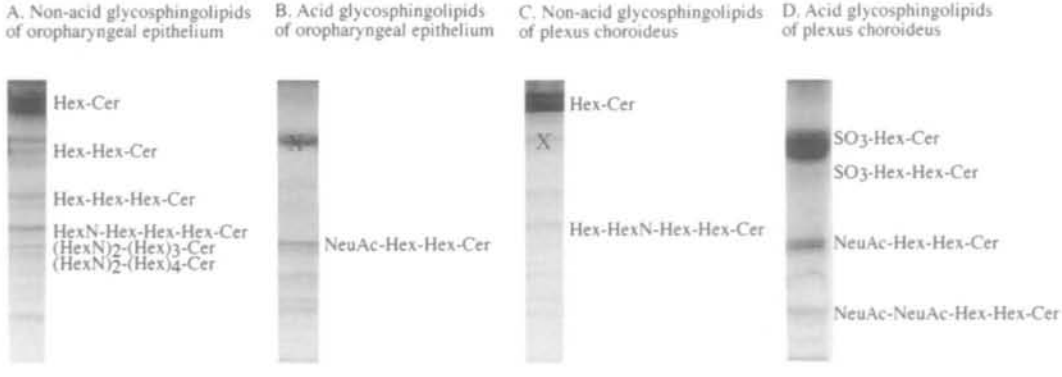

Fig. 10. **Summary of the glycosphingolipid structures in human oropharyngeal epithelium and plexus choroideus, target tissues for *Haemophilus influenzae* and *Neisseria meningitidis*.** Thin-layer chromatograms of acid (A) and non-acid (B) glycosphingolipids of human oropharyngeal epithelium, and acid (C) and non-acid (D) glycosphingolipids of plexus choroideus. Simplified formulae, as interpreted based on mass spectrometry, have been assigned to the different bands. The samples (40 μg each) were chromatographed using chloroform/methanol/water, 60:35:8 (by volume), as the solvent system, and stained with anisaldehyde. The band denoted by X is a non-glycosphingolipid contaminant.

TABLE II. **Summary of *Haemophilus influenzae/Neisseria meningitidis*-binding glycosphingolipids in human target tissues.**

No.	Trivial name	Structure	Granulocytes[a]	Oropharyngeal epithelium[b]	Plexus choroideus[b]
1.	LacCer (t18:0-h16:0-h24:0)	Galβ4Glcβ1Cer	−	(+)	−
2.	Neolactotetra	Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	+	−
3.	Neolactohexa	Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	−	−
4.	NeuAcα3neolactohexa	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer	+	−	−

[a]Refs. *58*, *61*, and *67*. [b]Present paper.

be utilized by *H. influenzae* and *N. meningitidis* as a receptor for adherence to the oropharyngeal epithelium.

Interestingly, *Streptococcus pneumoniae*, another meningitis-associated bacterium, is also capable of recognizing neolactotetraosylceramide, with a preference for the GlcNAcβ3Galβ saccharide (*71*).

The binding of *H. influenzae* and *N. meningitidis* to neolactotetraosylceramide also suggests a potential for the bacteria to interact with the *N*-acetyllactosamine chains of glycoproteins and polyglycosylceramides. However, polyglycosylceramides from human erythrocytes, having branched *N*-acetyllactosamine chains (*72*), were not recognized by the bacteria (data not shown). Furthermore, when proteins from human oropharyngeal epithelial cells were extracted (*73*), and the binding of ^{35}S-labeled *H. influenzae* and *N. meningitidis* was examined on blotting membranes, no specific binding of the bacteria was observed (data not shown). Neither was any binding of the bacteria to reference glycoproteins (human lactoferrin, transferrin, orosomucoid, glycophorin, secretory IgA, neolactotetra-BSA, lactotetra-BSA, and GalNAcβ3Galβ-BSA) on blotting membranes observed. However, the *N*-acetyllactosamine chains of glycoprotein are often branched (*60*), and the glycosphingolipid binding data shows that while the bacteria are capable of interacting with linear *N*-acetyllactosamine chains, such as NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer, branches, such as in Galβ4GlcNAcβ6(Galβ4GlcNAcβ3)Galβ4Glcβ1Cer, are not tolerated.

Dihexaosylceramide, most likely lactosylceramide, with both sphingosine and phytosphingosine, and non-hydroxy and hydroxy fatty acids, was also present in oropharyngeal cells, as demonstrated by mass spectrometry. Lactosylceramide with sphingosine or phytosphingosine and hydroxy fatty acids is recognized by both *H. influenzae* and *N. meningitidis*, but no consistent binding of the bacteria to the diglycosylceramide region of the non-acid fraction of human oropharyngeal cells was observed. The dominant molecular species of dihexaosylceramide had sphingosine and non-hydroxy fatty acids, while the binding-active molecular species were relatively minor, and thus presumably below the detection level of the chromatogram binding assay.

The binding-active glycosphingolipids found in the target tissues of *H. influenzae* and *N. meningitidis* are summarized in Table II.

All four *H. influenzae* strains studied were hemagglutinating, which suggests that they are fimbriated (*74*). The fimbriae of *H. influenzae* mediate hemagglutination through interaction with the AnWj antigen on adult human erythrocytes and also bind to an unidentified receptor on human oropharyngeal epithelial cells (*75*). Non-fimbrial adhesins of *H. influenzae* have also been partly characterized (*16*, *76*).

The binding patterns of *H. influenzae* type b and nontypable *H. influenzae* showed no differences. The same binding patterns were also obtained for the *N. meningitidis* strains with different serogroups, and these results strengthen the indication that the capsules of *H. influenzae* and *N. meningitidis* are of minor importance in the pathogenic events involved in target cell adhesion proceeding systemic disease. However, encapsulated bacteria are more resistant to host defence of bactericidal and phagocytic nature (*5*).

Several of the binding-active carbohydrate sequences defined in this study are also present in the lipooligosaccharides in the outer membrane of the bacteria. The Galβ4GlcNAcβ3Galβ4Glc sequence of neolactotetraosylceramide is found in both *N. meningitidis* and *H. influenzae* lipooligosaccharides (*77*, *78*). In addition, oligosaccharides with a terminal Galβ4Glc sequence, such as lactosylceramide, have been identified in a non-typable *H. influenzae* strain (*79*), whereas the major lipooligosaccharide of *H. influenzae* serotype b has terminal Glc1-4Glc disaccharides (*80*).

Thus, in addition to the binding of bacterial adhesins to target cell surface glycoconjugates, these carbohydrate structures on the bacterial surface may be utilized for attachment to host cells through interaction with Galβ-binding lectins on mammalian cells (*81*). Alternatively, the presence of the same carbohydrate sequences on bacteria and host cells might provide the bacteria with a means of escaping the host immune response through host mimicry. Further studies are obviously required to determine the role(s) of carbohydrate binding in the pathogenic mechanisms of these bacteria.

Binding activities similar to those observed for whole bacterial cells were found using preparations of outer membrane proteins of *N. meningitidis* and *H. influenzae*, suggesting that these preparations may be utilized for the isolation and characterization of the carbohydrate-binding adhesins.

REFERENCES

1. Greenwood, B.M., Blakebrough, I.S., Bradley, A.K., Wall, S., and Whittle, H.C. (1984) Meningococcal disease and season in sud-Saharan Africa. *Lancet* **i**, 1339-134221
2. Noah, N.D. (1987) Epidemiology of bacterial meningitis: UK and USA in *Bacterial Meningitis* (Williams, J.D. and Burnie, J., eds.) pp. 93-115, Academic Press, London
3. Sell, S.H.W. (1987) *Hemophilus influenzae* type b meningitis: manifestations and long-term sequelae. *Pediatr. Infect. Dis. J.* **6**, 775-778
4. Smith, A.L. (1988) Neurologic sequelae of meningitis. *N. Engl. J. Med.* **319**, 1012-1014
5. Hugosson, S., Fredlund, H., and Olcén, P. (1996) Serum opsonic and bactericidal activity after *Neisseria meningitidis* group A+C and *Hemophilus influenzae* type b vaccination: a comparison of three functional assays. *Serodiagn. Immunother. Inf. Dis.* **8**, 213-220

6. Beachey, E.H. (1981) Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.* **143**, 325-345
7. Mirelman, D. and Ofek, I. (1986) Introduction to microbial lectins and agglutinins in *Microbial Lectins and Agglutinins* (Mirelman, D., ed.) pp. 1-19, John Wiley & Sons, New York
8. Karlsson, K.-A. (1989) Animal glycosphingolipids as membrane attachment sites for bacteria. *Annu. Rev. Biochem.* **58**, 309-350
9. Ofek, I. and Doyle, R.J. (1994) Bacterial lectins as adhesins in *Bacterial Adhesion to Cells and Tissues*, pp. 94-135, Chapman & Hall, New York
10. Firon, N., Ofek, I., and Sharon, N. (1984) Carbohydrate-binding of the mannose specific fimbrial lectins of enterobacteria. *Infect. Immun.* **43**, 1088-1090
11. Leffler, H. and Svanborg-Edén, C. (1980) Chemical identification of a glycosphingolipid receptor for *Escherichia coli* attaching to human urinary tract epithelial cells and agglutinating human erythrocytes. *FEMS Microbiol. Lett.* **8**, 127-134
12. Källenius, G., Möllby, R., Svensson, B., Winberg, J., Lundblad, A., Svensson, S., and Cedergren, B. (1980) The P^k antigen as receptor for the haemagglutinin of pyelonephritic *Escherichia coli*. *FEMS Microbiol. Lett.* **7**, 297-302
13. Bock, K., Breimer, M.E., Brignole, A., Hansson, G.C., Karlsson, K.-A., Larson, G., Leffler, H., Samuelsson, B.E., Strömberg, N., Svanborg-Edén, C., and Thurin, J. (1985) Specificity of binding of a strain of uropathogenic *Escherichia coli* to Galα1-4Gal-containing glycosphingolipids. *J. Biol. Chem.* **260**, 8545-8551
14. Strömberg, N., Marklund, B.-I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K.-A., and Normark, S. (1990) Host-specificity of uropathogenic *Escherichia coli* depends on differences in binding specificity to Galα4Gal-containing isoreceptors. *EMBO J.* **9**, 2001-2010
15. Krivan, H.C., Roberts, D.D., and Ginsburg, V. (1988) Many pathogenic bacteria bind specifically to the carbohydrate sequence GalNAcβ1-4Gal found in some glycolipids. *Proc. Natl. Acad. Sci. USA* **85**, 6157-6161
16. Busse, J., Hartmann, E., and Lingwood, C.A. (1996) Receptor affinity purification of a lipid-binding adhesin from *Hemophilus influenzae*. *J. Infect. Dis.* **175**, 77-83
17. van Alphen, L., Geelen-van den Broek, L., Blaas, L., van Ham, M., and Dankert, J. (1991) Blocking of fimbria-mediated adherence of *Hemophilus influenzae* by sialyl gangliosides. *Infect. Immun.* **59**, 4473-4477
18. Fakih, M.G., Murphy, T.F., Pattoli, M.A., and Berenson, C.S. (1997) Specific binding of *Hemophilus influenzae* to minor gangliosides of human respiratory epithelial cells. *Infect. Immun.* **65**, 1695-1700
19. Hartmann, E. and Lingwood, C. (1997) Brief heat shock treatment induces a long-lasting alteration in the glycolipid receptor binding specificity and growth rate of *Hemophilus influenzae*. *Infect. Immun.* **65**, 1729-1733
20. Olcén, P., Danielsson, D., and Kjellander, J. (1976) The use of protein A-containing staphylococci sensitized with antimeningococcal antibodies for grouping *Neisseria meningitidis* and demonstration of meningococcal antigen in cerebrospinal fluid. *Acta Pathol. Microbiol. Scand. B* **83**, 387-396
21. Dahlberg, T. and Branefors, P. (1980) Enzyme-linked immunosorbent assay for titration of *Hemophilus influenzae* capsular and O antigen antibodies. *J. Clin. Microbiol.* **12**, 185-192
22. Danielsson, D. and Johannisson, G. (1973) Culture diagnosis of gonorrhoea. A comparison of the yield with selective and non-selective gonococcal culture media inoculated in the clinic and after transport of specimens. *Acta Dermatovener.* **53**, 75-80
23. Tam, M.R., Buchanan, T.M., Sandström, E.G., Holmes, K.K., Knapp, J.S., Siadak, A.W., and Nowinski, R.C. (1982) Serological classification of *Neisseria gonorrhoeae* with monoclonal antibodies. *Infect. Immun.* **36**, 1042-1053
24. Pesce, M. and Strande, C.S. (1973) A new micromethod for determination of protein in cerebrospinal fluid and urine. *Clin. Chem.* **19**, 1265-1267
25. Aggarwal, B.B., Eessalu, T.E., and Hass, P.E. (1985) Characterization of receptors for human tumor necrosis factor and their regulation by γ-interferon. *Nature* **318**, 665-667
26. Waldi, D. (1962) Sprühreagentien für die dünnschicht-chromatographie in *Dünnschicht-Chromatographie* (Stahl, E., ed.) pp. 496-515, Springer-Verlag, Berlin
27. Hansson, G.C., Karlsson, K.-A., Larson, G., Strömberg, N., and Thurin, J. (1985) Carbohydrate-specific adhesion of bacteria to thin-layer chromatograms: a rationalized approach to the study of host cell glycolipid receptors. *Anal. Biochem.* **146**, 158-163
28. Teneberg, S., Ångström, J., Jovall, P.-Å., and Karlsson, K.-A. (1994) Characterization of binding of Galβ4GlcNAc-specific lectins from *Erythrina cristagalli* and *Erythrina corallodendron* to glycosphingolipids. *J. Biol. Chem.* **269**, 8554-8563
29. Karlsson, K.-A. (1987) Preparation of total non-acid glycolipids for overlay analysis of receptors for bacteria and viruses and for other studies. *Methods Enzymol.* **138**, 212-220
30. Handa, S. (1963) Blood group active glycolipid from human erythrocytes. *Jpn. J. Exp. Med.* **33**, 347-360
31. Samuelsson, B.E., Pimlott, W., and Karlsson, K.-A. (1990) Mass spectrometry of mixtures of intact glycosphingolipids. *Methods Enzymol.* **193**, 623-646
32. Falk, K.-E., Karlsson, K.-A., and Samuelsson, B.E. (1979) Proton nuclear magnetic resonance analysis of anomeric structure of glycolipids. The globoseries (one to five sugars). *Arch. Biochem. Biophys.* **192**, 164-176
33. Falk, K.-E., Karlsson, K.-A., and Samuelsson, B.E. (1979) Proton nuclear magnetic resonance analysis of anomeric structure of glycolipids. Blood group ABH-active substances. *Arch. Biochem. Biophys.* **192**, 177-190
34. Falk, K.-E., Karlsson, K.-A., and Samuelsson, B.E. (1979) Proton nuclear magnetic resonance analysis of anomeric structure of glycolipids. Lewis-active and Lewis-like substances. *Arch. Biochem. Biophys.* **192**, 191-202
35. Koerner Jr., T.A.W., Prestegard, J.H., Demou, P.C., and Yu, R.K. (1983) High-resolution proton NMR studies of gangliosides. 1. Use of homonuclear spin-echo J-correlated spectroscopy for determination of residue composition and anomeric configurations. *Biochemistry* **22**, 2676-2687
36. Yang, H.-j. and Hakomori, S.-i. (1971) A sphingolipid having a novel ceramide and lacto-*N*-fucopentose III. *J. Biol. Chem.* **246**, 1192-1200
37. Stellner, K., Saito, H., and Hakomori, S.-i. (1973) Determination of aminosugar linkages in glycolipids by methylation. Aminosugar linkages of ceramide pentasaccharides of rabbit erythrocytes and of Forssman antigen. *Arch. Biochem. Biophys.* **155**, 464-472
38. Ångström, J., Teneberg, S., Abul Milh, M., Larsson, T., Leonardsson, I., Olsson, B.-M., Ölwegård Halvarsson, M., Danielsson, D., Ljungh, Å., Wadström, T., and Karlsson, K.-A. (1998) The lactosylceramide binding specificity of *Helicobacter pylori*. *Glycobiology* **8**, 297-309
39. Teneberg, S., Miller-Podraza, H., Lampert, H.C., Evans Jr., D.J., Evans, D.G., Danielsson, D., and Karlsson, K.-A. (1997) Carbohydrate binding specificity of the neutrophil-activating protein of *Helicobacter pylori*. *J. Biol. Chem.* **272**, 19067-19071
40. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509
41. Larson, G., Karlsson, H., Hansson, G.C., and Pimlott, W. (1987) Application of a simple methylation procedure for the analysis of glycosphingolipids. *Carbohydr. Res.* **161**, 281-290
42. Karlsson, K.-A. (1974) Carbohydrate composition and sequence analysis of a derivative of brain disialo ganglioside by mass spectrometry, with molecular weight ions at m/e 2245. Potential use in the specific microanalysis of cell surface components. *Biochemistry* **13**, 3643-3647
43. Breimer, M., Hansson, G.C., Karlsson, K.-A., Larson, G., Leffler, H., Pascher, I., Pimlott, W., and Samuelsson, B.E. (1980) Fingerprinting of lipid-linked oligosaccharides by mass spectrometry in *Advances in Mass Spectrometry* (Quayle, A., ed.) Vol. 8, pp. 1097-1108, Heyden & Son, London
44. Teneberg, S., Lönnroth, I., Torres Lopez, J.F., Galili, U., Ölwegård Halvarsson, M., Ångström, J., and Karlsson, K.-A. (1996) Molecular mimicry in the recognition of glycosphingolipids by Galα3Galβ4GlcNAcβ-binding *Clostridium difficile* toxin A, human natural anti α-galactosyl IgG and the monoclonal antibody Gal-13; characterization of a binding-active human glycosphingolipid, non-identical with the animal receptor. *Glycobiology* **6**, 599-609

45. Karlsson, K.-A. (1976) Microscale fingerprinting of blood-group fucolipids by mass spectrometry in *Glycolipid Methodology* (Witting, L.A., ed.) pp. 97-122. Am. Oil. Soc., Champaign, IL
46. Karlsson, K.-A. (1978) Mass-spectrometric sequence studies of lipid-linked oligosaccharides, blood-group fucolipids, gangliosides and related cell-surface receptors. *Progr. Chem. Fats Other Lipids* **16**, 207-230
47. Virji, M., Watt, S.M., Barker, S., Makepeace, K., and Doyonhas, R. (1996) The N-domain of the human CD66a adhesin molecule is a target for Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Mol. Microbiol.* **22**, 929-939
48. Virji, M., Makepeace, K., Fergusson, D.J.P., and Watt, S.M. (1996) Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol. Microbiol.* **29**, 941-950
49. de Vries, F.P., Cole, R., Dankert, J., Frosch, M., and van Putten, J.P.M. (1998) *Neisseria meningitidis* producing the Opc adhesin binds epithelial cell proteoglycan receptors. *Mol. Microbiol.* **27**, 1203-1212
50. Jimenez-Lucho, V., Ginsburg, V., and Krivan, H.C. (1990) *Cryptococcus neoformans, Candida albicans*, and other fungi bind specifically to the glycosphingolipid lactosylceramide (Galβ1-4Glcβ1-1Cer), a possible adhesion receptor for yeasts. *Infect. Immun.* **58**, 2085-2090
51. Sporsem Orø, H., Kolstø, A.-B., Wennerås, C., and Svennerholm, A.-M. (1990) Identification of asialo GM1 as a binding structure for *Escherichia coli* colonization factor antigens. *FEMS Microbiol. Lett.* **72**, 289-292
52. Lingwood, C.A., Huesca, M., and Kuksis, A. (1992) The glycerolipid receptor for *Helicobacter pylori* (and Exoenzyme-S) is phosphatidylethanolamine. *Infect. Immun.* **60**, 2470-2474
53. Willoughby, R.E., Yolken, R.H., and Schnaar, R.L. (1990) Rotaviruses specifically bind to the neutral glycosphingolipid asialo-GM1. *J. Virol.* **64**, 4830-4835
54. Baker, N., Hansson, G.C., Leffler, H., Riise, G., and Svanborg-Edén, C. (1990) Glycosphingolipid receptors for *Pseudomonas aeruginosa*. *Infect. Immun.* **58**, 2361-2366
55. Lee, K.K., Sheth, H.B., Wong, W.Y., Sherburne, R., Paranchynch, W., Hodges, R.S., Lingwood, C.A., Krivan, H., and Irvin, R.T. (1994) The binding of *Pseudomonas aeruginosa* pili to glycosphingolipids is a tip-associated event involving the C-terminal region of the structural pilin subunit. *Mol. Microbiol.* **11**, 705-713
56. Sheth, H.B., Lee, K.K., Wong, W.Y., Srivastava, G., Hindsgaul, O., Hodges, R.S., Paranchynch, W., and Irvin, R.T. (1994) The pili of *Pseudomonas aeruginosa* strains PAK and PAO bind specifically to the carbohydrate sequence βGalNAc(1-4)βGal found in glycosphingolipids asialo-GM_1 and asialo-GM_2. *Mol. Microbiol.* **11**, 715-723
57. Hakomori, S.-i. (1983) Chemistry of glycosphingolipids in *Sphingolipid Biochemistry* (Kanfer, J.N. and Hakomori, S.-i., eds.) Vol. 3, pp. 1-165, Plenum Press, New York
58. Macher, B.A. and Klock, J.C. (1980) Isolation and chemical characterization of neutral glycosphingolipids of human neutrophils. *J. Biol. Chem.* **255**, 2092-2096
59. Ritter, G., Krause, W., Geyer, R., Stirm, S., and Wiegandt, H. (1987) Glycosphingolipid composition of human semen. *Arch. Biochem. Biophys.* **257**, 370-378
60. Montreuil, J. (1984) Spatial conformations of glycans and glycoproteins. *Biol. Cell* **51**, 115-132
61. Stroud, M.R., Handa, K., Salyan, M.E.K., Ito, K., Levery, S.B., Hakomori, S.-i., Reinhold, B.B., and Reinhold, V.N. (1996) Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 1. Separation of E-selectin binding from nonbinding gangliosides, and absence of sialosyl-Le^x having tetraosyl to octaosyl core. *Biochemistry* **35**, 758-769
62. Taki, T., Matsuo, K.-i., Yamamoto, K., Matsubara, T., Hayashi, A., Abe, T., and Matsumoto, M. (1988) Human placenta gangliosides. *Lipids* **23**, 192-198
63. Karlsson, K.-A. and Larson, G. (1979) Structural characterization of lactotetraosylceramide, a novel glycosphingolipid isolated from human meconium. *J. Biol. Chem.* **254**, 9311-9316
64. Björk, S., Breimer, M.E., Hansson, G.C., Karlsson, K.-A., and Leffler, H. (1987) Structures of blood group glycosphingolipids in human small intestine. A relation between the expression of fucolipids of epithelial cells and the ABO, Le and Se phenotype of the donor. *J. Biol. Chem.* **262**, 6758-6765
65. Hansson, G.C., Karlsson, K.-A., Larson, G., McKibbin, J.M., Strömberg, N., and Thurin, J. (1983) Isoglobotriaosylceramide and the Forssman glycolipid of dog small intestine occupy separate tissue compartments and differ in ceramide composition. *Biochim. Biophys. Acta* **750**, 214-216
66. Gillard, B.K., Jones, M.A., and Marcus, D.M. (1987) Glycosphingolipids of human umbilical vein endothelial cells and smooth muscle cells. *Arch. Biochem. Biophys.* **256**, 435-445
67. Iglesias, J.L., Lis, H., and Sharon, N. (1982) Purification and properties of a D-galactose/*N*-acetyl-galactosamine-specific lectin from *Erythrina cristagalli*. *Eur. J. Biochem.* **123**, 247-252
68. Sharon, N. and Lis, H. (1989) Carbohydrate specificity in *Lectins*, pp. 37-46, Chapman and Hall, London
69. Stroud, M.R., Handa, K., Salyan, M.E. K., Ito, K., Levery, S.B., Hakomori, S.-i., Reinhold, B.B., and Reinhold, V.N. (1996) Monosialogangliosides of human myelogenous leukemia HL60 cells and normal human leukocytes. 2. Characterization of E-selectin binding fractions, and structural requirements for physiological binding to E-selectin. *Biochemistry* **35**, 770-778
70. Symington, F.W., Murray, W.A., Bearman, S.I., and Hakomori, S.-i. (1987) Intracellular localization of lactosylceramide, the major human neutrophil glycosphingolipid. *J. Biol. Chem.* **262**, 11356-11363
71. Andersson, B., Dahmén, J., Frejd, T., Leffler, H., Magnusson, G., Noori, G., and Svanborg Edén, C. (1983) Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. *J. Exp. Med.* **158**, 559-570
72. Koscielak, J., Miller-Podraza, H., and Zdebska, E. (1978) Isolation and characterization of polyglycosylceramides (megaloglycolipids) with A, H and I blood-group activities. *Eur. J. Biochem.* **71**, 9-18
73. Yang, Z., Bergström, J., and Karlsson, K.-A. (1994) Glycoproteins with Galα4Gal are absent from human erythrocyte membranes, indicating that glycolipids are the sole carriers of blood group P activities. *J. Biol. Chem.* **269**, 14620-14624
74. Geme III, J.W. St. (1996) Molecular determinants of the interaction between *Haemophilus influenzae* and human cells. *Am. J. Respir. Crit. Care Med.* **154**, 5192-5196
75. van Alphen, L., Poole, J., and Overbeeke, M. (1986) The Anton blood group antigen is the erythrocyte receptor for *Haemophilus influenzae*. *FEMS Microbiol. Lett.* **37**, 69-71
76. Gilsdorf, J.R., McCrea, K.W., and Marrs, C.F. (1997) Role of pili in *Haemophilus influenzae* adherence and colonization. *Infect. Immun.* **65**, 2997-3002
77. Mandrell, R.E., Griffiss, J.M., and Macher, B.A. (1988) Lipooligosaccharides (LOS) of *Neisseria gonorrhoeae* and *Neisseria meningitidis* have components that are immunologically similar to precursors of human blood group antigens. Carbohydrate sequence specificity of the mouse monoclonal antibodies that recognize crossreacting antigens on LOS and human erythrocytes. *J. Exp. Med.* **168**, 107-126
78. Mandrell, R.E., McLaughlin, R., Kwaik, Y.A., Lesse, A., Yamasaki, R., Gibson, B., Spinola, S.M., and Apicella, M.A. (1992) Lipooligosaccharides (LOS) of some *Haemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. *Infect. Immun.* **60**, 1322-1328
79. Phillips, N.J., Apicella, M.A., Griffiss, J.M., and Gibson, B.W. (1992) Lipooligosaccharides (LOS) of some *Hemophilus* species mimic human glycosphingolipids, and some LOS are sialylated. *Biochemistry* **31**, 4515-4526
80. Phillips, N.J., Apicella, M.A., Griffiss, J.M., and Gibson, B.W. (1993) Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. *Biochemistry* **32**, 2003-2012
81. Mandrell, R.E., Apicella, M.A., Lindstedt, R., and Leffler, H. (1994) Possible interaction between animal lectins and bacterial carbohydrates. *Methods Enzymol.* **236**, 231-254