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Different glycosphingolipid composition in human neutrophil subcellular compartments

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The binding of a number of carbohydrate-recognizing ligands to glycosphingolipids and polyglycosylceramides of human neutrophil subcellular fractions (plasma membranes/secretory vesicles of resting and ionomycin-stimulated cells, specific and azurophil granules) was examined using the chromatogram binding assay. Several organelle-related differences in glycosphingolipid content were observed. The most prominent difference was a decreased content of the GM3 ganglioside in plasma membranes of activated neutrophils. Gangliosides recognized by anti-VIM-2 antibodies were detected mainly in the acid fractions of azurophil and specific granules. Slow-migrating gangliosides and polyglycosylceramides with Helicobacter pylori-binding activity were found in all acid fractions. A non-acid triglycosylceramide, recognized by Galα4Gal-binding Escherichia coli, was detected in the plasma membrane/secretory vesicles but not in the azurophil and specific granules. Although no defined roles of glycosphingolipids have yet been conclusively established with respect to neutrophil function, the fact that many of the identified glycosphingolipids are stored in granules, is in agreement with their role as receptor structures that are exposed on the neutrophil cell surface upon fusion of granules with the plasma membrane. Accordingly, we show that neutrophil granules store specific carbohydrate epitopes that are upregulated to the plasma membrane upon cell activation.

Keywords: glycosphingolipids, polyglycosylceramides, human neutrophils, neutrophil granules

Abbreviations: CFU, colony forming units; DFP; diisopropyl fluorophosphate; FAB, fast atom bombardment; HPNAP, neutrophil-activating protein of Helicobacter pylori; PBS, phosphate-buffered saline.

Introduction

The neutrophil granulocytes play a key role in innate immunity as they have a great capacity to eliminate invading microorganisms and proper cell function depends largely on adhesion-related events involving lectin binding to glycoconjugate receptors [1]. Important neutrophil functions such as adhesion to the endothelial lining during diapedesis and specific recognition of chemoattractants and foreign materials are dependent on a dynamic expression of cell surface receptors. Many of these neutrophil receptors and surface effector molecules required for phagocytic uptake and elimination of intruders, are present in internal stores and can be mobilized to the neutrophil plasma membrane during cell activation [2].

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The neutrophil contains several types of subcellular organelles, granules, which take part in fusion processes with the plasma membrane (for a review, see ref. 3). The dominating granule subtypes are the lysosome-like, peroxidase-positive granules (primary or azurophil granules) and the peroxidase-negative granules (secondary or specific/gelatinase granules). A number of membrane glycoproteins have been identified in neutrophils, of which only two (CD63 and CD68) are localized in the azurophil granule membrane. In contrast, the membrane of the specific granules is rich in glycosylated proteins of importance for neutrophil diapedesis and phagocytosis [3]. The granules start forming during neutrophil maturation in the bone marrow, probably by aggregation of immature transport vesicles that bud off from the Golgi network [4,5]. The azurophilic granules are formed from the *cis*-Golgi at the promyelocyte stage, whereas the peroxidase-negative granules are formed from the *trans*-Golgi network at a later stage in the maturation process. The refinement of carbohydrates from simple to complex side chains takes place

in the intermediate and *trans*-Golgi stacks [6], suggesting that the glycosylation patterns of carbohydrate-bearing membrane molecules might differ between the granule subtypes.

A large number of microbial adhesins use glycosphingolipids rather than glycoproteins as their host target [7], but very little is known about the identity, intracellular storage and surface exposure of glycosphingolipid moities in neutrophils. There are reports showing that neutrophils contain a complex mixture of glycosphingolipids [8–10] and that the major glycosphingolipid, lactosylceramide, is found mainly in intracellular compartments [11,12].

The aim of the present study was to determine the subcellular distribution of complex glycosphingolipids in neutrophils by probing with a panel of defined carbohydrate-binding proteins and microbes. We found that the pattern of expression of glycosphingolipids differs between the two mobilizable granule compartments and the plasma membrane, respectively.

Materials and methods

Isolation of human neutrophil granulocytes

Human neutrophils were isolated from buffy coats from healthy blood donors using dextran sedimentation and Ficoll-Paque gradient centrifugation [13]. Remaining erythrocytes were carefully removed by hypotonic lysis repeated four times.

Neutrophils from 60 buffy coats (prepared on 12 consecutive days; 5 buffy coats in each preparation) were washed, resuspended in physiological saline and treated with diisopropyl fluorophosphate (DFP, 5 mM) for 10 min on ice. After washing and resuspension in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM Pipes, pH 7.4), half of the cells were used for subcellular fractionation (see below) while the remaining part was used for direct preparation of glycosphingolipids (3.3×10^{10} cells; designated whole cell preparation).

To prepare activated cells, neutrophils from 30 buffy coats (prepared on 6 consecutive days; 5 buffy coats in each preparation) were washed and resuspended in Krebs-Ringer phosphate buffer containing glucose (10 mM), Ca^{2+} (1 mM), and Mg^{2+} (1.5 mM) (KRG, pH 7.3). The neutrophils (3.0×10^{10}) were incubated at 37°C for 10 min in the presence of cytochalasin B (5 mg/ml) after which ionomycin (10^{-7} M) was added, and the incubation was continued for another 10 min. During this incubation, the cells mobilize their granules to the cell surface, thereby upregulating receptors and releasing granule matrix constituents. After sedimentation, the cells were washed twice in physiological saline, treated with DFP, washed again, resuspended in relaxation buffer and subjected to subcellular fractionation.

Isolation of neutrophil subcellular organelles

Non-activated or ionomycin-activated neutrophils (in total 2.8×10^{10} cells) were subjected to disintegration and subcellular fractionation using the technique described by Borregaard *et al*. [14]. In summary, the cells were suspended in relaxation buffer supplemented with ATP (1 mM), phenylmethanesulfonyl fluoride (0.5 mM) and Pefabloc (1 mM), and were disintegrated by cavitation in a nitrogen bomb (Parr instrument company, Moline, IL). The cavitate was collected in EGTA (1.5 mM final concentration) and the post nuclear supernatant was centrifuged on two-step density gradients of Percoll. Three bands were visible in the gradients and these were denoted α, β and γ in order of decreasing density. The gradients were collected from the bottom of the tube in 1.5-ml fractions and the content of myeloperoxidase (marker for the azurophil granules), vitamin B_{12} binding protein (marker for the specific granules) and alkaline phosphatase (marker for the plasma membrane and secretory vesicles) was measured in these fractions [15–17]. The γ fraction from the ionomycin-activated neutrophils is designated γ_{ACT}.

To isolate granule membranes, the fractions were freeze-thawed five times and washed free of matrix constitutents and Percoll by ultracentrifugation ($100{,}000 \times g$, 2 h). The membranes and whole cell preparation were kept at −70°C until lyophilization.

Glycosphingolipid preparations

Total acid and non-acid glycosphingolipid fractions were isolated as described [18]. 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 separated on a second silicic acid column, followed by deacetylation and dialysis. Final purification was achieved by chromatography on DEAE-cellulose and silicic acid columns.

Isolation of polyglycosylceramides

Polyglycosylceramides were isolated from lyophilized material by the peracetylation method [19], after extraction with chloroform and methanol. The material was purified by Sephadex LH chromatography, followed by deacetylation and dialysis. The polyglycosylceramides from the whole neutrophil cell preparation were further purified by extraction with 2-propanol/hexane/water 55:25:20 (by volume).

Reference glycosphingolipids

Total acid and non-acid glycosphingolipid fractions were obtained by standard procedures [18]. The individual glycosphingolipids were isolated by repeated chromatography on silicic acid columns of the native glycosphingolipid fractions, or acetylated derivatives thereof. The identity of the purified

glycosphingolipids was confirmed by mass spectrometry [20], ^{1}H NMR [21–24], and degradation studies [25,26].

Thin-layer chromatography

Thin-layer chromatography of glycosphingolipids was performed on glass- or aluminum-backed silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). The non-acid glycosphingolipids were chromatographed using chloroform/methanol/water 60:35:8 (by volume) as solvent system. The acid glycosphingolipids were first chromatographed using chloroform/methanol/water 60:35:8 (by volume) as solvent system, and after drying re-chromatographed once using the same solvent system. Chemical detection was done with anisaldehyde [27].

Carbohydrate binding ligands

The binding specificities of the ligands used are summarized in Table 1. The Galα4Gal-binding recombinant *Escherichia coli* strain HB101/pPIL291-15, carrying a plasmid-born *pap* gene cluster with a class II *pap*G allele, was a kind gift from Dr. Irma van Die, Vrije University, The Netherlands, and Dr. Benita Westerlund, University of Helsinki, Finland. *Helicobacter pylori* strain 17874 was from CCUG (Culture Collection University of Göteborg) and the strain 032 was a gift from Dr. Dan Danielsson, Örebro Medical Centre, Sweden. *H. pylori* neutrophil-activating protein (HPNAP) was obtained by the courtesy of Dr. Dolores G. Evans, Houston, Texas.

Solanum tuberosum lectin was a kind gift of Dr. Jeana Ciopraga, Romanian Academy, Bucharest, Hungary. *Erythrina corallodendron* lectin was purchased from Sigma, St. Louis, MO.

The monoclonal antibodies TH2 and FH6 were kind gifts of Dr. Henrik Clausen, University of Copenhagen, Denmark. Mouse monoclonal anti-Le^x antibodies were purchased from Calbiochem, San Diego, CA. Mouse monoclonal anti-VIM-2 antibodies were from Dianova GmbH, Hamburg, Germany. Rabbit polyclonal anti-mouse antibodies were from Dakopatts a/s, Glostrup, Denmark.

Labeling

Lectins, HPNAP, and anti-mouse antibodies were labeled with ^{125}I, using Na^{125}I (100 mCi/ml; Amersham Pharmacia Biotech, Little Chalfont, U.K.), according to the IODO-GEN protocol of the manufacturer (Pierce, Rockford, IL). Approximately 5×10^3 cpm/μg protein was obtained.

Culture and labeling of bacteria

The recombinant *E. coli* strain HB101/pPIL291-15 was cultured in Luria broth supplemented with ampicillin (100 μg/ml) and ^{35}S-methionine (400 μCi/10 ml; Amersham Pharmacia Biotech) at 37°C for 12 h. Conditions for culture and labeling of *H. pylori* were as described [28]. The bacteria were harvested by scraping or centrifugation, washed three times with phosphate-buffered saline (PBS), pH 7.3, and thereafter resuspended in PBS to 1×10^8 CFU/ml. The

Table 1. Carbohydrate binding specificities of bacteria, lectins and monoclonal antibodies used in thin-layer chromatogram binding assays

Ligand	*Specificity*	*Reference*
I. Bacteria		
1. *Escherichia coli* HB 101/pPIL291-15	Galα4Gal	[32]
2. *Helicobacter pylori* CCUG 17874	NeuAcα3Galβ[a]	[35]
3. *Helicobacter pylori* 032	NeuAcα3Galβ[a]	[35]
II. Lectins		
1. *Erythrina corallodendrom*	Galβ4GlcNAcβ and Fucα2Galβ4GlcNacβ	[42]
2. *Solanum tuberosum*	(Galβ4GlcNAcβ)n and Galβ4Glcβ1Cer[b]	[43]
3. Neutrophil-activating protein of *H. pylori*	NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ	[44]
III. Monoclonal antibodies		
1. Anti-Le^x	Galβ4(Fucα3)GlcNAcβ	
2. TH2	GalNAcβ3Galβ4GlcNAcβ	[45]
3. Anti-VIM-2	NeuAcα3Galβ4GlcNAcβ3Galβ4(Fucα3)GlcNAcβ	[38]
4. FH6	NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ	[46]

[a] *H. pylori* strain CCUG 17874 and strain 032 bind to gangliosides with terminal NeuAcα3Galβ. When cultured in broth the bacteria also bind to polyglycosylceramides in a sialic-dependent manner.

[b] *S. tuberosum* lectin binds to glycosphingolipids with one or several *N*-acetyllactosamine units, and in addition selectively interacts with lactosylceramide with sphingosine and non-hydroxy fatty acids.

specific activities of the suspensions were approximately 1 cpm per 100 bacteria.

Glycosphingolipid binding assays

Binding of radiolabeled bacteria to human neutrophil glycosphingolipids separated on thin-layer chromatograms was done as reported previously [28,29]. Dried chromatograms were dipped for 1 min in diethylether/*n*-hexane 1:5 (by volume) containing 0.5% (w/v) polyisobutylmethacrylate (Aldrich Chem. Comp. Inc., Milwaukee, WI). After drying, the chromatograms were soaked in PBS containing 2% bovine serum albumin (w/v), 0.1% NaN_3 (w/v) and 0.1% Tween 20 (by volume) for 2 h at room temperature. The chromatograms were subsequently covered with radiolabeled bacteria diluted in PBS ($2–5 \times 10^6$ cpm/ml). Incubation was done for 2 h at room temperature, followed by repeated washings with PBS. The chromatograms were thereafter exposed to XAR-5 X-ray films (Eastman Kodak, Rochester, NY) for 12 h.

Binding of lectins and monoclonal antibodies to neutrophil glycosphingolipids on thin-layer chromatograms was done as described [30].

Negative ion FAB mass spectrometry

Negative ion FAB mass spectra were recorded on a JEOL SX-102A mass spectrometer (JEOL, Tokyo, Japan). The ions were produced by 6 keV xenon atom bombardment, using triethanolamine (Fluka, Buchs, Switzerland) as matrix, and an accelerating voltage of −10 kV.

Results

Subcellular granule membranes of human neutrophils

Human neutrophil subcellular organelles were isolated on Percoll gradients. Three bands were visible in the gradients and these were denoted α, β and γ in order of decreasing density (Figure 1A). The gradients were collected in 1.5-ml

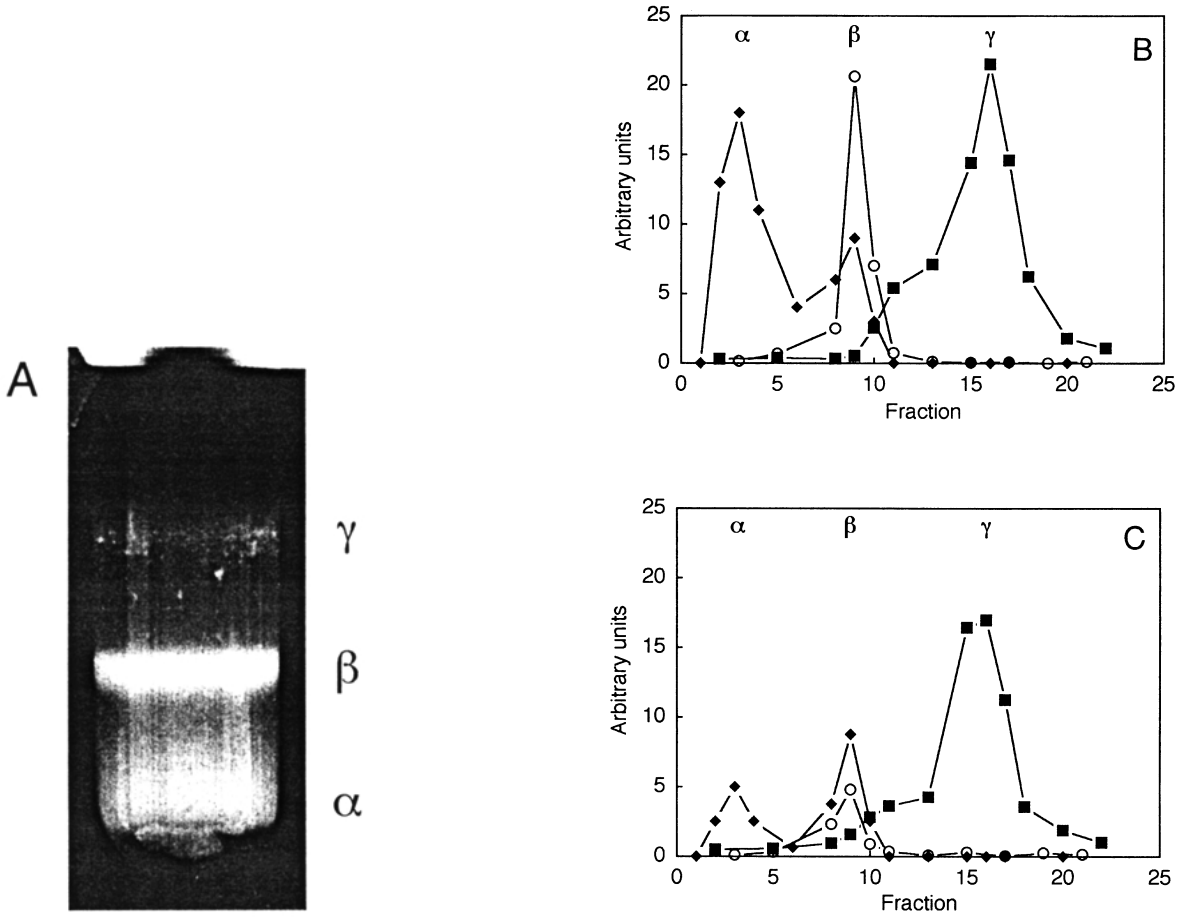

Figure 1. Subcellular fractionation of resting and activated neutrophils. The postnuclear supernatant of a neutrophil homogenate was centrifuged on a two-step Percoll gradient (A) and fractions of 1.5-ml were collected from the bottom of the centrifuge tube. The fractions were analyzed for myeloperoxidase (marker for azurophil granules; α; ◆), vitamin B_{12}-binding protein (marker for the specific granules; β; ○), and total alkaline phosphatase (marker for secretory vesicles and plasma membrane; γ; ■). The distribution of markers in resting neutrophils is shown in (B) , while (C) shows the distribution in ionomycin-activated neutrophils. Abscissa, fraction number; ordinate, amount of marker (arbitrary units).

fractions, and the fractions containing the azurophil granules (α fraction), specific granules (β fraction), and plasma membrane/secretory vesicles (γ fraction) were pooled according to content of marker proteins (Figure 1B). The plasma membrane fraction from ionomycin-activated neutrophils (γ_{ACT}) was isolated accordingly. Ionomycin activation induces degranulation of specific and azurophil granules, seen in Figure 1C as loss of myeloperoxidase (approximately 68%) and vitamin B_{12} binding protein (approximately 73%) from the α and β fractions, respectively.

Glycosphingolipid preparations

The amounts of acid and non-acid glycosphingolipids obtained from membrane preparations of human neutrophils and their subcellular compartments are shown in Table 2. The yields from the subcellular fractions were approximately equal. A noteworthy feature is, however, the increased glycosphingolipid content in the plasma membranes from activated cells (γ_{ACT}), reflecting the transfer of granule membrane to the plasma membrane due to granule fusion with the cell surface.

The amounts of polyglycosylceramides obtained were very low and could not be accurately determined.

Non-acid glycosphingolipids

The patterns of the non-acid glycosphingolipid fractions of human neutrophils, and the subcellular compartments thereof, were very similar when analyzed by thin-layer chromatography and chemical detection (Figure 2A). The fractions were dominated by a compound migrating in the diglycosylceramide region, and minor bands migrating as mono- and tetraglycosylceramides were also vizualized. The one exception was the non-acid fraction of specific granules (β fraction; lane 5), having relatively less of diglycosylceramides, and thereby approximately equal amounts of di- and tetraglycosylceramides.

The major non-acid glycosphingolipid of human neutrophils is lactosylceramide (Galβ4Glcβ1Cer), with sphingosine and non-hydroxy 16:0 and 24:1 fatty acids, which constitutes 75% of the total non-acid fraction [11,31]. In addition, the non-acid fraction of human neutrophils contains lactotriaosylceramide (GlcNAcβ3Galβ4Glcβ1Cer), neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer), neolactohexaosylceramide (Galβ4GlcNAcβ3Galβ4GlcNAcβ3-Galβ4Glcβ1Cer), and a series of glycosphingolipids carrying the Le^x determinant (Galβ4(Fucα3)GlcNAcβ).

The glycosphingolipids were probed with a number of carbohydrate binding ligands in the chromatogram binding assay. The binding specificities of the ligands used are summarized in Table 1.

The binding of the Galβ4GlcNAcβ-recognizing lectins from *E. corallodendron* (Figure 2B) and *S. tuberosum* (Figure 2C) to the tetraglycosylceramide region confirmed the presence of neolactotetraosylceramide in all preparations. The *E. corallodendron* lectin also bound to a more slow-migrating compound, migrating as neolactohexaosylceramide, in all fractions. In the specific granules (β fraction; lane 5) several more slow-migrating *E. corallodendron* lectin-binding compounds were also observed.

The *S. tuberosum* lectin also binds selectively to lactosylceramide with sphingosine and non-hydroxy fatty acids, and the binding pattern obtained with this lectin confirmed that compared with the other non-acid fractions the specific granules (β fraction) had relatively less of lactosylceramide (Figure 2C, lane 5).

Symington *et al*. demonstrated that the majority of lactosylceramide of human neutrophils is present in intracellular granules [11]. This finding was recently confirmed by Kneip and Skubitz [12]. Both groups reported that the specific granules had the highest content of glycosphingolipids, and also the highest content of lactosylceramide, which differs from the results of the present study. The reason for the discrepancy between their findings and our results is unclear.

Monoclonal antibodies directed against the Le^x determinant (Figure 2D) bound to slow-migrating compounds present in all fractions, with most pronounced binding to the specific granules (β fraction; lane 5).

When using the monoclonal antibody TH2, recognizing the x_2 glycosphingolipid (GalNAcβ3Galβ4GlcNAcβ3Galβ4 Glcβ1Cer), a distinction between the neutrophil subcellular compartments was obtained (Figure 2E). The x_2 glycosphingolipid was readily detected in the non-acid fractions

Table 2. Amounts (mg) of acid and non-acid glycosphingolipids obtained from human neutrophils and subcellular compartments thereof

	Cells	γ_{ACT}	α	β	γ
Starting material (No. of cells × 10^{10})	3.3	3.0	2.8	2.8	2.8
Dry weight (g)	3.22	0.5	0.56	0.71	0.36
Total acid glycosphingolipids (mg)	53.5	26.0	11.5	15.0	14.2
Total non-acid glycosphingolipids (mg)	46.1	15.3	15.4	12.6	9.6
mg acid glycosphingolipids per 10^{10} cells	16.2	8.7	4.1	5.3	5.1
mg non-acid glycosphingolipids per 10^{10} cells	13.9	5.1	5.5	4.5	3.4

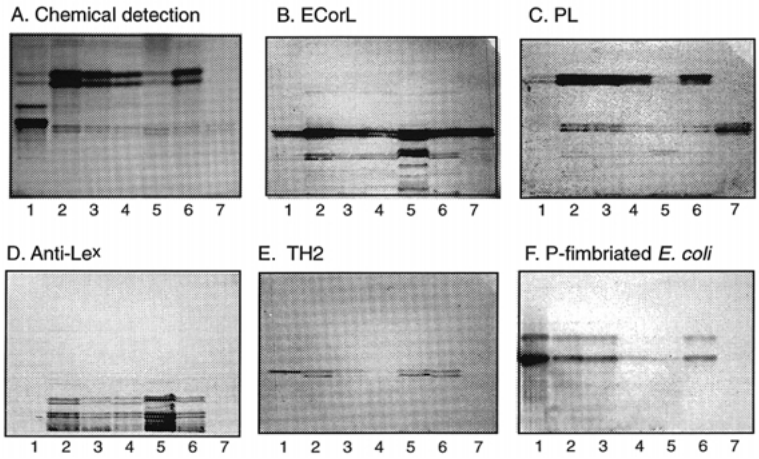

Figure 2. Chromatogram binding experiment showing the binding of *Erythrina corallodendron* lectin, *Solanum tuberosum* lectin, anti-Le^x and TH2 monoclonal antibodies, and P-fimbriated *Escherichia coli*, to non-acid glycosphingolipids of human neutrophil granulocytes. The glycosphingolipids were chromatographed on aluminum-backed silica gel plates and visualized with anisaldehyde (A). Duplicate chromatograms were incubated with *E. corallodendron* lectin (ECorL) (B), *S. tuberosum* lectin (PL) (C), anti-Le^x (D) and TH2 (E) monoclonal antibodies, and P-fimbriated *E. coli* strain HB101/pPIL291-15 (F), followed by autoradiography for 12 h, as described in "Materials and methods". The solvent system used was chloroform/methanol/water 60:35:8, (by volume). The lanes were: lane 1, non-acid glycosphingolipids of human blood group A erythrocytes, 40 μg; lane 2, non-acid glycosphingolipids of human neutrophils whole cells, 40 μg; lane 3, non-acid glycosphingolipids of plasma membrane of activated human neutrophils, 40 μg; lane 4, non-acid glycosphingolipids of azurophilic granules of human neutrophils, 40 μg; lane 5, non-acid glycosphingolipids of specific granules of human neutrophils, 40 μg; lane 6, non-acid glycosphingolipids of plasma membrane/secretory vesicles of resting human neutrophils, 40 μg; lane 7, neolactotetraosylceramide (Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of human granulocytes, 4 μg.

of whole cells (lane 2), plasma membrane of activated cells (γ_{ACT}, lane 3), specific granules (β fraction; lane 5) and plasma membrane/secretory vesicles of resting cells (γ fraction; lane 6). However, the binding to the non-acid fraction of azurophil granules (α fraction; lane 4) was very weak, indicating that this compartment contains only trace amounts of the x_2 glycosphingolipid.

A distinction between the neutrophil subcellular compartments was also detected by using P-fimbriated *E. coli* (Figure 2F). These Galα4Gal-specific bacteria bound to a compound migrating in the tetraglycosylceramide region in all fractions, albeit the binding to the azurophil granules (α fraction; lane 4) and specific granules (β fraction; lane 5) was less pronounced. Binding of P-fimbriated *E. coli* to a compound migrating in the triglycosylceramide region was also detected in the non-acid fractions of whole neutrophil cells (lane 2), plasma membrane of activated cells (γ_{ACT}; lane 3) and plasma membrane/secretory vesicles of resting cells (γ fraction; lane 6), but no binding to the triglycosylceramide region of azurophil granules (α fraction; lane 4) and specific granules (β fraction; lane 5) was obtained.

The compound migrating as triglycosylceramide recognized by P-fimbriated *E. coli* is most likely globotriaosylceramide (Galα4Galβ4Glcβ1Cer), while globoside (GalNAcβ3Galα4-Galβ4Glcβ1Cer) is the binding-active tetraglycosylceramide [32]. The detection of glycosphingolipids interacting with P-fimbriated *E. coli* was unexpected since it has been reported that P-fimbriated *E. coli* do not bind to human neutrophils [33,34]. It should, however, be noted that the presence of globotriaosylceramide and globoside in human neutrophils was inferred based on the binding of Galα4Gal-specific bacteria, but not conclusively demonstrated by solid chemical criteria. Isolation and characterization of the compounds with P-fimbriated *E. coli*-binding activity from human neutrophils will be reported separately (Karlsson *et al.*, manuscript in preparation).

Acid glycosphingolipids

Detailed structural characterization of human neutrophil acid glycosphingolipids have established that these cells contain a very complex ganglioside mixture [8–10]. Apart from the GM3 ganglioside (NeuAcα3Galβ4Glcβ1Cer), the acid glycosphingolipids have one or several *N*-acetyllactosamine moieties, where one or more of the *N*-acetylglucosamines may be substituted with α3-linked fucose(s). The terminal sialic acid may be α6- or α3-linked.

The initial analysis with thin-layer chromatography and chemical detection (Figure 3A) showed a band migrating as the GM3 ganglioside in the preparation from whole neutrophil cells (lane 1) and plasma membrane/secretory vesicles of resting cells (γ fraction; lane 5). However, this band was not seen in the acid fractions of plasma membrane of activated neutrophils (γ_{ACT}; lane 2), azurophil granules (α fraction; lane 3) or specific granules (β fraction; lane 4).

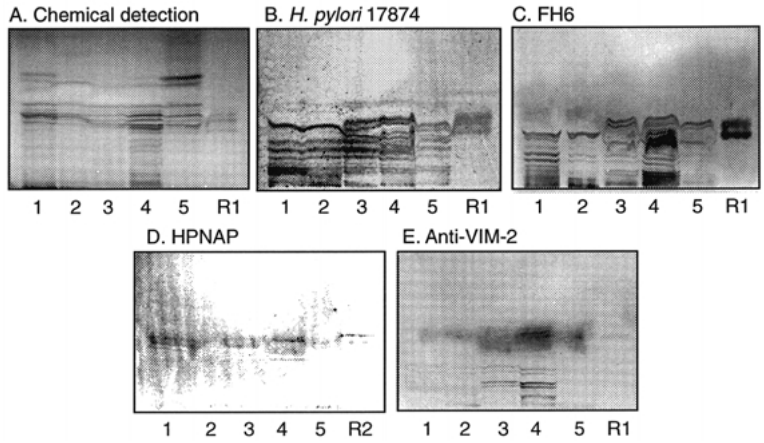

Figure 3. Chromatogram binding experiment showing the binding of *Helicobacter pylori* CCUG 17874, FH6 monoclonal antibodies, the neutrophil-activating protein of *H. pylori*, and anti-VIM-2 monoclonal antibodies to acid glycosphingolipids of human neutrophil granulocytes. The glycosphingolipids were chromatographed on aluminum-backed silica gel plates and visualized with anisaldehyde (A). Duplicate chromatograms were incubated with *H. pylori* strain CCUG 17874 (B), FH6 monoclonal antibodies (C), the neutrophil-activating protein of *H. pylori* (HPNAP) (D), and anti-VIM-2 monoclonal antibodies (E), followed by autoradiography for 12 h, as described under "Materials and methods". The chromatograms were developed two times in chloroform/methanol/water 60:35:8 (by volume). The lanes were: lane 1, acid glycosphingolipids of human neutrophil whole cells, 40 μg; lane 2, acid glycosphingolipids of plasma membrane of activated human neutrophils, 40 μg; lane 3, acid glycosphingolipids of azurophilic granules of human neutrophils, 40 μg; lane 4, acid glycosphingolipids of specific granules of human neutrophils, 40 μg; lane 5, acid glycosphingolipids of plasma membrane/secretory vesicles of resting human neutrophils, 40 μg; lane 6, R1, reference sialyl dimeric Le^x nonaglycosylceramide (NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer) of human gallbladder adenocarcinoma, 4 μg/R2, reference sialylneolactohexaosylceramide (NeuGcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ3Galβ4Glcβ1Cer) of rabbit thymus, 4 μg.

A specific interaction of certain *H. pylori* strains with gangliosides of human neutrophil granulocytes has previously been described [35,36]. Binding of *H. pylori* strain CCUG 17874 (Figure 3B) to the acid fractions of human neutrophil whole cells and subcellular compartments confirmed the complex binding pattern, with a number of minor binding-positive bands migrating at and below the level of the reference sialyl dimeric Le^x ganglioside (lane *R1*). The distribution of *H. pylori*-binding bands varied somewhat between the subcellular fractions. A very similar binding pattern was obtained with the sialyl dimeric Le^x-binding monoclonal antibody FH6 (Figure 3C). The parallel binding patterns thus suggests that the structural element recognized by *H. pylori* on human neutrophils is the sialyl dimeric Le^x sequence. The picture is, however, still confounded since it was recently reported that the FH6 antibody also binds to linear sialylneolactohexaosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβGalβ4Glcβ1Cer) [9]. Chemical determination of the *H. pylori*-binding sequence(s) is thus of crucial importance, although this is difficult to achieve due to the extensive structural heterogeneity of human neutrophil gangliosides.

The binding pattern obtained with the NeuAcα3Galβ4GlcNAcβ3Galβ4GlcNAcβ-recognizing neutrophil-activating protein of *H. pylori* (HPNAP; Figure 3D) was distinct from the pattern of the bacterial cells. HPNAP-binding gangliosides were detected mainly in the whole cell preparation (lane 1), azurophil granules (α fraction; lane 3) and specific granules (β fraction; lane 4). Interestingly, only weak binding of HPNAP to the gangliosides of plasma membrane/secretory vesicles of resting cells (γ fraction; lane 5) and plasma membrane of activated neutrophils (γ_{ACT}; lane 2) was detected.

A striking difference in the expression of glycosphingolipids carrying the VIM-2 epitope was found by using anti-VIM-2 antibodies in the chromatogram binding assay (Figure 3E). The VIM-2 epitope (NeuAcα3Galβ4GlcNAcβ3-Galβ4(Fucα3)GlcNAcβ) is on human neutrophil gangliosides predominantly found on decaglycosyl- and dodecaglycosylceramides [37]. Although some degree of cross-reactivity was noted, the antibodies preferentially bound to three slow-migrating double bands in the preparation from specific granules (β fraction; lane 4) and to two double bands in the sample from azurophil granules (α fraction; lane 3). No binding of the anti-VIM-2 antibody to the preparation of plasma membrane/secretory vesicles of resting neutrophils (γ fraction; lane 5) was obtained, while the binding to the plasma membrane of activated cells (γ_{ACT}; lane 2) and to the whole cell preparation (lane 1) was very weak, indicating that VIM-2-carrying glycosphingolipids are only to a minor degree exposed on the cell surface, even on activated neutrophils.

Negative ion FAB mass spectrometry of acid glycosphingolipids of human neutrophil subcellular compartments

Negative ion FAB mass spectra of the total acid glycosphingolipid fractions of the subcellular compartments of human neutrophils are shown in Figure 4. All spectra had two $[M\text{-}H]^-$ ions at m/z 1517 and 1627, corresponding to a NeuAc-hexose-*N*-acetylhexosamine-hexose-hexose sequence with d18:1-16:0 and d18:1-24:1 ceramides. Fragment ions, obtained by loss of terminal carbohydrate units, were found at m/z 1226 and 1337. The spectra of acid glycosphingolipids of the whole cell preparation (Figure 4A), plasma membrane of activated cells (Figure 4B), azurophil granules (Figure 4C), and plasma membrane/secretory vesicles of resting neutrophils (Figure 4E), also had $[M\text{-}H]^-$ ions at m/z 1151 and 1263, indicating a NeuAc-hexose-hexose sequence (NeuAc-GM3) with d18:1-16:0 and d18:1-24:0 ceramides. These ions were not found in the spectrum of the acid glycosphingolipids of specific granules (Figure 4D). Also the relative intensities differed between the spectra, *i.e.* the ions at m/z 1517 and 1627, and fragment ions thereof, where predominant in the spectra of the acid glycosphingolipids of plasma membrane of activated neutrophils (Figure 4B) and azurophil granules (Figure 4C), while in the spectra of the acid glycosphingolipids of the whole cell preparation (Figure 4A), and plasma membrane/secretory vesicles of resting neutrophils (Figure 4E) the ions at at m/z 1151 and 1263 were in dominance.

Polyglycosylceramides

Polyglycosylceramides are complex glycosphingolipids with 15 or more carbohydrate units. The amount of polyglycosylceramides in granulocytes is approximately 20 times lower than the amount of common gangliosides [36]. Due to these low levels a precise chemical quantitation of the amounts of polyglycosylceramides obtained from the neutrophil subcellular compartments was not possible. However, Figure 5A, where the amounts in lanes 2–6 correspond to 12 mg starting material, allows a visual evaluation and comparison of the concentrations of polyglycosylceramides in different neutrophil subfractions. Well defined fractions were seen in the preparation from whole cells (lane 6), plasma membrane of activated cells (γ_{ACT}; lane 2), specific granules (β fraction; lane 4), and plasma membrane/secretory vesicles of resting neutrophils (γ fraction; lane 5), while only trace amounts of polyglycosylceramides were obtained from the azurophil granules (α fraction; lane 3).

When cultured in liquid medium *H. pylori* binds selectively to a unique sialylated epitope on polyglycosylceramides of human origin [38]. By binding of liquid-cultured *H. pylori* to the isolated polyglycosylceramides of human neutrophil subcellular fractions, binding-active compounds were detected in all fractions (Figure 5B), *i.e.* even in the minute polyglycosylceramide fraction of azurophil granules (α fraction; lane 3).

Discussion

The specific granule fraction (comprising both the lactoferrin- and the gelatinase-containing granule subsets) is particularly rich in membrane proteins. In addition, these granules store a number of different glycosphingolipids. Some of these are shared with the azurophil granules and/or the plasma membrane, whereas others are unique to the specific granule membrane (Table 3). This membrane had a predominance of slow-migrating acid as well as non-acid glycosphingolipids, giving pronounced binding of ligands recognizing slow-migrating compounds, such as the neutrophil-activating protein of *H. pylori* (HPNAP), anti-Le^x antibodies and anti-VIM-2 antibodies.

The neutrophil azurophil granules resemble in many aspects the lysosomes in other cells, but they lack one of the classical lysosomal membrane markers, the highly glycosylated lysosomal associated membrane glycoprotein, LAMP [39]. There are to date only three membrane proteins identified in the azurophil granules, CD63, CD68 and a V-type H^+-ATPase [3]. With respect to glycosphingolipids we show that lactosylceramide is a major component of the azurophil granule membrane. The membrane also contains relatively high amounts of gangliosides carrying the VIM-2 epitope. In addition we found slow-migrating acid and non-acid glycosphingolipids as well as polyglycosylceramides, mediating significant binding of several of the used probes, but not of ligands such as the HPNAP or the anti-TH2 antibodies. Neutrophil granules are believed to be formed by aggregation of membrane vesicles that are formed in the ER-Golgi transport pathway. It has been suggested that the membrane vesicles forming the azurophil granules bud off from the *cis*-Golgi. However, the addition of β-linked galactose residues during glycosphingolipid synthesis (giving lactosylceramide) takes place in the intermediate and *trans* cisterna of the Golgi stacks. Further elongation giving rise to a wide variety of different cores also takes place in the *trans*-Golgi network. The fact that the azurophil granule membrane contains glycosphingolipids with complex oligosaccharides suggests that the membrane of these storage granules formed during the promyelocyte stage, have received membranes also from the *trans*-Golgi, possibly achieved through fusion of immature granules with some type of transport vesicles.

The binding characteristics of the plasma membrane fraction isolated from activated neutrophils are very similar to that of the plasma membrane of resting cells, with a few exceptions. One single binding structure, recognized by the anti-VIM-2 antibody, was absent in the plasma membrane of resting cells but present in the activated plasma membrane. This suggests that granules not only store the reserve pools of plasma membrane glycosphingolipids, but that they also store specific carbohydrate epitopes that are upregulated to the plasma membrane upon cell activation and thus are exposed only on activated cells. As compared to the plasma membrane

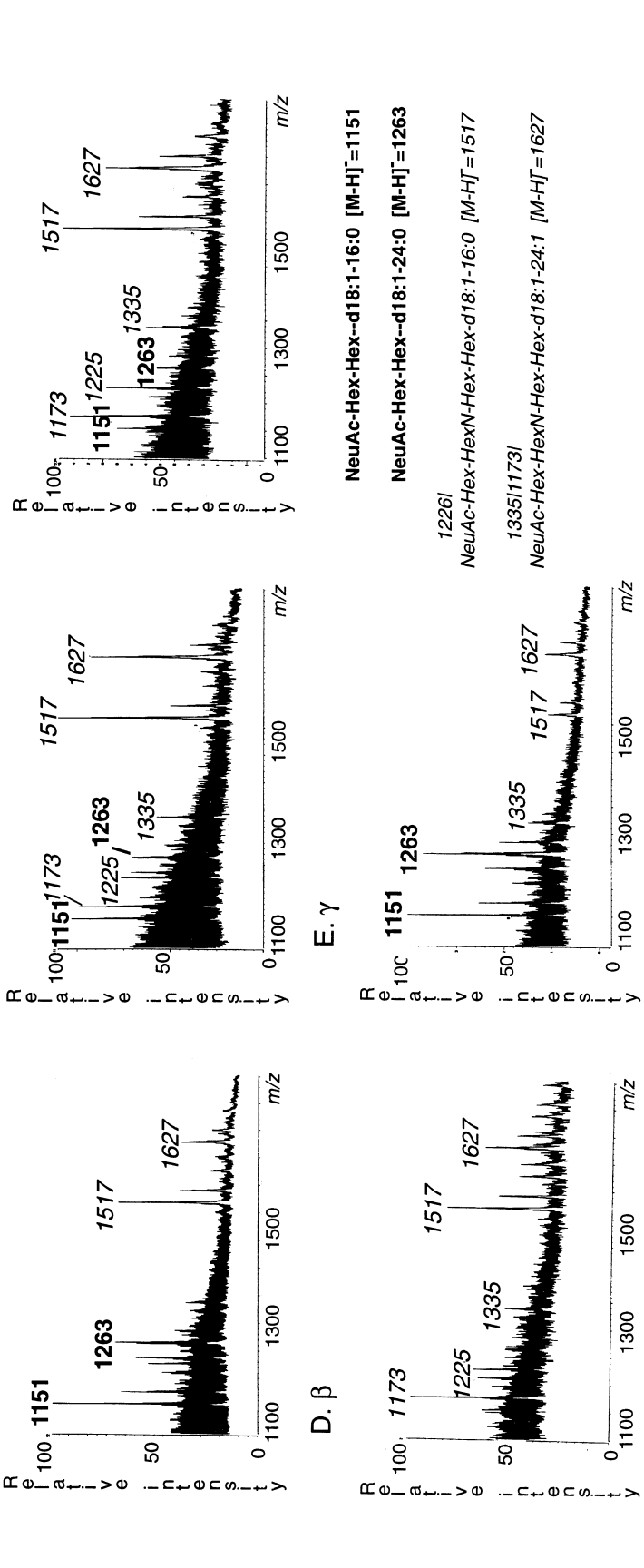

Figure 4. Negative ion FAB mass spectra of the acid glycosphingolipid fractions isolated from human neutrophil subcellular compartments. The analyses were done as described in "Materials and methods".

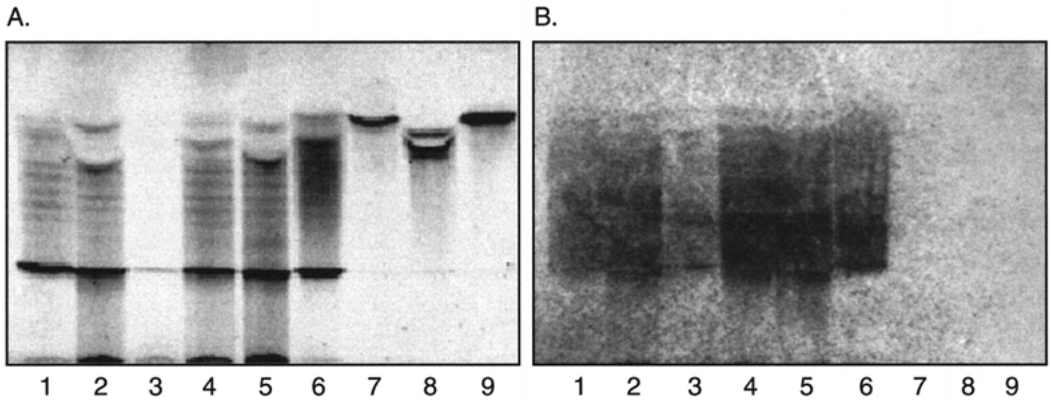

Figure 5. Chromatogram binding experiment showing the binding of *Helicobacter pylori* to polyglycosylceramides of human neutrophil subcellular compartments. The polyglycosylceramides were chromatographed on silica gel plates, using chloroform/methanol/water 50:55:19 (by volume) as solvent system. The chromatogram in (A) was visualized with anisaldehyde. The duplicate chromatogram in (B) was incubated with radiolabeled *H. pylori* strain 032 cultured in broth, followed by autoradiography for 12 h, as described under ''Materials and methods''. The lanes were: lane 1, reference polyglycosylceramides of human granulocytes, 10 μg; lane 2, polyglycosylceramides of plasma membrane of activated human neutrophils; lane 3, polyglycosylceramides of azurophilic granules of human neutrophils; lane 4, polyglycosylceramides of specific granules of human neutrophils; lane 5, polyglycosylceramides of plasma membrane/secretory vesicles of resting human neutrophils; lane 6, polyglycosylceramides of total human neutrophil cells; lane 7, reference sialylneolactotetraosylceramide (NeuAcα3Galβ4GlcNAcβ3Galβ4Glcβ1Cer), 1.5 μg; lane 8, reference bovine brain gangliosides 5 μg; lane 9, reference sialyl-Le^x hexaglycosylceramide (NeuAcα3Galβ4(Fucα3)GlcNAcβ3Galβ4Glcβ1Cer), 1 μg. The amounts applied in lanes 2–6 correspond to 12 mg of dry starting material.

Table 3. Summary of results from thin-layer chromatogram binding assays

Ligand	*Cells*	γ_{ACT}	α	β	γ	*Comments*
I. Non-acid glycosphingolipids						
1. *Erythrina corallodendron* lectin	++	+	+	+++	+	Binding in tetraglycosylceramide region
	+++	+	+	+++	+	Binding in hexaglycosylceramide region
2. *Solanum tuberosum lectin*	+++	+++	+++	+	+++	Binding in diglycosylceramide region
	++	++	+	+	+	Binding in tetraglycosylceramide region
3. Anti-Le^x	++	++	++	+++	++	
4. TH2	+++	+++	−	+++	+++	
5. *Escherichia coli* HB 101/pPIL291-15	+	+	−	−	+	Binding in triglycosylceramide region
	++	++	+	+	++	Binding in tetraglycosylceramide region
II. Acid glycosphingolipids						
6. *Helicobacter pylori* CCUG 17874	+++	+++	+++	+++	+++	
7. FH6	+++	++	++	+++	++	
8. Anti-VIM-2	(+)	(+)	++	+++	−	
9. Neutrophil-activating protein of *H. pylori*	++	(+)	++	+++	(+)	
II. Polyglycosylceramides						
10. *Helicobacter pylori* 0.32[a]	+++	+++	+++	+++	+++	

[a]Binding to polyglycosylceramides was tested after culture of the bacteria in liquid medium

of resting cells, the plasma membrane of activated neutrophils contained relatively low amounts of the GM3 ganglioside, not detectable by chemical staining, but only by mass spectrometry. Ions derived from the GM3 ganglioside were also present in the mass spectra of the acid fractions of the plasma membrane/secretory vesicles of resting cells and of the azurophil granules. However, no ions corresponding to the GM3 ganglioside were found in the spectrum of the acid

fraction of the specific granules. Although negative ion FAB mass spectrometry does not allow definite quantitation, the relative abundance of the ions corresponding to the GM3 ganglioside and the NeuAc-hexose-*N*-acetylhexosamine-hexose-hexose-Cer compound (sialyl-neolactotetraosylceramide) in the spectra of the different acid fractions were in agreement with the thin-layer chromatogram analysis. The reduction of GM3 content in activated plasma membrane may thus be explained by a mechanism where incorporation of membranes with low or lacking content of GM3, *i.e*., the azurophil and specific granule membranes, into the plasma membrane dilutes the GM3 ganglioside content of this membrane. A parallel finding is the reduced level of lactosylceramide on the cell surface of activated neutrophils [40]. This event was primarily associated with exocytosis of specific granules, and is in agreement with the relatively low levels of lactosylceramide found in specific granules in the present study.

No functions of glycosphingolipids have yet been conclusively established with respect to neutrophil function, but there are many putative roles such as the specific binding to endothelial cells upon cell migration. The inflammatory response is initialized by rolling of the neutrophils on the endothelium, a process that involves interactions between P- and E-selectins on the endothelial cells and glycoconjugate ligands on the neutrophil. Among the potential ligands for E-selectin are sialyl-fucosyl-poly-*N*-acetyllactosamine residues which may be exposed on glycosphingolipids or polyglycosylceramides on myeloid cells [41]. These binding epitopes may be partially shared with *H. pylori* binding sites, demonstrating the complex roles of glycoconjugates in neutrophil activation. With respect to the function of neutrophil granules, these are not just simple storage bags for proteolytic or bactericidal matrix proteins, but are also important reservoirs of membrane components that are exposed on the neutrophil cell surface upon fusion of these organelles with the plasma membrane. Granule mobilization may thus fundamentally regulate/alter the interaction pattern of the neutrophil with its environment.

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