Glycoconjugate Journal (1994) **11**:486–492

A comparative assessment of TLC overlay technique and microwell adsorption assay in the examination of influenza A and Sendai virus specificities towards oligosaccharides and sialic acid linkages of gangliosides

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Received 17 May 1994, revised 25 July 1994

Influenza A and Sendai viruses bind to *neo*lacto-series gangliosides isolated from human granulocytes. Differences in receptor specificity of influenza viruses A/PR/8/34 (H1N1), A/X-31 (H3N2), and parainfluenza Sendai virus (HNF1, Z-strain) were determined by two direct solid phase binding assays: the overlay technique, which combines high-resolution in the separation of gangliosides on thin-layer chromatograms with direct binding; and the microwell adsorption assay as a convenient binding assay which is performed in microtitre wells to estimate the avidity of binding to an isolated ganglioside. Both methods were applied for comparative binding studies. Viruses were found to exhibit specificity for oligosaccharides and sialic acids as well as for chain length of the neutral carbohydrate backbone, whereas differing fatty acids ($C_{24:1}$ and $C_{16:0}$) in the ceramide portion had no impact on virus adsorption. Terminal sialyloligosaccharides Neu5Acα2-3Galβ1-4Glc-R of G_{M3}, and Neu5Acα2-3Galβ1-4GlcNAc-R as well as Neu5Acα2-6Galβ1-4GlcNAc-R of *neo*lacto-series gangliosides with nLcOse$_4$Cer and nLcOse$_6$Cer backbone, exhibited significant specific receptor activity towards the different viruses. To compare the data revealed from both test systems, values of virus binding were ascertained by a non-parametric statistical approach based on rank correlation. The rank correlation coefficient r_s was calculated according to Spearman from each virus binding towards G_{M3}, IV^{3}Neu5Ac-nLcOse$_4$Cer, IV^{6}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$SCer. The rank correlation coefficients 0.74, 0.95 and 0.92, which were determined for A/PR/8/34 (H1N1), A/X-31 (H3N2) and Sendai virus (HNF1, Z-strain), respectively, indicated that both assays generate highly correlated experimental data. Based on these results, analyses of virus binding on thin-layer chromatograms as well as in microwells were found equivalent tools for ganglioside receptor studies.

Keywords: gangliosides; receptors; human granulocytes; TLC overlay technique; microwell adsorption assay; influenza A virus, Sendai virus.

Abbreviations: BSA, bovine serum albumin; GSL(s), glycosphingolipids; HPTLC, high performance thin-layer chromatography; PBS, phosphate buffered saline; Neu5Ac, *N*-acetylneuraminic acid [35]; r_s = rank correlation coefficient according to Spearman. The designation of the glycosphingolipids follows the IUPAC-IUB recommendations [36]. LacCer or lactosylceramide, Galβ1-4Glcβ1-1Cer; lacto-*N*-*neo*tetraosylceramide or nLcOse$_4$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; lacto-*N*-*nor*hexaosylceramide or nLcOse$_6$Cer, Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; G_{M3} (according to Svennerholm [37]) or II^{3}Neu5Ac-LacCer.

Introduction

Gangliosides are glycosphingolipids (GSLs) containing one or more sialic acid residues. They are characteristic constituents of the outer surface of animal cells in which they are located with the ceramide portion embedded in the lipid layer and the sialooligosaccharide residue facing the extracellular environment. Structures and functions of gangliosides have been widely reviewed [1–4]. Sialic acids as characteristic substituents of gangliosides are known to play important roles in various cellular processes [5]. First found in brain, gangliosides are now known to be ubiquitous compounds of all mammalian cells studied so far. They act, for instance, as markers for development and differentiation and as regulators for cell proliferation [6]. Also the involvement of gangliosides in cell–cell recognition phenomena as well as their function as receptors for toxins and bacteria [7], viruses [8] and other ligands have been reported. During the initial phase of infection, myxoviruses

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such as influenza and Sendai viruses interact with receptors on the host cell surface, followed by fusion with surface membranes. Terminally sialylated gangliosides are important receptor binding sites for viruses [9] and are able to mediate virus attachment [10]. Several assay systems have been found to be convenient to investigate the accessibility of viruses to cells, based on the adsorption to [11], fusion with [12] or infection of target cells [13]. To minimize the interference of other cell or virus membrane components, direct solid phase binding assays have been developed. These tests offer the opportunity to measure directly the interaction of the virus to its receptor ganglioside. To estimate the avidity of virus binding to a purified ganglioside, plastic microtitre wells to which receptors have been adsorbed have been used [14]. The solid phase approach was extended by employment of virus binding to thin-layer chromatograms on which the GSLs were separated [15]. Both the solid phase binding assay in microtitre wells as well as the overlay technique are now well established tools in receptor binding studies [16]. In a previous work we have shown the virus receptor function of neolacto-series gangliosides from human granulocytes to influenza A and Sendai viruses [17]. In this study the binding capacities of influenza A and Sendai viruses are systematically examined, comparing experimental values obtained by exposure of radiolabelled viruses to plastic fixed thin-layer chromatograms of GSLs (overlay technique) and to GSLs adsorbed to polystyrene microtitre wells (microwell adsorption assay). Data obtained by both assays were found to highly correlate by use of a non-parametric statistical approach.

Materials and methods

Gangliosides from human granulocytes

The isolation of gangliosides from granulocytes was performed according to standard procedures as recently reported [17]. Briefly, the GSLs were extracted with chloroform:methanol (2:1) and (1:2), each by vol. The combined extracts were evaporated and partitioned according to Folch *et al.* [18]. 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.* [19]. 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) according to Ueno *et al.* [20]. 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

Gangliosides were separated on glass-backed silica gel 60 precoated HPTLC plates (size 10 cm × 10 cm, thickness 0.24 mm, E. Merck, Darmstadt, Germany) in chloroform: methanol:water (120:85:20, by vol) containing 2 mM $CaCl_2$ and visualized by resorcinol according to Svennerholm [21]. Lipid bound sialic acid was determined by densitometry. Resorcinol stained ganglioside chromatograms were scanned with a Desaga CD60 scanner (Desaga, Heidelberg, Germany) equipped with an IBM compatible personal computer and densitometric software. Intensities of bands were measured in reflectance mode at 580 nm with a light beam slit of 0.1 mm × 2 mm.

Preparative GSL amounts were applied to HPTLC plates with an automatic sample applier Linomat IV (CAMAG, Muttenz, Switzerland). After chromatography, gangliosides were visualized by thoroughly spraying with 0.002% (w/v) pyrene-1-aldehyde in acetone:methanol (60:40, each by vol) for nondestructive detection [22]. Zones containing GSLs were localized under UV light, scraped off and the silica gel was transferred to small columns with sintered glass plugs. Gangliosides were extracted from the silica gel with chloroform:methanol:water (30:60:8, each by vol) followed by anion exchange chromatography as previously described [22], which enables separation of gangliosides from the fluorochrome. Finally, impurities were removed by Iatrobeads chromatography (see above).

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 [23]. The multiplicity of infection was about five plaque forming units per cell and metabolic labelling with L-[^{35}S]-methionine (Amersham Buchler, Braunschweig, Germany) was carried out from 5 to 24 h after infection. For each virus 27.75 MBq (750 μCi) 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 25000 rpm (120000 × **g**). The sediment was resolved in 1 ml phosphate buffered saline (PBS).

Binding of viruses to methacrylate fixed gangliosides on silica gel precoated plates (TLC overlay technique)

Binding assays were performed according to Magnani *et al.* [24] with slight modifications. Gangliosides were chromatographed as described above. The plates were thoroughly dried for 0.5 h over P_2O_5 under vacuum in a desiccator and the silica gel was then fixed by chromatography in hexane saturated polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt, Germany). 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 1% bovine serum albumin (BSA) in PBS (= solution A) to block unspecific binding. The solution was thoroughly withdrawn by suction and 80 µl of radiolabelled virus suspension was applied per lane (about 2×10^5 cpm). The strips were covered with small pieces of parafilm™ and incubated in a humdified 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. Virus autoradiographies were scanned as described above and ratios of bound viruses towards single gangliosides were calculated from the average of three scans of autoradiography and resorcinol stained parallel ganglioside chromatograms, respectively.

Binding of viruses to polystyrene fixed gangliosides (microwell adsorption assay)

Microwells of polystyrene microtitre plates (Linbro 7601804, ICN, Meckenheim, Germany) were each loaded with 1 nmol of purified individual gangliosides in 100 µl of methanol and the solvent was evaporated by incubation in a dry atmosphere for 90 min at 37 °C. After washing each well five times with solution A (see above), 50 µl of radiolabelled virus suspension (about 1.3×10^5 cpm) were applied, each, and incubated for 2 h at +4 °C. Unbound virus was carefully removed and the wells were washed five times with 100 µl PBS, each. Pooled unbound virus containing supernatants and washing solutions were transferred to scintillation vials. The washed wells were cut from the flexible microtitre plates and transferred to scintillation vials. Unbound as well as bound radioactivity was determined in a Tri-Carb 1900 CA liquid scintillation spectrometer (Packard Instrument Co., Downers Grove, IL).

Calculation of the rank correlation coefficient r_s

Eight individuals (= gangliosides) are ranked by two criteria: (1) binding capacity to virus on a methacrylate fixed silica gel precoated TLC plate (overlay technique); and (2) binding to virus on the surface of a polystyrene microtitre plate well (microwell adsorption assay). Are the results obtained significantly alike?

The assignment of ranks to experimental values of n ranked variables is the first step in this non-parametric test. The variables are on the one hand the values of bound virus to gangliosides adsorbed on to microwells and on the other hand relative binding capacities of viruses towards gangliosides on TLC plates. Next, the rank differences d_i and the sums from rank square differences $\sum d_i^2$ have to be calculated. The formula for the rank correlation coefficient r_s is given by

$$r_s = 1 - \frac{6 \sum d_i^2}{n^3 - n}$$

according to Spearman [25, 26, and references therein]. r_s has a range from −1 to 1 and values are tabled [27]. On the 5% significance level ($p = 0.95$) and $n = 8$, r_s values $\leqslant 0.643$ indicate no significant correlation.

Results and discussion

The resorcinol stained HPTLC chromatogram of human granulocyte gangliosides is shown in Fig. 1. The mixture used for TLC overlaying, contains eight major gangliosides (Fig. 1, lane R). The structures have previously been shown to be G_{M3}(Neu5Ac), IV^{3}Neu5Ac-nLcOse$_4$Cer, IV^{6}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer [17] and are listed in Table 1. Ganglioside pairs differ only in their respective ceramide portions, composed mainly of $C_{24:1}$ and $C_{16:0}$ fatty acids and in some cases substituted to a minor extent by $C_{22:0}$ fatty acid (see Table 1). Purified single gangliosides, shown in lanes 1 to 8 of Fig. 1, were used for the microwell adsorption assay to quantify specific binding of viruses to individual gangliosides bound to a polystyrene support.

Virus binding determined by TLC overlay technique

The autoradiograms from TLC overlay assays of human granulocytes gangliosides with influenza A/PR/8/34,

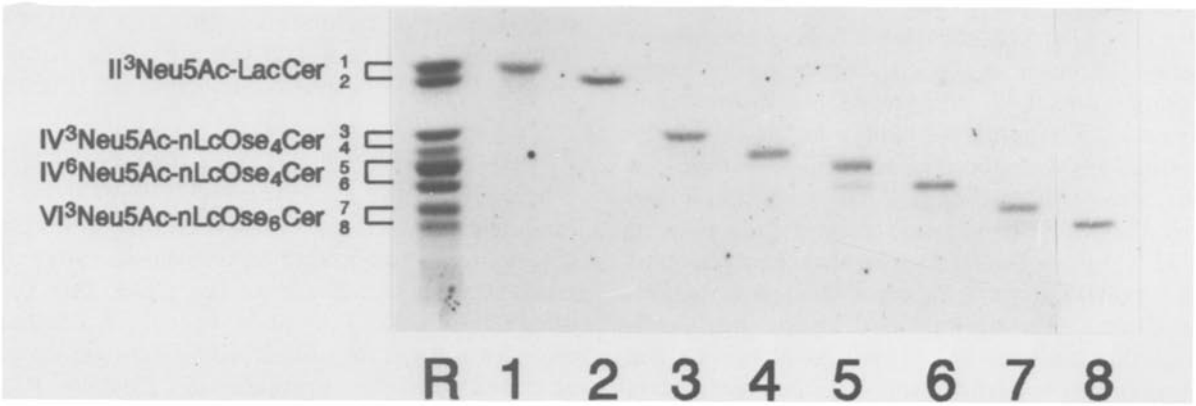

Figure 1. Resorcinol stain of individual gangliosides isolated from human granulocytes by preparative HPTLC. R, 20 µg of total granulocytes gangliosides (references). 1 µg of gangliosides 1 to 8, respectively, were chromatographed. Their structures are listed in Table 1.

Table 1. Relative binding capacities of influenza A and Sendai viruses to gangliosides from human granulocytes revealed by the TLC overlay technique.

Ganglioside no.[a]	*Major fatty acid*	*Structure*	*Relative binding*[b]		
			Influenza A/PR/8/34	*Influenza A/X-31*	*Sendai virus*
1	$C_{24:1}$	II^{3}Neu5Ac-LacCer	5.4	0.0	0.0
2	$C_{16:0}$	II^{3}Neu5Ac-LacCer	5.6	0.0	0.0
3	$C_{24:1}$	IV^{3}Neu5Ac-nLcOse$_4$Cer	9.9	0.0	0.8
4	$C_{16:0}$	IV^{3}Neu5Ac-nLcOse$_4$Cer	8.2	0.0	1.1
5	$C_{24:1}$	IV^{6}Neu5Ac-nLcOse$_4$Cer	1.0	1.0	0.0
6	$C_{16:0}$	IV^{6}Neu5Ac-nLcOse$_4$Cer	1.8	1.8	0.0
7	$C_{24:1}$	VI^{3}Neu5Ac-nLcOse$_6$Cer	4.6	4.9	3.2
8	$C_{16:0}$	VI^{3}Neu5Ac-nLcOse$_6$Cer	4.5	7.1	4.6

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

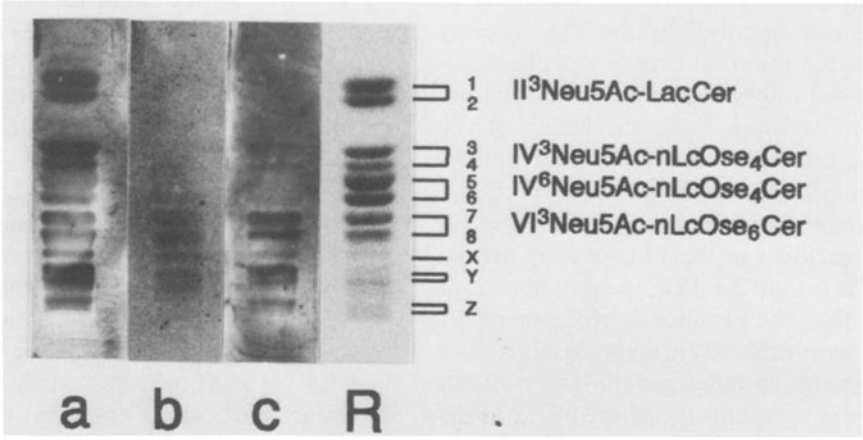

Figure 2. Autoradiographies of TLC overlay assays of gangliosides from human granulocytes with ^{35}S labelled influenza A/PR/8/34 (lane a), influenza A/X-31 (lane b) and Sendai virus (lane c), exposure time 20 d. Lane R shows the resorcinol stained ganglioside mixture (references). In each lane 20 μg of gangliosides were applied. Gangliosides are enumerated according to Fig. 1 and Table 1.

influenza A/X-31 and Sendai viruses are shown in Fig. 2 (lanes a to c, respectively) in parallel to the resorcinol stained control chromatogram (Fig. 2, lane R). Twenty μg of gangliosides per lane were chromatographed and the relative binding capacities were determined as the quotients of densitometric values of resorcinol stained ganglioside bands and autoradiographic bands, obtained after exposure of the TLC plate to labelled viruses (see Table 1).

Influenza A/PR/8/34 preferently bound to the terminally Neu5Acα2,3 sialylated gangliosides G_{M3}, IV^{3}Neu5Ac-nLcOse$_4$Cer and VI^{3}Neu5Ac-nLcOse$_6$Cer and to a lesser extent to IV^{6}Neu5Ac-nLcOse$_4$Cer as deduced from the overlay assay (Fig. 2, lane a) respectively, calculated from relative binding capacities (Table 1). The preferential binding of influenza A/PR/8/34 to gangliosides bearing Neu5Acα2,3 sialic acids was also found by Suzuki *et al.* [10] by use of a similar overlay assay system. This preference of influenza A/PR/8/34 to Neu5Acα2,3 sialylated glycoconjugates, determined in adhesion studies of influenza virions towards resialylated erythrocytes according to the technique originally described by Paulson and Rogers [28], was also found by Nobusawa *et al.* [29]. In contrast to influenza A/PR/8/34, influenza A/X-31 did not bind to G_{M3} and IV^{3}Neu5Ac-nLcOse$_4$Cer, but showed almost identical relative binding capacities towards VI^{3}Neu5Ac-nLcOse$_6$Cer and IV^{6}Neu5Ac-nLcOse$_4$Cer (Fig. 2, lane b) compared to influenza A/PR/8/34 (see Table 1). The difference in the receptor specificities of both viruses is caused by the different types of haemagglutinins: H1 in the case of influenza A/PR/8/34 and H3 in the case of influenza A/X-31. Influenza A/X-31 is a recombinant virus whose haemagglutinin originates from influenza A/Aichi/2/68 (H3N2) [30]. By use of a haemolysis assay, Suzuki *et al.* [30] demonstrated that both influenza A/X-31 and influenza A/Aichi/2/68 used Neu5Acα2,3 as well as Neu5Acα2,6 sialylated gangliosides as virus receptors. The same result was also obtained by Nobusawa *et al.* [29] for influenza A/Aichi/2/68 in adsorption studies with resialylated erythrocytes.

In the TLC overlay assay (Fig. 2, lane c), Sendai virus exhibited strong binding to VI^{3}Neu5Ac-nLcOse$_6$Cer and faint adhesion towards IV^{3}Neu5Ac-nLcOse$_4$Cer, but the virus did not recognize Neu5Acα2,3 sialylated G_{M3} nor IV^{6}Neu5Ac-nLcOse$_4$Cer (see Table 1). These results coincide with data from Markwell and Paulson [9], employing specifically Neu5Acα2,3 and Neu5Acα2,6 resialylated erythrocytes. Results of Umeda *et al.* [31] and Hansson *et al.* [15] also implicate Neu5Acα2,3 sialylated neolacto-series gangliosides as the major receptors of Sendai virus.

All three strains of viruses used in this study showed considerable adhesion to more polar minor gangliosides X, Y and Z (see Fig. 2). Their structures were not elucidated due to their low incidence within the ganglioside mixture (see Fig. 2, lane R). Although the virus receptor specificities could not be clearly deduced due to the lack of structural data, somewhat different binding towards these gangliosides by the three virus strains analysed is obvious (Fig. 2, lanes a to c).

The results in this study obtained by the TLC overlay technique demonstrate that the specificity of virus haemagglutinin is not determined only by the type of sialylation. Thus, in the case of influenza A/X-31, the length of the carbohydrate backbone modulated the receptor strength of the virus haemagglutinin paratope; VI^{3}Neu5Ac-nLcOse$_6$-Cer, but neither IV^{3}Neu5Ac-nLcOse$_4$Cer nor II^{3}Neu5Ac-LacCer were suitable receptors in the TLC overlay assays.

It should be mentioned that the TLC overlay approach does not necessarily reflect the ganglioside receptor repertoire in a plasma membrane accurately because the adhesion of viruses towards isolated gangliosides without surrounding membrane components is analysed. Due to the absence of the natural environment (phospholipids, membrane proteins, etc.) the TLC overlay assay might not provide an ideal *in vivo* model. On the other hand, the formation of ganglioside clusters, i.e. tightly arranged GSLs in the membrane, is a well known phenomenon and exposure of the gangliosides in the TLC overlay assay would simulate this *in vivo* situation quite well. However, the microwell adsorption assays described in the following section were performed without phospholipids, since otherwise the data would not be comparable to the results of the TLC overlay tests.

Virus binding determined by microwell adsorption assay

Data about binding capacities of virus-gangliosides interactions obtained by the above described TLC overlay technique should be compared and correlated to alternative studies carried out in polystyrene microwells. For this purpose, 1 nmol of each ganglioside (listed from 1 to 8 in Table 1) was applied to microwells followed by virus incubation. As shown in Fig. 3a, the strength of the binding of influenza A/PR/8/34 to individual gangliosides declined in the order IV^{3}Neu5Ac-nLcOse$_4$Cer (gangliosides 3 and 4) > VI^{3}Neu5Ac-nLcOse$_6$Cer (gangliosides 7 and 8) > II^{3}Neu5Ac-LacCer (gangliosides 1 and 2). These results were found to be highly correlated with the data obtained by TLC overlay technique (see above). In both systems, the preference of influenza A/PR/8/34 for Neu5Acα2,3 sialylated gangliosides was detected and IV^{3}Neu5Ac-nLcOse$_4$Cer was found to be the most adhesive ganglioside. The exact calculation of the correlation coefficient r_s will be given in the next section.

The incubation of II^{3}Neu5Ac-LacCer (gangliosides 1 and 2) as well as IV^{3}Neu5Ac-nLcOse$_4$Cer (gangliosides 3 and 4) with influenza A/X-31 revealed almost the same amount of bound virus compared to uncoated microwells (Fig. 3b). As established in the overlay binding assay (see above), VI^{3}Neu5Ac-nLcOse$_6$Cer was the strongest receptor (gangliosides 7 and 8), whereas much less virus adhered to the IV^{6}Neu5Ac-nLcOse$_4$Cer coated wells.

Maximum adhesion of Sendai virus was found towards VI^{3}Neu5Ac-nLcOse$_6$Cer (gangliosides 7 and 8) in the microwell adsorption assay, whereas IV^{3}Neu5Ac-nLcOse$_4$-Cer (gangliosides 3 and 4) were weak receptors. The binding to II^{3}Neu5Ac-LacCer (gangliosides 1 and 2) as well as IV^{6}Neu5Ac-nLcOse$_4$Cer did not exceed the controls in uncoated wells (Fig. 3c). Again, the data obtained by the overlay technique and the microwell adsorption assay coincided.

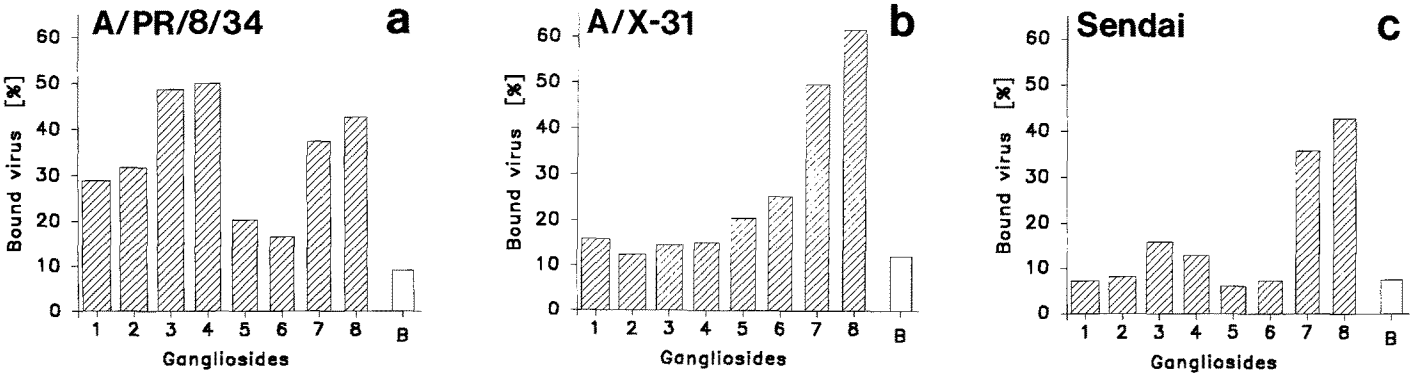

Figure 3. Binding of ^{35}S labeled influenza A/PR/8/34 (a), influenza A/X-31 (b) and Sendai virus (c) to polystyrene fixed gangliosides determined by microwell adsorption assays. 1 nmol of each ganglioside was applied per microwell. In control experiments the adsorption of viruses to uncoated wells was counted (B = background). Gangliosides were numerated according to Fig. 1 and Table 1.

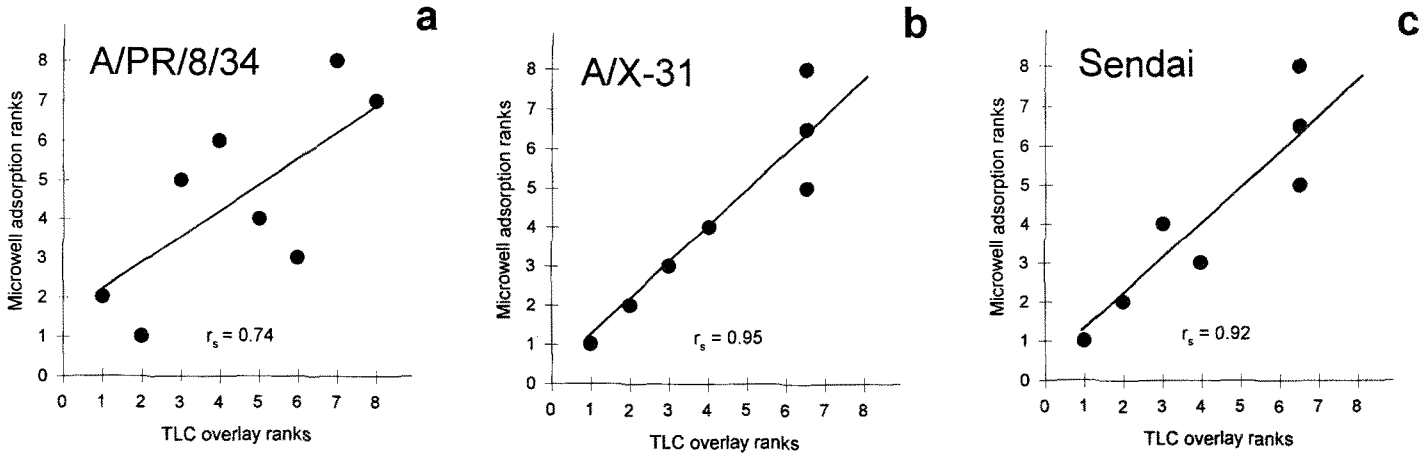

Figure 4. Rank correlation coefficients calculated from virus binding towards gangliosides determined by TLC overlay and microwell adsorption assay. The rank correlation coefficients r_s were calculated for influenza A/PR/8/34 (a), influenza A/X-31 (b) and Sendai virus (c).

Comparative statistical approach of TLC overlay and microwell adsorption assay of virus binding

Virus binding was calculated by a non-parametric statistical test based on rank correlation. Values of virus binding, deduced from TLC overlay and microwell adsorption assay, were ranked. The rank correlation coefficient r_s for each virus strain in both assay systems was calculated (see Materials and methods). High correlation with $r_s = 0.92$ was found for Sendai virus, graphically demonstrated in Fig. 4c. Comparison of binding data from influenza A/X-31 and influenza A/PR/8/34 resulted in $r_s = 0.95$ (Fig. 4b) and $r_s = 0.74$ (Fig. 4a), respectively. In all cases, $r_s > 0.643$ was obtained, indicating high correlation of the data obtained by the TLC overlay technique and the microwell adsorption assay.

Conclusion

The overlay assay is a very convenient detection method for the assessment of virus receptor function of gangliosides. Major advantages are easy handling and the applicability of complete GSL mixtures which can be used after thin-layer chromatography for simultaneous estimation of virus adhesion. The range of application of the microwell adsorption assay is limited to the availability of single gangliosides, in many cases a hard to fulfil prerequisite due to small quantities of purified material. Both assays are based on solid phase anchorage of GSLs, on the one hand by methacrylate fixation of silica gel on TLC plates (overlay technique) and on the other hand by adhesion to a polystyrene support (microwell assay). There is some evidence that binding of ligands to their receptors might be influenced by the plastic coating of silica gel precoated HPTLC plates [32]. However, in our comparative study of virus binding in two different solid phase binding assays, no significant differences were found as proved by a non-parametric statistical analysis. Viruses were found in both systems to exhibit specificity towards oligosaccharides and type of sialic acid linkage as well as the chain length of the carbohydrate backbone, whereas differing ceramide portions had no impact. The TLC overlay assays were performed with purified ganglioside mixtures and the microwell adsorption assays with isolated single gangliosides. To make both test systems comparable, no phospholipids or other membrane constituents in addition to GSLs were used. Any possible fatty acid effect would most likely be manifest only in a phospholipid matrix or plasma membrane.

The overlay technique has become a well established screening method for virus gangliosides specificities. Also the development of the overlay technique should lead to a well established test system to estimate the pathogeneity of infectious viruses. Furthermore, the overlay assay may be suitable to simulate virus-cell interaction in the early infection event. Several groups [34, 35] have suggested the involvement of neutral GSLs in the fusion process of myxoviruses with target membranes. The distinction of receptors mediating the adsorption from those responsible for the fusion process should be possible by use of the overlay technique. More insight into the early phases of the infection processes might lead to new strategies in the prevention of virus infections.

Acknowledgements

We express our warmest thanks to Professor Dr-Ing J. Lehmann for his generous support and Dr H. Ziehr (GBF, Braunschweig) for critical reading of the manuscript. The expert technical assistance of Mrs H. Doedens is also gratefully acknowledged. We would like to thank Professor Dr R. Rott and Mrs M. Orlich (University of Giessen) for production of viruses and Professor Dr H. Tschesche (University of Bielefeld) for providing with human granulocytes. This work was financed by grants from the Ministry

of Science and Research (Nordrhein-Westfalen, Germany, AZ IV A6-108 411 89) and was in parts supported by the Deutsche Forschungsgemeinschaft SFB 223.

References

1. Hakomori S (1981) *Ann Rev Biochem* **50**:733–64.
2. Thompson TE, Tillack TW (1985) *Ann Rev Biophys Biophys Chem* **14**:361–86.
3. Stults CLM, Sweeley CC, Macher BA (1989) *Methods Enzymol* **179**:167–214.
4. Zeller BC, Marchase RB (1992) *Am J Physiol* **31**:C1341–55.
5. Schauer R (1988) *Adv Exp Med Biol* **228**:47–72.
6. Hakomori S (1984) In *The Cell Membrane* (Habes E, ed.) pp. 181–201. New York: Plenum Press.
7. Karlsson KA (1989) *Annu Rev Biochem* **58**:309–50.
8. Paulson JC (1985) In *The Receptors*, Vol. II (Conn PM, ed.) pp. 131–219. Orlando: Academic Press.
9. Markwell MAK, Paulson JC (1980) *Proc Natl Acad Sci USA* **77**:5693–97.
10. Suzuki Y, Nakao T, Ito T, Watanabe N, Toda Y, Guiyun X, Suzuki T, Kobayashi T, Kimura Y, Yamada A, Sugawara K, Nishimura H, Kitame F, Nakamura K, Deya E, Kiso M, Hasegawa A (1992) *Virology* **189**:121–31.
11. Markwell MAK, Moss J, Hom BE, Fishman PH, Svennerholm L (1986) *Virology* **155**:356–64.
12. Wilschut J (1991) In *Membrane Fusion* (Wilschut J, Hoekstra D, eds.) pp. 89–126. New York: Marcel Dekker.
13. Bergelson LD, Bukrinskaya AD, Prokazova NV, Shaposhnikova GI, Kocharov SL, Shevchenko VP, Kornilaeva GV, Fomina-Ageeva EV (1982) *Eur J Biochem* **128**:467–74.
14. Holmgren J, Elwing H, Fredman P, Svennerholm L (1980) *Eur J Biochem* **106**:371–79.
15. Hansson GC, Karlsson KA, Larson G, Strömberg N, Thurin J, Örvell C, Norrby E (1984) *FEBS Lett* **170**:15–18.
16. Karlsson KA, Strömberg N (1987) *Methods Enzymol* **138**: 220–32.
17. Müthing J, Unland F, Heitmann D, Orlich M, Hanisch FG, Peter-Katalinić J, Knäuper V, Tschesche H, Kelm S, Schauer R, Lehmann J (1993) *Glycoconjugate J* **10**:120–26.
18. Folch J, Lees M, Sloane Stanley GH (1957) *J Biol Chem* **226**:497–509.
19. Müthing J, Egge H, Kniep B, Mühlradt PF (1987) *Eur J Biochem* **163**:407–16.
20. Ueno K, Ando S, Yu RK (1978) *J Lipid Res* **19**:863–711.
21. Svennerholm L (1957) *Biochim Biophys Acta* **24**:604–11.
22. Müthing J, Heitmann D (1993) *Anal Biochem* **208**:121–24.
23. Orlich M, Khatchikian D, Teigler A, Rott R (1990) *Virology* **176**:531–38.
24. Magnani JL, Smith DF, Ginsburg V (1980) *Anal Biochem* **109**:399–402.
25. Olds EG (1938) *Ann Math Statist* **9**:133–48.
26. Olds EG (1949) *Ann Math Statist* **20**:117–18.
27. Siegel S, Castellan NJ (1988) In *Nonparametric Statistics*, 2nd ed., hardcover text edition. New York: McGraw-Hill, Inc.
28. Paulson JC, Rogers GN (1987) *Methods Enzymol* **138**:162–68.
29. Nobusawa E, Aoyama T, Kato H, Suzuki Y, Tateno Y, Nakajima K (1991) *Virology* **182**:475–85.
30. Suzuki Y, Nagao Y, Kato H, Matsumoto M, Nerome K, Nakajima K, Nobusawa E (1986) *J Biol Chem* **36**:17057–61.
31. Umeda M, Nojima S, Inoue K (1984) *Virology* **133**:172–82.
32. Yiu SCK, Lingwood CA (1992) *Anal Biochem* **202**:188–92.
33. Sakkar DP, Blumenthal R (1987) *Membr Biochem* **7**:231–47.
34. Huang RTC (1983) *Lipids* **18**:489–92.
35. Reuter G, Schauer R (1988) *Glycoconjugate J* **5**:133–35.
36. IUPAC-IUB, Commission on Biochemical Nomenclature (1977) *Eur J Biochem* **79**:11–21.
37. Svennerholm L (1963) *J Neurochem* **10**:613–23.