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# Epitope dissection of receptor-active gangliosides with affinity for *Helicobacter pylori* and influenza virus*

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**Receptor-active gangliosides with affinity for *Helicobacter pylori* and influenza virus were chemically modified and analyzed by negative ion fast atom bombardment mass spectrometry (FAB MS) or electron ionization mass spectrometry (EI MS) after permethylation. Derivatizations included mild periodate oxidation of the sialic acid glycerol tail or conversion of the carboxyl group to primary alcohol or amides. The modified gangliosides were then tested for binding affinity using thin-layer plates overlaid with labeled microbes or microbe-derived proteins.**

**Mild periodate oxidation, which shortens sialic acid tail without destruction of sugar cores, abolished or drastically reduced binding of *H. pylori* and avian influenza virus to sialyl-3-paragloboside (S-3-PG). The same effect was observed in the case of binding of the human influenza virus to receptor-active gangliosides of human leukocytes. Conversion of S-3-PG or leukocyte gangliosides to primary alcohols or amides also abolished the binding. However, mild periodate oxidation had no effect on binding of NAP (neutrophil-activating protein of *H. pylori*) to the active ganglioside.**

There are many examples of the involvement of glycosphingolipids in receptor-associated phenomena [1-5], the classical being binding of cholera toxin B subunit to ganglioside $G_{M1}$. Among different sugars present in glycosphingolipids, sialic acid provides the best

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**Abbreviations:** FAB MS, fast atom bombardment mass spectrometry; EI MS, electron ionization mass spectrometry; PGCs, polyglycosylceramides; S-3-PG, sialyl-3-paragloboside (sialyl$\alpha$3neolactotetraosylceramide); NAP, neutrophil-activating protein of *H. pylori*; HRP, horseradish peroxidase. The carbohydrate and glycosphingolipid nomenclatures are according to recommendations of IUPAC-IUB Commission on Biochemical Nomenclature (*Lipids* 1977, **12**, 455-468; *J. Biol. Chem.* 1982, **257**, 3347-3351; and *J. Biol. Chem.* 1987, **262**, 13-18).

model for receptor function because of the presence of the glycerol side chain („glycerol tail") and the carboxyl group. This acidic saccharide can easily undergo minor chemical modifications like acetylation at the glycerol tail hydroxyl groups, lactylation, methylation, sulfation, phosphorylation or lactonization [6], which, if reversible, may be the basis for regulatory events *in vivo*, ensuring rapid switching off and on of receptor activities.

Several gangliosides have been characterized as being receptor-active for influenza virus [7–10] and *Helicobacter pylori* [11–17], the binding being dependent on the presence of sialic acid. Sialic acid is known to be involved in binding of various microbes to target cells, however, the mechanisms of the interactions have usually not been adequately understood. In order to establish which particular groups are involved in the recognition, we performed epitope dissection, in other words, a specific enzymatic or chemical cleavage or an other modification of an active compound followed by binding studies. In this paper we show some examples of the epitope dissection of receptor-active gangliosides using chemical methods. We chemically modified the sialic acid glycerol tail or the sialic acid carboxyl group and tested the derivatized compounds for binding activities using thin-layer plates overlaid with labeled microbes or microbe-derived proteins.

## MATERIALS AND METHODS

Gangliosides used in these studies were obtained in our laboratory as described previously [18]. Polyglycosylceramides were prepared according to Miller-Podraza *et al.* [19]. Mild periodate oxidation/reduction [20] was performed as follows: Gangliosides (0.05–0.1 mM) were incubated in 1–2 mM $NaIO_4$ in 0.05 mM acetate buffer, pH 5.5, for 40 min on ice. To terminate the reaction an excess of $Na_2SO_3$ was added. The sample was concentrated by freeze-drying (about 5-fold) and again reduced with an excess of $NaBH_4$ at room temperature overnight. Finally, the sample was dialyzed against distilled water for two days. Modifications of the carboxyl group were performed as described by Lanne *et al.* [21]. The derivatives were purified by Sephadex LH 20 (Pharmacia, Uppsala, Sweden) column chromatography. The column was packed in methanol. After application of the sample, the column was eluted with methanol and the glycolipid recovered from sugar-positive fractions (monitored by TLC plates sprayed with anisaldehyde). *H. pylori* cultivation and overlaying of silica gel TLC plates with $^{35}S$-labeled bacterium were performed as described earlier [14]. Overlaying with HRP-labeled influenza virus was done according to Matrosovich *et al.* [22] and labeling and binding of NAP according to Teneberg *et al.* [17]. The bacterial and viral strains were as follows: *H. pylori* 032 (a gift from Dr. D. Danielsson, Örebro Medical Centre, Sweden), HRP-labeled avian influenza virus A/ duck/ Czechoslovakia/56 ($H_4N_6$) and HRP-labeled human influenza virus A/X-113 ($H_1N_1$). FAB MS analyses of glycolipids were performed on a JEOL SX102 mass spectrometer. Ions were produced by collision with Xe atoms (6 keV) using triethanolamine as matrix. EI MS was performed on the same mass spectrometer as described by Miller-Podraza *et al.* [23].

## RESULTS

Chemical modifications performed in these studies included: (a) mild periodate oxidation of the sialic tail followed by reduction with sodium borohydride, (b) reduction of the carboxyl group to primary alcohol or (c) conversion of the carboxyl group to various amides. All chemical derivatizations were confirmed by mass spectrometry. Less complex gangliosides were tested by FAB MS. More complex species including polyglycosylceramides were permethylated and investigated by EI MS in positive mode. After derivatizations the gan-

gliosides were tested by TLC followed by overlaying with labeled microbes or microbe-derived proteins to permit observation of changes in binding properties.

The following ganglioside fractions were used: (a) a mixture of gangliosides prepared from human leukocytes (receptor-active for *H. pylori* and human and avian influenza virus), (b) S-3-PG from human erythrocytes, NeuAc$\alpha$3Gal$\beta$4GlcNAc$\beta$3Gal$\beta$4Glc-Cer (receptor-active for *H. pylori* and avian influenza virus), and (c) seven-sugar monosialoganglioside from rabbit thymus, NeuGc$\alpha$3Gal$\beta$4GlcNAc-$\beta$3Gal$\beta$4GlcNAc$\beta$3Gal$\beta$4Glc-Cer (receptor-active for NAP).

### Mild periodate oxidation of the sialic acid tail

Mild periodate oxidation of sialic acid in gangliosides followed by reduction with sodium borohydride resulted in two derivatives with shortened sialic acid glycerol tail [20]. This treatment does not affect other sugar moieties in sugar chains.

$$CH_2OH\text{-}CHOH\text{-}CHOH\text{-}R \xrightarrow{NaIO_4/NaBH_4} CH_2OH\text{-}CHOH\text{-}R + CH_2OH\text{-}R$$

Figure 1 provides an example of the EI MS spectrum of complex gangliosides (polyglycosylceramides) from human leukocytes after mild periodate oxidation, reduction and permethylation. The ions characteristic for NeuAc (*m/z* 376 and 344) and NeuAcHexHexNAc (*m/z* 825 and 793) present in the control sample were replaced in the oxidized sample by ions at *m/z* 332, 300, 288, and 781, 749, 737, 705, as expected. Ions derived from other sugars were generally the same in controls and oxidized samples indicating, that the core structure remained undestroyed during the oxidation procedure (not shown wholly in Fig. 1). A similar result regarding sialic acid was obtained for polyglycosylceramides from human erythrocytes. EI MS after permethylation seems to be a convenient method to investigate terminal carbohydrate stuctures in highly complex glycolipids, however, it does not give much information about size of molecular species. The distribution of sialic acid among PGC molecules is now being investigated in our laboratory using matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI TOF MS). Our preliminary results indicate that NeuAc is associated with PGCs of different complexity, including highly complex molecules. Mild periodate oxidation of the glycerol tail of NeuAc completely abolished binding by *H. pylori* of polyglycosylceramides prepared from both human leukocytes and human erythrocytes, as exemplified in Fig. 2.

*H. pylori* binds also to less complex gangliosides of the neolacto-series including S-3-PG. This binding apparently represents a different specificity compared to the binding of PGCs, as described previously and confirmed using different solid-phase binding assays and hemagglutination inhibition tests [15, 16]. The binding of *H. pylori* to S-3-PG also turned out to be sensitive to mild periodate oxidation although some residual activity appeared to remain after the procedure. Thus, in this case the glycerol tail also proved to be important for the receptor function. Similarly, the shortening of the glycerol tail abolished the binding of avian and human influenza viruses to the active gangliosides. However, the binding by NAP [24] of gangliosides was not affected by mild periodate oxidation, although the dependence of the binding on the presence of sialic acid was proven [17].

### Modifications of the carboxyl group

Gangliosides were modified on their COOH group by reduction to alcohol or by conversion to various amides [21, 25, 26], as shown below.

**(a)** $R\text{-}COOH \rightarrow R\text{-}CH_2OH$

**(b)** $R\text{-}COOH \rightarrow R\text{-}CONH_2$

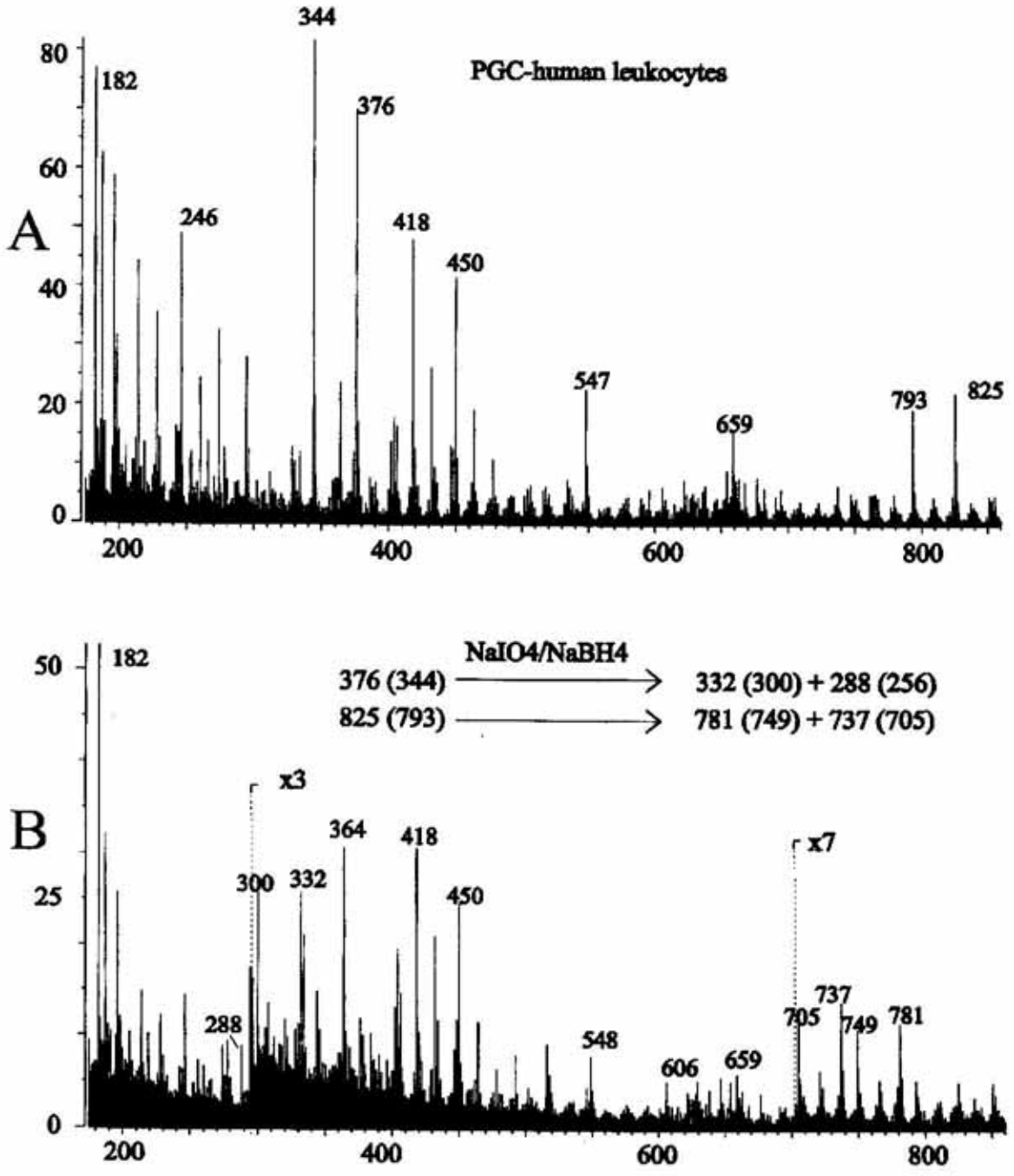


**Figure 1. Electron impact ionization mass spectrometry (EI MS) of complex glycosphingolipids prepared from human leukocytes.**

The glycolipids were permethylated before analysis [23]. Spectrum A, unmodified glycolipids; spectrum B, glycolipids oxidized with $NaIO_4$ and reduced with $NaBH_4$.

**(c)** R-COOH → R-CONHCH$_3$
**(d)** R-COOH → R-CONHCH$_2$CH$_3$
**(e)** R-COOH → R-CONHCH$_2$CH$_2$CH$_3$
**(f)** R-COOH → R-CONHCH$_2$C$_6$H$_5$

Spectra of S-3-PG and its *N*-ethylamide are presented in Fig. 3. As expected, there was no destruction of the core chain as indicated by fragment ions at *m/z* 649, 811, 973, 1176,

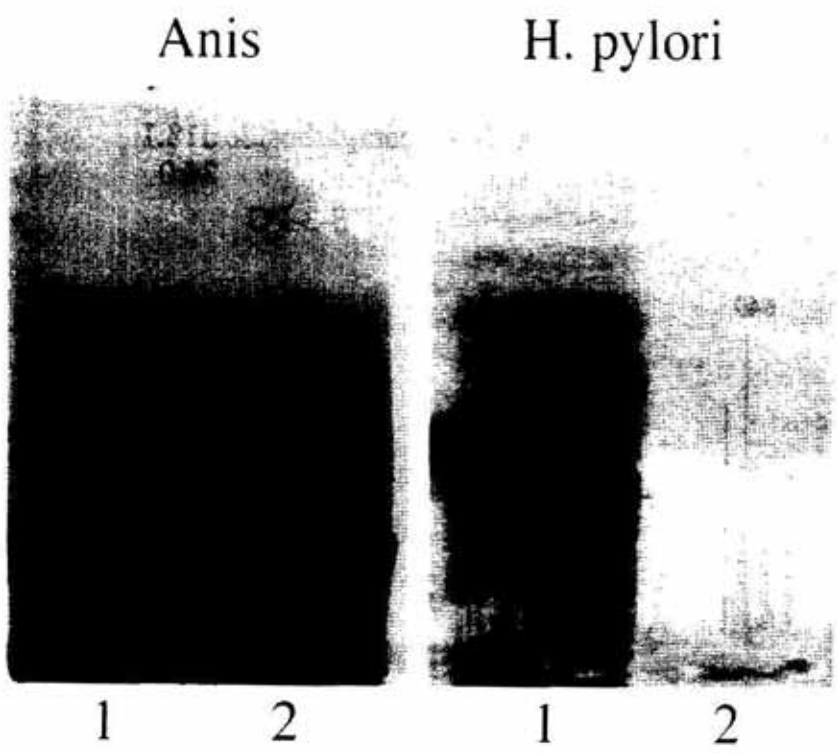


**Figure 2. Binding of *Helicobacter pylori* (strain 032, see ref. [14]) to polyglycosylceramides of human erythrocytes after mild periodate oxidation.**

ANIS, TLC plate visualized with anisaldehyde; *H. pylori*, autoradiogram of the TLC plate overlaid with $^{35}$S-labeled *H. pylori*. The chromatograms were developed in propanol/0.25% KCl in water/methanol/chloroform (7:5:1:0.5, by vol.). Lane 1, control PGCs; lane 2, oxidized and reduced PGCs.

1338, which confirmed the sequence of Cer(d18:1,24:0)-Hex-Hex-HexNAc-Hex. The peaks of molecular ions [$(M-H)^-$] in the derivatized sample were by 27 units higher compared to control, which is in agreement with the reaction shown above **(d)**. Thus, the ions at *m/z* 1601 and 1629 in the control sample resulted in ions at 1628 and 1656 in the derivatized sample. The structures of all derivatives of sialylparagloboside, as well as of other less complex glycosphingolipids used in these studies were confirmed in a similar way by negative ion FAB spectra.

Figure 4 shows, as an example, binding of avian influenza virus to various modified S-3-PG. This virus binds strongly to native S-3-PG which is in accordance with previous reports showing its affinity for 3-linked *N*-acetylneuraminic acid. The modifications of the carboxyl group abolished the binding, confirming the crucial role of this group in the interaction. Similar results were obtained for human influenza virus documenting strict dependence of the binding on both glycerol tail and the carboxyl group. The fraction used in the experiments with human influenza virus was a mixture of gangliosides prepared from human leukocytes. It has been shown recently that human leukocytes contain a series of complex gangliosides which display a strong binding affinity for human-specific influenza virus [10].

The results presented in this paper are summarized in Table 1.

## DISCUSSION

In this work we investigated sialic acid-dependent binding of different virulence factors to gangliosides in relation to sialic acid glycerol tail and carboxyl group. The results presented for the avian influenza virus are in agreement with the previous reports in this

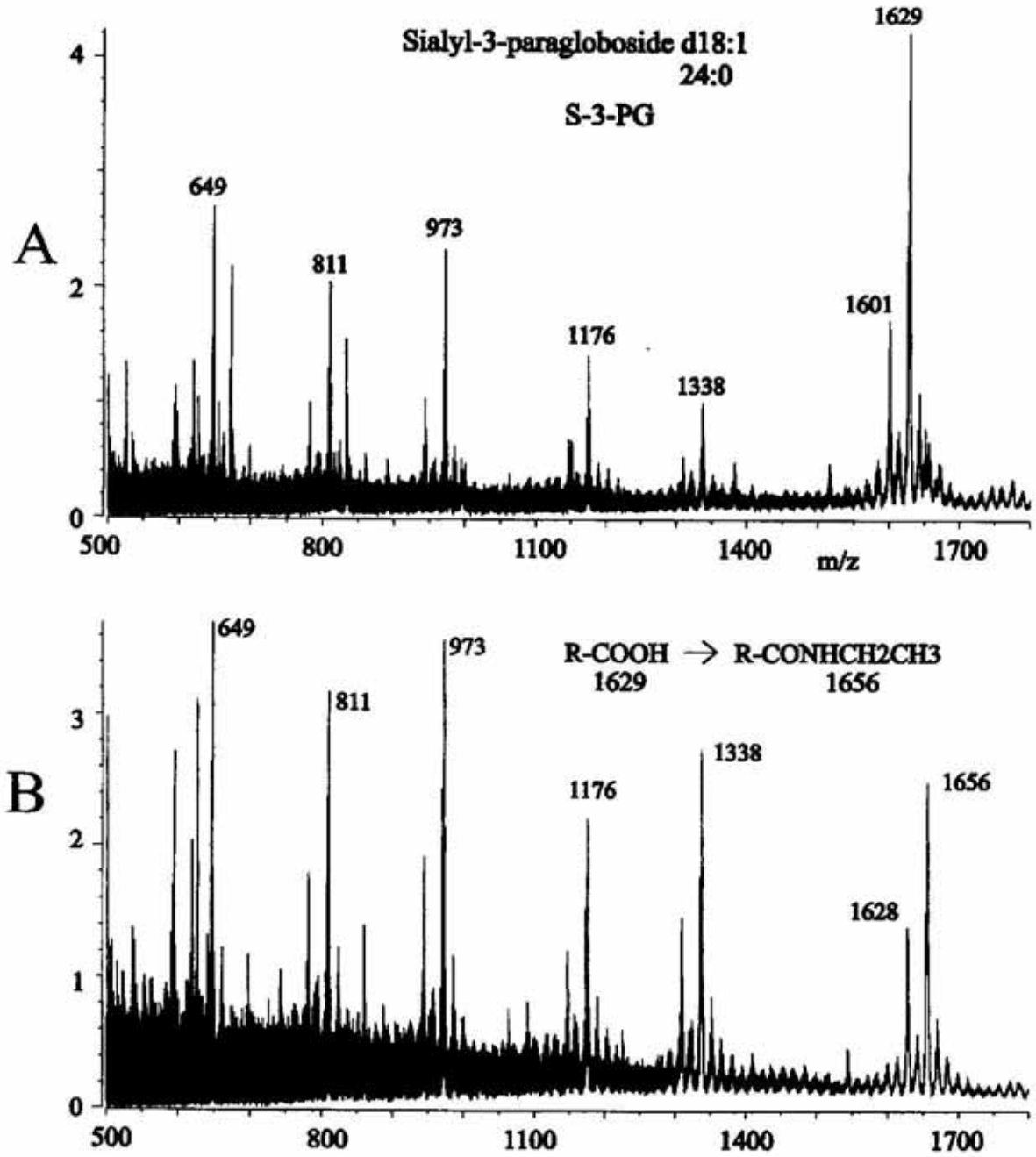


**Figure 3. Negative ion fast atom bombardment mass spectra (FAB MS) of sialyl-3-paragloboside (S-3-PG) and its *N*-ethylamide.**

Spectrum A, unmodified S-3-PG; spectrum B, *N*-ethylamide of S-3-PG.

field, showing preferential recognition of sialyl-3-oligosaccharides as receptor determinants [27, 28]. In our studies the avian virus bound selectively to gangliosides containing terminal NeuAc$\alpha$3Gal, like S-3-PG, ganglioside $G_{D1a}$ or ganglioside $G_{T1b}$, but not to gangliosides with 6-linked sialic acid or gangliosides with non-terminal sialic acid. The bind-

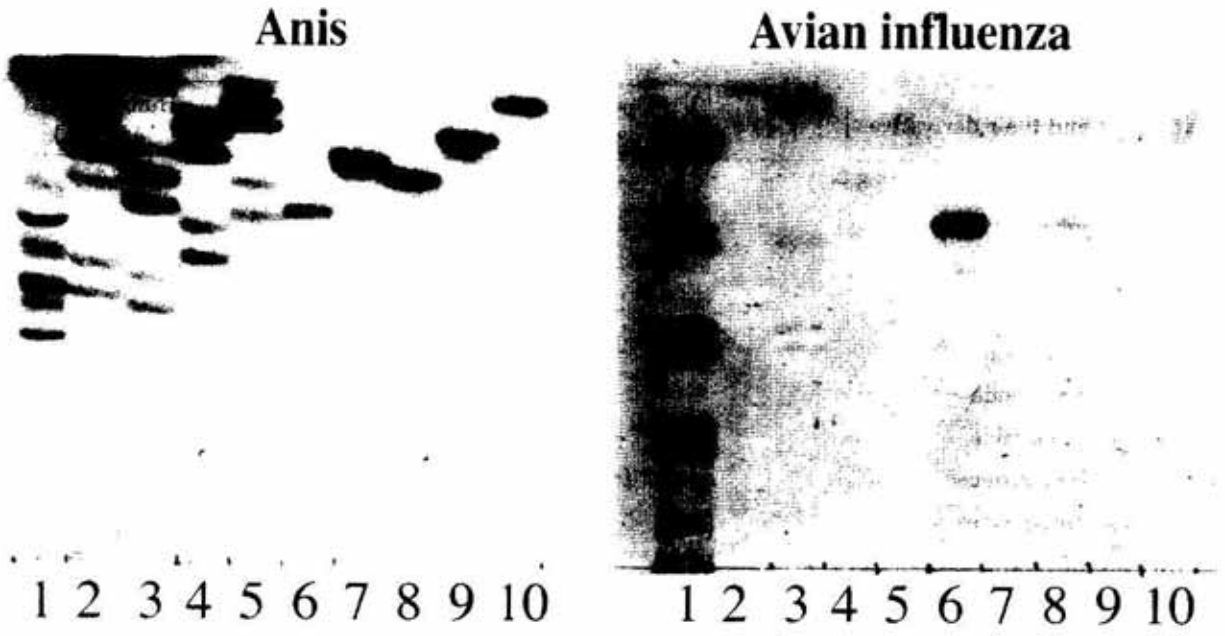


**Figure 4. Binding of the avian influenza virus (A/duck/Czechoslovakia/56, $H_4N_6$) to gangliosides after chemical modifications.**

Anis, TLC chromatogram visualized with anisaldehyde; Avian influenza, the TLC plate overlaid with the HRP-labeled virus. Lanes 1–5, upper phase gangliosides from human leukocytes converted to particular derivatives; Lane 1, underivatized fraction; Lane 2, converted to alcohols; Lane 3, to amides; Lane 4, to *N*-methylamides; Lane 5; to *N*-ethylamides. Lanes 6–10, sialylparagloboside (S-3-PG) from human erythrocytes: Lane 6, underivatized S-3-PG; Lane 7, converted to alcohol; Lane 8, to amide; Lane 9, to *N*-methylamide; Lane 10, to *N*-ethylamide. The chromatograms were developed in chloroform/methanol/0.25% KCl, (50:40:10, by vol).

ing was strictly dependent on the presence of the glycerol tail and the carboxyl group. These findings support previous reports based on inhibition studies in which different sialic acid analogs were tested, and the importance of both the glycerol tail and the carboxyl group was demonstrated [29, 30]. The binding of the human influenza virus to leukocyte gangliosides also turned out to be totally dependent on the tail and the carboxyl group, which again was not unexpected. However, the binding of human virus in these studies could be based on a larger epitope with new characteristics, since the high affinity interaction found in this case required more than simply NeuAc$\alpha$3Gal or NeuAc$\alpha$6Gal (to be published).

The results on gangliosides with affinity for *H. pylori* are new. *H. pylori* is a widespread bacterium implicated in development of human gastric diseases such as gastritis, peptic ulcers and gastric carcinoma [31–33]. The bacterium affects more than half of the global population and the only effective way of its eradication known today is a therapy using a proton pump inhibitor together with antibiotics. Considering the universality of *H. pylori* infection, such a treatment constitutes a danger regarding development of antibiotic-resistant bacterial strains. An alternative way of treatment would be the use of receptor analogs competing for *H. pylori* with binding receptors on target cells and efforts are being made to develop such a therapeutic procedure [34]. Therefore a detailed knowledge of *H. pylori* binding specificities is highly needed.

We have previously shown that *H. pylori* carries three sialic acid-dependent specificities, one represented by binding of *H. pylori* cells to S-3-PG [15, 16] probably identical with the previously described „sialyllactose/fetuin" binding activity [35–37], another represented

**Table 1. Changes of binding specificities of gangliosides after chemical modifications**

| Glycolipids and their derivatives | Binding activity | | |
|---|---|---|---|
| | *H. pylori* 032 | Avian virus $A(H_4N_6)$ | Human virus $A(H_1N_1)$ |
| Sialyl-3-paragloboside (S-3-PG) | + | + | - |
| S-3-PG-alcohol | - | - | - |
| S-3-PG-amide | - | - | - |
| S-3-PG-*N*-Methylamide | - | - | - |
| S-3-PG-*N*-Ethylamide | - | - | - |
| S-3-PG-*N*-Propylamide | - | - | - |
| S-3-PG-*N*-Benzylamide | - | - | - |
| S-3-PG-oxidized/reduced | (+) | - | - |
| Leukocyte gangliosides (L-Gangl) | + | + | + |
| L-Gangl-alcohol | - | - | - |
| L-Gangl-amide | (+) | - | - |
| L-Gangl-*N*-Methylamide | - | - | - |
| L-Gangl-*N*-Ethylamide | - | - | - |
| PGCs | + | + | + |
| PGCs-oxidized/reduced | - | n.d. | n.d. |
| NAP-positive gangl. | + | n.d. | n.d. |
| NAP-positive gangl-oxidized/reduced | + | n.d. | n.d. |

+Indicates strong and reproducible binding; (+) indicates weak or occasional binding; n.d. not determined.

by binding to PGCs of human origin and being expressed selectively after bacterial growth in liquid cultures [14, 15], and the third manifested by binding of NAP, the neutrophil-activating protein extracted from *H. pylori*, to selected gangliosides [17]. An important finding of this work was that the first and the second specificity were more or less dependent on the presence of the sialic acid glycerol tail, while the third was not, which indicates that *H. pylori* produces proteins that recognize sialic acids in different ways. This is not surprising since the presentation of the functional parts of sialic acids may be dependent on vicinal sugars in oligo- and polysaccharide structures as well as on other membrane constituents, and may be different in linear and branched molecules.

The binding properties of glycosphingolipids mentioned in this paper represent a pathological activity, where a microbe takes advantage of a structure whose real function in the body remains unknown. However, one can imagine that the same glycosphingolipid may participate in recognition of biologically active messengers of the parent cells. Especially glycosphingolipids with large and branched carbohydrate parts containing repeated antigenic structures seem to be created for this purpose. For complex glycolipids multivalency may be an important factor ensuring high affinity binding, and, compared with glycoproteins they may provide binding sites in closer proximity to the membrane surface. We already know that some glycosphingolipids carry blood group determinants and interact with specific antibodies and lectins. Polyglycosylceramides from human red cells which display ABH [38], Ii [39, 40] and Lewis x and Lewis y (H. Miller-Podraza *et al.*, unpub-

lished) blood group activities, carry their specific determinants on both terminal and internal portions of the saccharide chains. The same polyglycosylceramides, apart from *H. pylori* binding [14–16], were shown to be receptor-active for *Mycoplasma pneumoniae* [41] and *Streptococcus suis* [42], and were found to interact after desialylation with heat labile toxin (LT) of *Escherichia coli* (S. Teneberg, *et al.*, unpublished). The above facts provide a good example of the volume of the encoded information which these compounds may carry. However, the understanding of this information and of its translation into biological events requires a better knowledge of glycolipids and carbohydrates than the present data can provide.

We are indebted to Dr. D. Danielsson for providing us with 032 *H. pylori* strain.

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