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Different binding capacities of influenza A and Sendai viruses to gangliosides from human granulocytes

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The structures of gangliosides from human granulocytes were elucidated by fast atom bombardment mass spectrometry and by gas chromatography/mass spectrometry as their partially methylated alditol acetates. In human granulocytes besides G_{M3} (II^3Neu5Ac-LacCer), neolacto-series gangliosides (IV^3Neu5Ac-nLcOse$_4$Cer, IV^6Neu5Ac-nLcOse$_4$Cer and VI^3Neu5Ac-nLcOse$_6$Cer) containing $C_{24:1}$, and to some extent $C_{22:0}$; and $C_{16:0}$ fatty acid in their respective ceramide portions, were identified as major components. In this study we demonstrate that gangliosides from human granulocytes, the second most abundant cells in peripheral blood, can serve as receptors for influenza viruses A/PR/8/34 (H1N1), A/X-31 (H3N2), and a parainfluenza virus Sendai virus (HNF1, Z-strain). Viruses were found to exhibit specific adhesion to terminal Neu5Acα2-3Gal and/or Neu5Acα2-6Gal sequences as well as depending on the chain length of ganglioside carbohydrate backbones from human granulocytes, these important effector cells which represent the first line of defence in immunologically mediated reactions.

Keywords: Gangliosides, human granulocytes, TLC overlay assay, receptor, influenza A virus, Sendai virus

Abbreviations: FAB-MS, fast atom bombardment mass spectrometry; GC/EIMS, gas chromatography/electron impact mass spectrometry; GSL(s) glycosphingolipids; HPTLC, high performance thin-layer chromatography; Neu5Ac, *N*-acetylneuraminic acid [26], PFU, plaque forming unit. The designation of the following glycosphingolipids follows the IUPAC-IUB recommendations, and the ganglioside nomenclature system of Svennerholm was used. LacCer or lactosylceramide, Galβ1-4Glcβ1-1Cer; gangliotetraosylceramide or GgOse$_4$Cer, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-1Cer; lacto-*N*-tetraosylceramide or nLcOse$_4$Cer, Galβ1-4GlcNAcβ1-3Galβ-1-4-Glcβ1-1Cer; lacto-*N*-norhexaosylceramide or nLcOse$_6$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ 1-4-Glcβ1-1Cer; G_{M3}, II^3Neu5Ac-LacCer; G_{M1}, II^3Neu5Ac-GgOse$_4$Cer; G_{D1a}, IV^3Neu5Ac, II^3Neu5Ac-GgOse$_4$-Cer; G_{D1b}, II^3(Neu5Ac)$_2$-GgOse$_4$Cer; G_{T1b}, IV^3Neu5Ac, II^3(Neu5Ac)$_2$-GgOse$_4$Cer; G_{Q1b}, IV^3(Neu5Ac)$_2$, II^3(Neu5Ac)$_2$-GgOse$_4$Cer; sialyllacto-*N*-tetraosylceramide, IV^3Neu5Ac/IV^6Neu5Ac-nLcOse$_4$Cer; sialyllacto-*N*-norhexaosylceramide or i-active ganglioside, VI^3Neu5Ac-nLcOse$_6$Cer.

Introduction

Granulocytes are the second most abundant cells in peripheral blood, and their primary function is phagocytosis and the engulfing of pathogenic microorganisms. Gangliosides, glycosphingolipids (GSLs) containing sialic acids [1], are common constituents of the cell surface. Peripheral blood lymphocytes, monocytes and granulocytes have distinct 'GSL fingerprints' [2]. The predominant ganglioside species of granulocytes belong to the neolacto-series based on the neutral core (Galβ1-4GlcNAcβ1-3)$_n$-Galβ1-4Glcβ1-1Cer (where $n = 0$–2) [3]. One sialic acid is attached in the Neu5Acα2-3Gal or Neu5Acα2-6Gal sequence to the terminal galactose residue. Terminally sialylated gangliosides are important receptor binding sites for viruses

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and able to mediate virus attachment [4], a prerequisite for virus penetration into host cells. Orthomyxoviruses exhibit specificity of the sialooligosaccharide sequence in binding to cells which is mediated by influenza virus haemagglutinin (HA) and Sendai virus haemagglutinin-neuraminidase (HN) protein, respectively [4, 5]. The interaction of influenza A and Sendai virus with ganglio-series gangliosides from brain and gangliosides of the neolacto-series, isolated from human and bovine erythrocytes and meconium, has been reported by several groups [6–10]. In this study we demonstrate that neolacto-series gangliosides from human granulocytes are receptors of influenza A and Sendai virus using a direct solid-phase binding assay (overlay-assay) [11].

Materials and methods

Gangliosides from human granulocytes

Human leucocytes enriched with granulocytes were prepared from buffy coats as described [12]. Gangliosides were isolated and purified by standard procedures [13,14]. The GSLs were extracted with chloroform:methanol (2:1) and (1:2), each by vol. The combined extracts were evaporated and partitioned as described by Folch *et al.* [15]. Gangliosides of Folch upper phases were separated from neutral GSLs by anion exchange chromatography on DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals, Freiburg, Germany) as described by Müthing *et al.* [16]. Gangliosides were eluted with 0.45 M ammonium acetate in methanol. After evaporation and desalting by dialysis, the ganglioside fraction was incubated for 1 h at 37 °C in 1 N NaOH to hydrolyse phospholipids followed by neutralization with acetic acid and dialysis. Gangliosides were then further purified by Iatrobeads 6RS-8060 chromatography (Macherey-Nagel, Düren, Germany) as described by Ueno *et al.* [17]. Stepwise elution was performed with chloroform:methanol (85:15), (3:1), (2:1), (1:2), each by vol, and finally methanol. Gangliosides eluting with chloroform:methanol (2:1) and (1:2) were pooled.

Analytical and preparative thin-layer chromatography

High performance thin-layer chromatography plates (HPTLC silica plates, size 10 cm × 10 cm, thickness 0.24 mm, E. Merck, Germany) were used for analytical and preparative purposes. Gangliosides were separated in chloroform:methanol:water (120:85:20, each by vol) containing 2 mM $CaCl_2$ and visualized by resorcinol [18].

Individual gangliosides were isolated and purified by preparative HPTLC as recently published [19] using the fluorochrome pyrene-1-aldehyde for nondestructive detection. Briefly, gangliosides were applied to the plates with the automatic sample applier Linomat IV (CAMAG, Muttenz, Switzerland) and chromatographed as described above. After drying, the plates were sprayed thoroughly with 0.002% (w/v) fluorochrome in acetone:methanol (60:40, each by vol). Fluorescent zones containing the gangliosides were scraped off and gangliosides were extracted from the silica gel with chloroform:methanol:water (30:60:8, each by vol) followed by anion exchange chromatography which leads to separation of gangliosides from the fluorochrome. Finally, impurities were removed by Iatrobeads chromatography (see above).

HPTLC-immunostaining assay with cholera toxin B subunit (choleragenoid)

The HPTLC-binding method using choleragenoid for specific detection of G_{M1} has been developed by Magnani *et al.* [20]. We modified the described procedure as follows. Gangliosides were chromatographed on HPTLC plates as described above. The plate was dried for 0.5 h over P_2O_5 in a desiccator equipped with a vacuum pump and the silica gel was fixed by chromatography in hexane saturated polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany). The plate was incubated with choleragenoid (250 ng ml^{-1}, Sigma, No. C-7771, Deisenhofen, Germany) for 2 h at room temperature. Goat anti-choleragenoid antiserum (1:1000, Calbiochem, No. 227040, Frankfurt, Germany) and alkaline phosphatase conjugated rabbit anti-goat IgG antibody (1:1000, Dianova, Hamburg, Germany) were used for the immunostaining procedure (each 1 h at room temperature). Alkaline phosphatase activity was detected with 5-bromo-4-chloro-3-indolylphosphate. To reveal the presence of gangliosides with G_{M1}-core, i.e., G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b}, neuraminidase is employed to convert these gangliosides to G_{M1} prior to treatment with choleragenoid. Silica gel fixed plates were incubated with 50 mU ml^{-1} *V. cholerae* neuraminidase for 18 h at 37 °C in 0.05 M sodium acetate, 9 mM $CaCl_2$, pH 5.5. The subsequent immunostaining assay with choleragenoid was performed as described above. The technique was originally developed by Wu and Ledeen [21].

Fast atom bombardment mass spectrometry

Fast atom bombardment mass spectrometry (FAB-MS) was carried out on a ZAB-HF mass spectrometer (VG Analytical, Manchester, UK) essentially as described earlier [22]. The native samples of gangliosides were dissolved in chloroform:methanol (1:1 by vol) and desorbed from the matrix thioglycerol (EGA Chemie, Steinheim, Germany) using xenon as bombarding gas. The mass spectra were acquired as single scans in the upscan mode on an AMD DP10 data system fitted with SAMII (KWS) hardware and SUSY software (AMD Intectra, Beckeln, Germany). The mass values found were obtained after calibration with cesium iodide and do not represent nominal, but physical numbers.

Gas chromatography/mass spectrometry

Purified gangliosides were permethylated, hydrolysed and the resulting monosaccharide derivatives were reduced with NaB^2H_4 followed by acetylation according to Levery and

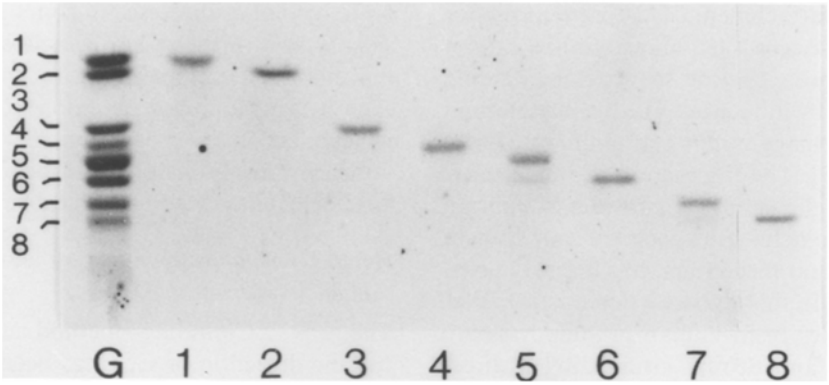

Figure 1. Resorcinol stain of individual gangliosides isolated from human granulocytes by preparative HPTLC. G, total granulocyte gangliosides. Gangliosides 1–8 are listed in Table 1.

Hakomori [23]. The derivatives were analysed by gas chromatography/mass spectrometry (GC/MS) essentially as described earlier [24]. Briefly, separation of the partially methylated alditol acetates was achieved on a capillary column (15 m) wall coated with SE54, heated from 100 °C to 300 °C at 10 °C min^{-1}. Detection of the derivatives was performed by single-ion monitoring using dwell times of 0.02 s on an MSD 5970.

Viruses

Human influenza virus A/PR/8/34 (H1N1) and a reassortant between A/PR/8/34 and A/Aichi/2/68, A/X-31 (H3N2) were propagated in embryonic chicken fibroblasts and Sendai virus (HNF1, Z-strain) was grown in MDCK cells [25]. The multiplicity of infection was about 5 PFU per cell and metabolic labelling with L-[^{35}S]methionine (Amersham Buchler, Braunschweig, Germany) was carried out from 5 h to 24 h after infection. 27.75 MBq (750 μCi) for each virus were used. Progeny virus was purified by adsorption to and elution from chicken erythrocytes and subsequent centrifugation in a Beckman SW 28 rotor for 90 min at 25 000 rev min^{-1} (120 000 × g). The sediment was resolved in 1 ml phosphate buffered saline (PBS, 100 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.3). Inactivation of influenza A/PR/8/34 was performed by heat treatment at 56 °C for 60 min.

HPTLC-overlay assay with ^{35}S-labelled viruses

Ganglioside chromatography and silica gel fixation were performed as described above. To reduce the amounts of labelled viruses, the plates were cut with a diamond glass cutter into strips of 1.5 cm × 10 cm per lane. The strips were soaked for 15 min in solution A (phosphate buffered saline, supplemented with 1% bovine serum albumin) to block unspecific binding sites. The solution was thoroughly withdrawn by suction and 80 μl labelled virus preparation was added per lane (about 2 × 10^5 counts min^{-1}). The strips were covered with small pieces of parafilm™ and kept in a humidified atmosphere for 2 h at +4 °C. After incubation the virus suspension was tipped off and the plate was washed six times with PBS. The dried plate was exposed to Hyperfilm™-^{3}H (Amersham Buchler, Braunschweig, Germany) for 20 days at +4 °C. Stained ganglioside chromatograms and virus autoradiographies were scanned with the Desaga CD60 scanner (Heidelberg, Germany) equipped with an IBM AT compatible personal computer and densitometric software. Intensities of resorcinol stained bands and autoradiographies were measured in reflectance mode at 580 nm with a light beam slit of 0.1 mm × 2 mm. Ratios of bound ^{35}S-labelled viruses to gangliosides were calculated from the average of three scans from each spot.

Results and discussion

Individual gangliosides from human granulocytes were isolated and purified from the whole ganglioside fraction by preparative HPTLC (see Fig. 1). The eight main gangliosides were structurally characterized by FAB-MS of native samples and GC/MS of respective partially methylated alditol acetates.

Structural assignment of major ganglioside fractions using FAB-MS of native samples and GC/EIMS of partially methylated alditol acetates

The migrational properties of ganglioside species containing the same sugar unit, appearing as double bands on TLC, are determined by the presence of different long chain fatty acids in their respective ceramide portions. The fractions with higher R_F values of ganglioside pairs are characterized by the presence of $C_{24:1}$ and to some extent by $C_{22:0}$ fatty acids, whereas $C_{16:0}$ fatty acids are characteristic for the fractions showing lower R_F values. From the negative ion FAB mass spectra of native gangliosides, the molecular weight, the number and sequence of monosaccharide units as well as the nature of the ceramide portion can be clearly deduced [22] (Table 1). For example, in the FAB-MS of

Table 1. Structural assignment of ions observed in negative ion FAB-MS of ganglioside samples isolated from human granulocytes.

Ganglioside fraction No.	$[M-H]^-$ *m/z*	Cer^- *m/z*	*Fatty acid*	$LacCer^-$ *m/z*	$Hex_3HexNAcCer^-$ *m/z*	*Composition*
1	1261	646	24:1	970	—	$Neu5AcHex_2Cer$
	1235	620	22:0	944	—	$Neu5AcHex_2Cer$
2	1151	536	16:0	860	—	$Neu5AcHex_2Cer$
3	1626	646	24:1	970	1335	$Neu5AcHex_3HexNAcCer$
	1600	620	22:0	944	1309	$Neu5AcHex_3HexNAcCer$
4	1516	536	16:0	860	1225	$Neu5AcHex_3HexNAcCer$
5	1626	646	24:1	970	1335	$Neu5AcHex_3HexNAcCer$
	1516	536	16:0	860	1225	$Neu5AcHex_3HexNAcCer$
6	1516	536	16:0	860	1225	$Neu5AcHex_3HexNAcCer$
7	1991	646	24:1	970	1335	$Neu5AcHex_4HexNAc_2Cer$
8	1881	536	16:0	860	1225	$Neu5AcHex_4HexNAc_2Cer$

the native fraction 4 (Fig. 2a) IVNeu5Ac-nLcOse$_4$Cer containing $C_{16:0}$ fatty acid in the ceramide portion was identified by its specific ions at $m/z = 1516$ ($[M-H]^-$), 1225 ($[M-$ H-Neu5Ac$]^-$), 1063 ($[M-$ H-Neu5Ac-Gal$]^-$), 860 ($[M-$ H-Neu5Ac-Gal-GlcNAc$]^-$), 698 ($[M-$ H-Neu5Ac-Gal-GlcNAc-Gal$]^-$), and 536 ($[Cer]^-$). The Neu5Ac-containing trisaccharide ion from the nonreducing terminus appeared at $m/z = 673/671$ ([Neu5Ac-Hex-HexNAc]$^-$). In fraction 8 the presence of VINeu5Ac-nLcOse$_6$Cer containing $C_{16:0}$ fatty acid was characterized by the same series of ions from the ceramide terminus as well as the molecular ion at $m/z = 1881$ (Fig. 2b).

The gangliosides of neolacto type are sialylated at position 3 or 6 of the galactose at the nonreducing terminus. The unequivocal assignment of the Neu5Ac attachment was analysed by GC/EIMS of partially methylated alditol acetates run in selective ion monitoring mode [23]. The 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol ($R_t = 9.0$ min) was present in all fractions investigated, but the 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol ($R_t = 9.4$ min) was only in fractions 5 and 6, indicating α(2-6) attachment of Neu5Ac in fractions 5 and 6 and α(2-3) linked Neu5Ac in the remaining fractions (Table 2). The neolacto core was deduced from the presence of 4-linked *N*-acetylglucosamine ($R_t = 11.6$ min) in fractions 3–8 (Table 2). In contrast to the neolacto type gangliosides of fractions 3–7, considerable amounts of 3-linked galactose were found in fraction 8 (see Table 2). Since contamination by ganglio series gangliosides can be excluded (see below), structural features in the inner portion of the carbohydrate remain obscure and will be the subject of further investigation.

Unidentified gangliosides which chromatographed below VI^{3}Neu5Ac-nLcOse$_6$Cer (see Fig. 3, lane 1) were designated X, Y and Z. Due to their chromatographic behaviour their presumed structures are VI^{6}Neu5Ac-nLcOse$_6$Cer, known to occur in human granulocytes, and Neu5Acα2-3 as well as Neu5Acα2-6 substituted neolacto-GSLs with carbohydrate backbones longer than that in nLcOse$_6$Cer which are, however, speculative at this point of research. The theoretical possibility that these bands contain higher sialylated gangliosides can be excluded due to previous studies [3] and our own results. Granulocyte gangliosides were examined for the presence of ganglio series gangliosides. G_{M1} can be visualized on the HPTLC plate with choleragenoid using the overlay technique [20]. The gangliosides G_{D1a}, G_{D1b}, G_{T1b} and G_{Q1b} can be detected by conversion with *V. cholerae* neuraminidase to G_{M1} prior to treatment with choleragenoid as described by Wu and Ledeen [21]. None of the mentioned ganglio series gangliosides could be detected in the granulocyte ganglioside fraction with this highly sensitive assay, indicating absence of these compounds in human granulocytes.

In summary, gangliosides with linear *N*-acetyllactosaminyl backbones substituted with *N*-acetylneuraminic acid by either α(2-3) and/or α(2-6) linkage i.e., II^{3}Neu5Ac-LacCer (G_{M3}), IV^{3}Neu5Ac-nLcOse$_4$Cer, IV^{6}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer were the major gangliosides of human granulocytes. Furthermore, we could show that different fatty acid substitution in the ceramide moiety caused additional structural heterogeneity. This is in agreement with the data of Fukuda *et al.* [3].

Overlay assay of ^{35}S-labelled viruses on thin-layer chromatograms

Virus binding to gangliosides was studied by using the overlay technique [11] (Fig. 3). A/PR/8/34 (H1N1) bound all gangliosides from human granulocytes to various extent (Fig. 3, lane 2). Heat treatment of A/PR/8/34 abolished

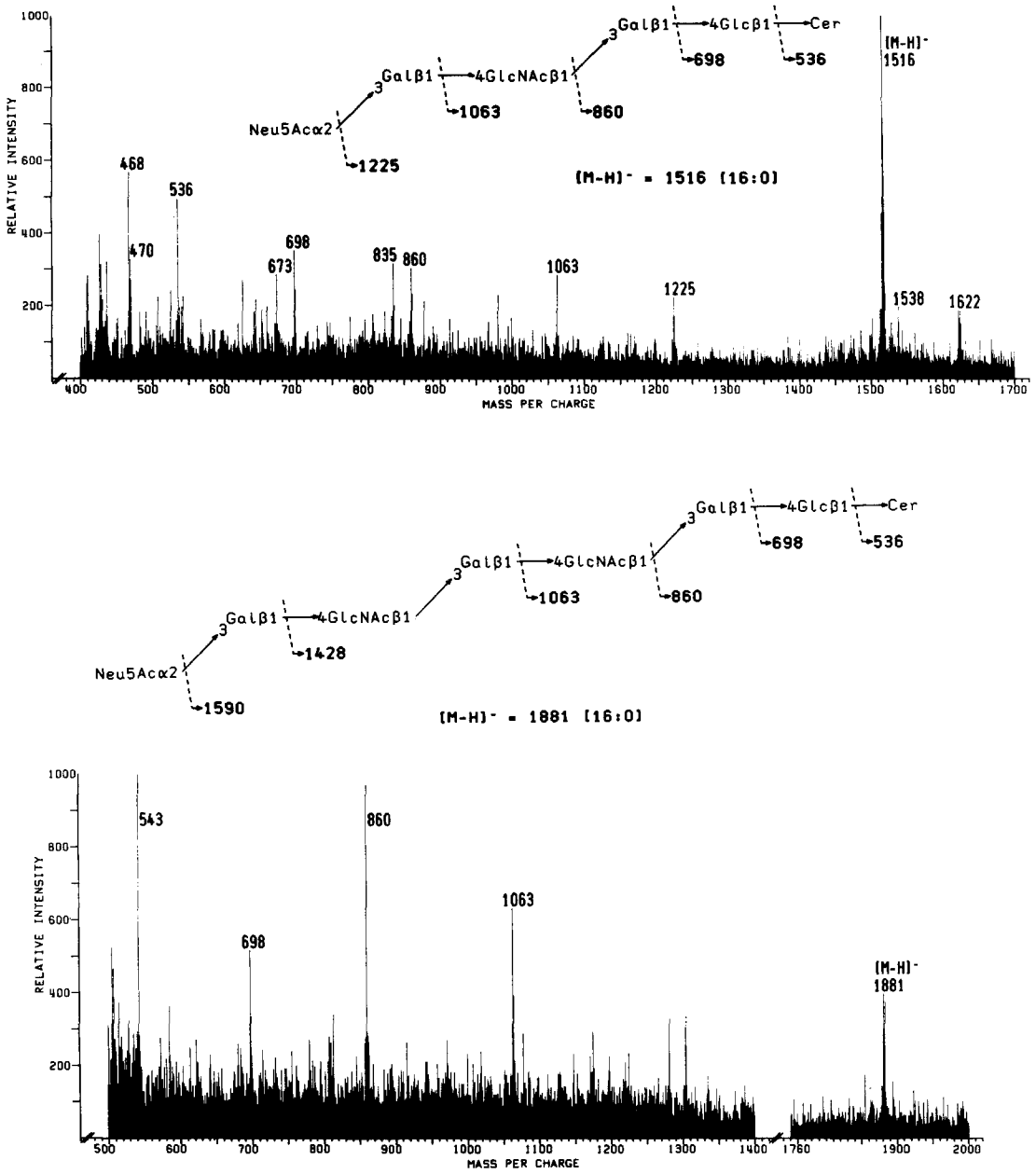

Figure 2. Negative ion FAB-MS of the native ganglioside fractions 4(a) and 8(b) from human granulocytes and their respective fragmentation patterns.

adhesion to all gangliosides (control, Fig. 3, lane 5). Relative binding capacities (see Table 3) indicate a preferential binding to α(2-3) linked Neu5Ac as in II^{3}Neu5Ac-LacCer, IV^{3}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer compared with α(2-6) linked Neu5Ac as in IV^{6}Neu5Ac-nLcOse$_4$Cer. This is in agreement with a previous study [10]. Influenza virus strains exhibit markedly different specificities for receptor determinants [4, 5]. This was also found in our study for gangliosides from human granulocytes. In contrast to A/PR/8/34, strain A/X-31 preferentially bound to α(2-6) linked Neu5Ac, as shown for the IVNeu5Ac-nLcOse$_4$Cer isomers (Table 3). However, A/X-31 also bound to α(2-3) linked Neu5Ac if presented on a longer backbone as in VI^{3}Neu5Ac-nLcOse$_6$Cer. This high binding could be due to a more accessible presentation of the ligand determinant on the TLC plate. This is in contrast to A/PR/8/34 which preferentially bound to gangliosides with α2-3Neu5Ac linked to the nLcOse$_4$ backbone. Sendai virus also clearly preferred gangliosides with longer backbones (Table 3). But, in contrast to influenza A/X-31, Sendai virus did not bind to α(2-6) linked Neu5Ac-containing structures. Binding specificities detected in overlay assays with whole ganglioside mixtures of human granulocytes were confirmed with individual gangliosides isolated by

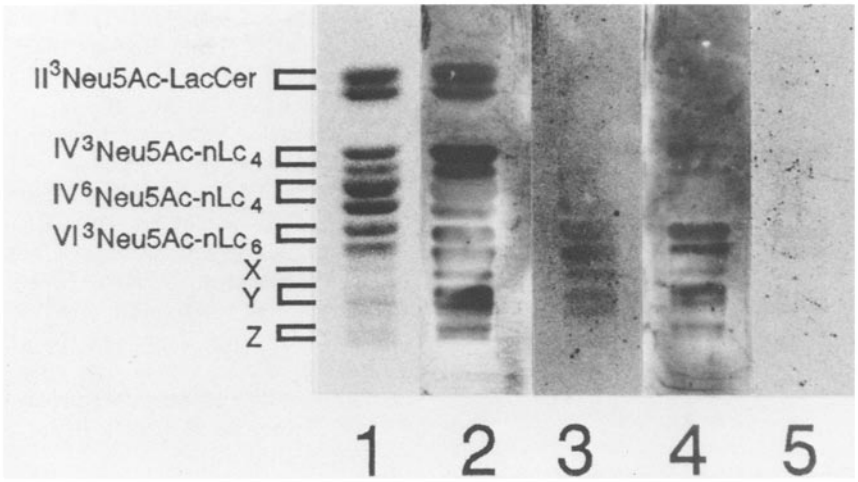

Figure 3. TLC-overlay assay of gangliosides from human granulocytes with ^{35}S-labelled influenza A/PR/8/34 (lane 2), influenza A/X-31 (lane 3) and Sendai virus (lane 4). Lane 1 shows the resorcinol stained ganglioside mixture and lane 5 the TLC overlay with heat inactivated influenza A/PR/8/34 virus. Exposure time 20 days. nLc_4 = $nLcOse_4Cer$; nLc_6 = $nLcOse_6Cer$.

Table 2. Partially methylated alditol acetates of gangliosides from human granulocytes.

Partially methylated alditol acetates	*Ganglioside No.*[a]			
	1/2	*3/4*	*5/6*	*7/8*
Glucitol				
1,4,5-Tri-*O*-acetyl	+	+	+	+/+
Galactitol				
1,4,5-Tri-*O*-acetyl	−	−	−	−/+[b]
1,3,5-Tri-*O*-acetyl	+	+	+	+/+
1,5,6-Tri-*O*-acetyl	−	−	+	−/−
N-Acetylglucosaminitol				
1,4,5-Tri-*O*-acetyl	−	+	+	+/+

[a] According to Fig. 1 and Table 1.
[b] About 30% molar amount compared to 1,3,5-tri-*O*-acetylgalactitol within the same fraction.

preparative HPTLC from the entire fractions (not shown). These data suggest that in the TLC overlay assay, receptor recognition extends beyond terminal sequences Neu5Acα2-3Gal and/or Neu5Acα2-6Gal and that the chain length of the carbohydrate may influence the recognition by influenza virus haemagglutinin and Sendai virus HN protein. However, it has to be considered that the presentation of the oligosaccharides of GSLs on the surface of silica gel fixed TLC plates is likely to differ from the presentation on the cell surface, and somewhat differing specificities may be seen in this type of assay. All three virus strains tested showed binding to the more polar bands X, Y and Z. However, since structural data were not available for these compounds due to lack of purified molecular gangliosides, no conclusion could be drawn from this observation.

Table 3. Relative binding capacities of influenza A and Sendai viruses to gangliosides from human granulocytes.

Gangl. No.[a]	*Major fatty acid*	*Structure*	*Relative binding*[b]		
			Influenza A/PR/8/34	*Influenza A/X-31*	*Sendai virus*
1,2	$C_{24:1,22:0}$, $C_{16:0}$	$II^3Neu5Ac$-LacCer	5.4, 5.6	0, 0	0, 0
3,4	$C_{24:1,22:0}$, $C_{16:0}$	$IV^3Neu5Ac$-$nLcOse_4Cer$	9.9, 8.2	0, 0	0.8, 1.1
5,6	$C_{24:1}$, $C_{16:0}$	$IV^6Neu5Ac$-$nLcOse_4Cer$	1.0, 1.8	1.0, 1.8	0, 0
7,8	$C_{24:1}$, $C_{16:0}$	$VI^3Neu5Ac$-$nLcOse_6Cer$	4.6, 4.5	4.9, 7.1	3.2, 4.6

[a] According to Fig. 1 and Table 1.
[b] Ratio of bound ^{35}S-labelled viruses to gangliosides, calculated from the intensities of autoradiographs and resorcinol stained bands by densitometric scanning.

Conclusion

In vivo, cytotoxic T cells and natural killer cells are able to kill virus infected cells whereas neutrophils, the most common type of granulocytes, phagocytose and destroy small particles. Since human granulocytes express gangliosides to which influenza A and Sendai virus bind, it seems feasible to speculate that these GSLs may be involved in receptor mediated phagocytosis of viruses, which could play a role in immunologically mediated reactions during virus infection and recovery.

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