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Studies on the Specificity and Sensitivity of the Influenza C Virus Binding Assay for 9-*O*-Acetylated Sialic Acids and Its Application to Human Melanomas

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The sensitivity and specificity of two influenza C virus assays, solid-phase and overlay assays, were investigated using naturally occurring 9-*O*-acetylated GD_3, rat serum glycoproteins containing 60% of *N*-acetyl-9-*O*-acetylneuraminic acid, and synthetically *O*-acetylated sialylated compounds. The sensitivity of the solid-phase assay was higher for glycoproteins containing *N*-acetyl-9-*O*-acetylneuraminic acid than for gangliosides, and also differed for various 9-*O*-acetylated gangliosides. The overlay assay was less sensitive for all glycoconjugates tested. For virus recognition the presentation of the sialic acid within the molecule and the structure of the sialic acid are essential. Investigation of gangliosides from human melanomas and normal skin with the influenza C virus assay showed an increase of *O*-acetylation of sialic acids in most tumour samples and the occurrence of several *O*-acetylated gangliosides.

Key words: 9-*O*-acetylated Neu5Ac, gangliosides, influenza C virus, melanoma, overlay assay, sialic acid analogues, solid phase assay.

Sialic acids comprise a family of about 40 related sugars derived from neuraminic acid. This diversity mainly arises from the enzymatic acetylation of hydroxyl groups at positions 4, 7, 8, and 9 of the molecule, the *O*-acetylation at C-9 prevailing (*2–4*). In most cases sialic acids are found at the terminal positions of glycoconjugates mainly linked to galactose, *N*-acetylgalactosamine or sialic acid itself. They are involved in many biological phenomena, especially with regard to the regulation of molecular and cellular recognition (*3*, *5–8*). Since many biological properties of sialic acids are associated with their 9-*O*-acetylation, the interest in this modification has increased. Examples are the partial or complete blocking of sialidase action (*9*, *10*), binding of influenza C viruses and coronaviruses to cells (*11*, *12*), antigenicity of bacterial capsules (*13*), modulation of the alternative pathway of complement activation (*14*), the expression of $Neu5,9Ac_2$ as a differentiation marker on chicken erythrocytes (*15*), the association of 9-*O*-acetylated gangliosides with cell migration in rat brain development (*16*), the modulation of the affinity of sialoadhesins to their counter-receptors (*8*) and the expression of the 9-*O*-acetylated ganglioside GD_3 in human melanoma (*17*) and basalioma (*18*, *19*) as a tumour-associated antigen. It has been shown that it also plays a crucial role in neurite outgrowth (*20*). In the cerebellum of the *nervous* mutant mouse, in which postnatally most Purkinje cells degenerate, there is a threefold increase in the ganglioside 9-*O*-acetyl GD_3 (*21*).

The most commonly used methods for the analysis of sialic acids, like TLC (*22*), HPLC with detection with ultraviolet light (*23*) or after the introduction of a fluorophor into the sialic acid molecule (*24*, *25*), GLC-MS (*26*), or NMR spectroscopy (*3*, *27*), require their previous release from glycosidic linkages through enzymatic or chemical hydrolysis, often followed by extensive purification. Since these procedures suffer from incomplete release of sialic acids and a remarkable loss or positional migration of *O*-acetyl groups (*3*, *28*, *29*), innovative methods which allow the detection of *O*-acetylated sialic acids without previous liberation are of great interest.

In contrast to influenza A and B viruses, influenza C viruses specifically recognise glycosidically linked $Neu5,9Ac_2$ (*30*), and the receptor-destroying enzyme of influenza C viruses has been characterised as sialate 9-*O*-acetylesterase [EC 3.1.1.53] (*31*, *32*). This property of influenza C viruses was used by Zimmer *et al.* (*33*) to detect 9-*O*-acetylated sialic acid in isolated glycoconjugates, in sialothioketosides synthesised by Roy *et al.* (*34*), and in tissue sections investigated by Harms *et al.* (*35*). Elegant tissue staining of

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Abbreviations: AGP, α_1-acid glycoprotein; BSA, bovine serum albumin; BSM, bovine submandibular gland mucin; DMB, 1,2-diamino-4,5-methylenedioxybenzene; GLC-MS, gas liquid chromatography–mass spectrometry; GSC, synthetic structure analogue of GM_3; HAU, hemagglutinating activity units; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MUAc, 4-methylumbelliferyl acetate; Neu5Ac, *N*-acetylneuraminic acid; $Neu5,8Ac_2$, *N*-acetyl-8-*O*-acetylneuraminic acid; $Neu5,7Ac_2$, *N*-acetyl-7-*O*-acetylneuraminic acid; $Neu5,9Ac_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; NMR, nuclear magnetic resonance spectroscopy; PBS, phosphate-buffered saline, pH 7.2; RR_D, relative retention time; TLC, thin-layer chromatography; TMOAc, trimethyl orthoacetate. The nomenclature for gangliosides used is according to Svennerholm (*1*).

9-*O*-acetylated sialoglycoconjugates is possible using chimeras of influenza C virus-hemagglutinin with the Fc portion of an IgG antibody (*36*). Although the binding site (hemagglutinin) is located on and the esterase of the virus is the same protein (*15*), these activities can be separated using different temperatures for binding (4°C) and the enzyme reaction (37°C or room temperature), respectively.

The present studies show that influenza C viruses recognise a variety of glycoconjugates containing Neu5,9Ac$_2$ regardless of the structure or nature of the total macromolecule. Though there are no qualitative differences in virus recognition, the different structures of the glycoconjugates may influence the efficiency of presentation of the carbohydrate receptor to the viral hemagglutinin or there may be steric hindrance, all of which could influence the extent of interaction. Apart from the general architecture of the molecule, the structure of the *O*-acetylated sialic acid moiety itself, especially that of its glycerol side-chain, may play a role. Using the method of Ogura *et al.* (*37*) for the *O*-acetylation of sialic acids at the primary hydroxyl group, it is possible to generate glycoconjugates with defined structures carrying the binding epitope, and to investigate their ability to mediate influenza C virus binding and the sensitivity of the assay for different receptors.

MATERIALS AND METHODS

Chemicals—All chemicals, if not indicated otherwise, were from Merck (Darmstadt, Germany) or Sigma-Aldrich (Deisenhofen, Germany). Glycine and Tris were purchased from Biomol (Hamburg, Germany). Iodogen was from Pierce (Rockford, Illinois/USA). For the blocking reaction, milk powder from Nestlé (Frankfurt, Germany) was used. Diaminomethylenedioxybenzene (DMB) was from Gerbu (Gaiberg, Germany). Human α_1-acid glycoprotein was purchased from Sigma-Aldrich. Glycoproteins from rat sera were prepared as described for horse serum (*38*). Total gangliosides from bovine brain were isolated and purified according to the method of Ledeen and Yu (*39*). The individual gangliosides, GM_1, GD_{1a}, and GT_{1b}, were separated from this mixture by HPLC (*40*). GM_3 was donated by Snow Brand Milk Products (Tokyo). ^{3}H-Labelled GM_3 and GM_1 were kindly provided by Prof. Dr. S. Sonnino (University of Milan, Italy). The synthetic structure analogues of GM_3 (GSC23, GSC24, GSC50, and GSC61; Fig. 1) were prepared as described (*41*). Melanoma samples and normal skin were kindly provided by Prof. Dr. E. Proksch, Priv.-Doz. Dr. U. Mrowietz and Dr. E. Stockfleth of the Department of Dermatology, University of Kiel.

Viruses—Influenza C viruses (strain Johannesburg/1/66) were cultivated as described (*30*). For binding assays an allantoic suspension of virions (32–64 HAU/ml) was used without any further preparation.

Esterase Assay—Substrate stock solutions were prepared by dissolving 4-methylumbelliferyl acetate (2 mM) in acetone/water (1 : 1, by volume) and α-naphthyl acetate (50 mM) in acetone. The activity of the influenza C virus sialate acetylesterase with MUAc as a substrate was determined as described previously (*33*) using a Hitachi F4010 fluorescence spectrophotometer (Colora, Lorch, Germany). Briefly, a solution comprising 10 µl virus suspension and 60 µl MUAc solution in 1,930 µl PBS was incubated at 37°C. The developing fluorescence was measured at an excitation

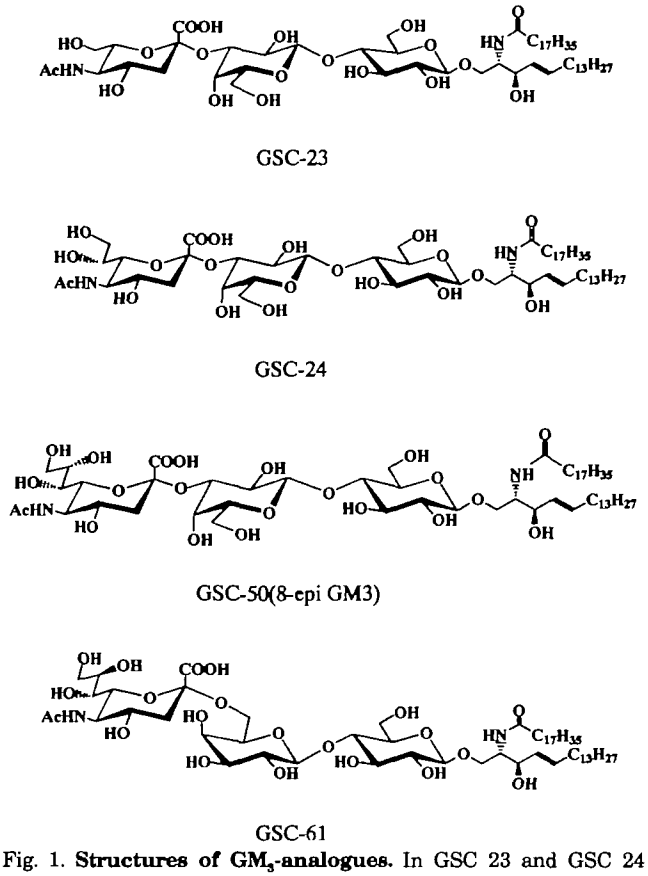

Fig. 1. **Structures of GM_3-analogues.** In GSC 23 and GSC 24 Neu5Ac has been substituted by the corresponding heptulosonic and octulosonic acids, respectively. The stereochemical arrangement at position 8 of Neu5Ac in GSC 50 has been changed from the *R*- to the *S*-configuration (8-epi-Neu5Ac), whereas the sialic acid in GSC 61 is linked to position 6 instead of position 3 of the penultimate galactose residue.

wavelength of 365 nm and an emission wavelength of 450 nm for 1–10 min. For determination of the spontaneous hydrolysis of the substrate, the assay was carried out with PBS containing 1% BSA instead of the virus suspension. After subtraction of this control value, the esterase activity was calculated and expressed in mU/ml (1 mU = 1 nmol methylumbelliferone liberated/min).

Hemagglutination Assay—Hemagglutinating activity of influenza C virus was examined according to the methods described by Herrler and Klenk (*30*), and Rogers *et al.* (*11*), using erythrocytes of mice or chicken, which contain surface-bound Neu5,9Ac$_2$.

Radioactive Labelling of Glycoproteins—A solution of α_1-acid-glycoprotein in PBS (50 pmol/90 µl) was transferred to an Iodogen-coated cap and then treated for 20 min at room temperature with 4.2 µl of a solution of ^{125}I-labelled sodium iodide in PBS (1.668 Mbq/µl). After the addition of 10 µl PBS containing 10% BSA, the solution was chromatographed over Sephadex G50 using 1,500 µl PBS/0.1% BSA for elution (*42*). The radioactive fractions were collected. Synthetically *O*-acetylated α_1-acid glycoprotein (5% of sialic acid was 9-*O*-acetylated, see below) was analogously labelled. The specific radioactivity was 4,118 cpm/fmol in the case of non-*O*-acetylated and 3,293 cpm/fmol in the case of acetylated glycoprotein.

Chemical O-Acetylation of Gangliosides and Glycoproteins—Gangliosides and glycoproteins were acetylated at position 9 of their sialic acids according to the method of Ogura *et al.* (*37*) by incubation with an up to 1,000-fold molar excess of the acetylating reagent trimethyl orthoacetate at room temperature overnight. In the case of gangliosides, the reaction products were analysed by TLC (see below). Sialic acids of the glycoproteins were analysed by fluorometric HPLC after acidic release as described below.

Analysis of Sialic Acid—Colorimetric quantification of sialic acids was carried out as described (*22*, *29*). For qualitative analysis the sialic acids were released from glycosidic linkages through acid hydrolysis with propionic acid at 80°C for 4 h (*43*), and then directly converted to fluorescent derivatives with DMB (*24*, *25*). The derivatives were separated by reversed phase chromatography on a RP-18 cartridge using acetonitrile/methanol/water (9 : 7 : 84, by volume) as the mobile phase. The elution was monitored with a 980-fluorescence detector, using an excitation wavelength of 343 nm and a cut-off emission filter of 389 nm. Pure Neu5Ac from Snow Brand and $Neu5,9Ac_2$ isolated from BSM (*44*) were used as standards. The relative retention times (RR_T) of the peaks were calculated relative to the retention time of Neu5Ac.

Thin-Layer Chromatography of Gangliosides—TLC of all glycolipids was performed on glass-backed HPTLC silica gel plates (Merck, Darmstadt, Germany) using the solvent systems chloroform/methanol/water (65 : 25 : 4, by volume), for GM_3 and its structural analogues, and chloroform/methanol/water containing 0.02% calcium chloride (60 : 40 : 9, by volume), for all other gangliosides. The gangliosides were visualised by staining with orcinol/Fe^{3+}/HCl spray reagent (*29*). The sialic acid contents of the stained bands were determined with a densitometer (CD, Desaga, Heidelberg, Germany).

Solid-Phase Assay for Glycoproteins and Gangliosides on Microtiter Plates—The solid-phase assay for *O*-acetylated glycoconjugates was performed on 96-well, flat-bottomed polystyrene microtiter plates (Greiner, Uertingen, Germany) using influenza C viruses and MUAc as the esterase substrate as described previously (*33*) with the following slight modifications. In order to avoid loss of activity, the virus suspension was not concentrated before use but adjusted to an esterase activity of level 15 mU/ml and to a hemagglutination activity level of 30 HAU/ml. Milk powder was used instead of BSA for the blocking reaction. In the cases of glycoproteins and gangliosides >0.2 pmol and 0.1–20 nmol were added to each well, respectively.

Immobilisation of Radioactive Glycoconjugates on Microtiter Plates—Synthetically *O*-acetylated ^{3}H-labelled GM_3 (0.078–20 nmol) and ^{125}I-labelled α_1-acid glycoprotein (0.129–132 pmol) was allowed to adsorb to the microtiter plates and then the assay was carried out as described above until the virus incubation step. All wash and blocking solutions were collected, lyophilised and resuspended in 150 or 500 μl water. In the case of GM_3, 100 μl of each suspension was added to 2 ml scintillation reagent and then the radioactivity was determined with a β-counter. In the case of the glycoprotein the radioactivity was measured directly with a γ-counter. After removal of the washing buffer, the plates were cut and the adsorbed amount of radioactive glycoconjugates in each well was determined.

TLC-Overlay—The procedure described by Zimmer *et al.* (*33*) for the staining of *O*-acetylated glycoconjugates involving influenza C viruses was also slightly modified as described for the solid-phase assay. 0.5–10 μg per lane of gangliosides were added. The esterase activity of the virus suspension used was 125 mU/ml.

Overlay Assay on Nitrocellulose Membranes—Glycoproteins containing $Neu5,9Ac_2$ were dissolved in PBS and then added directly to nitrocellulose membranes (100 μl/dot; BA 85, 0.45 μm, Scheicher-Schüll, Dassel, Germany) using a Bio-Dot apparatus (Bio-Rad, München, Germany) (*33*). Unspecific binding sites on the nitrocellulose were saturated by incubation with PBS containing 3% milk powder for 2 h at room temperature or alternatively overnight at 4°C. The binding and detection of influenza C virus were carried out as described for the TLC-overlay assay. In the case of rat serum glycoproteins, concentrations in the range of 0.075–0.75 μg/ml were analysed.

Furthermore, glycoproteins (1–10 μg/lane) were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (*45*), which involves a pH of 8.8 and may lead to a loss of *O*-acetyl groups. Therefore, this electrophoresis was also performed at a lower pH using phosphate buffer (50 mM sodium phosphate, 0.2% SDS, pH 7.2) (*46*). The separated proteins were transferred to nitrocellulose by electroblotting (Bio-Rad; 110 mA, 2 h) using ice-cold transfer buffer (25 mM Tris, 192 mM glycine, pH 8.5). To prevent loss of *O*-acetyl groups due to the alkaline conditions during transfer, blotting was performed alternatively by using phosphate buffer as the transfer buffer (25 mM, pH 6.5) at a current of 110 mA for 2 h (*47*). In both cases, transfer was controlled by reversible staining with Ponçeau S dye reagent. After saturation of unspecific binding sites on the nitrocellulose membranes by immersion in PBS containing 3% milk powder for 2 h at room temperature, the binding and detection of influenza C virus were carried out as described above.

Alkaline Treatment—For control experiments, glycoproteins and gangliosides were incubated in 0.1 N NaOH at room temperature for 1 h. Saponification of sialic acid *O*-acetyl groups was stopped by adding the same volume of 0.1 N HCl. Buffer exchange of glycoproteins was performed by gel chromatography on NAP-5 or NAP-10 columns (Pharmacia, Freiburg, Germany) equilibrated with 10-fold diluted PBS. The eluted solutions were lyophilised and then resuspended in a 10-fold smaller amount of water before use. Gangliosides were desalted by using Sephadex-LH-20 columns (Pharmacia) in methanol.

Alternatively, saponification of these glycoconjugates was carried out for 4 h at 4°C in 4 M ammonia. NH_3 was removed by lyophilisation. In some experiments, gangliosides were saponified on TLC-plates before chromatography by exposure to ammonia vapour (over conc. ammonia solution) at room temperature for about 12 h. After thorough evaporation, the plates were developed as described above, followed by staining with orcinol or influenza C virus.

Isolation and Analysis of Gangliosides from Human Melanoma—For investigation of the occurrence of *O*-acetylated gangliosides by means of the influenza virus assay, both embedded and fresh samples (8 and 6 probes, respectively) of human melanoma tissue (from skin or metastases) and normal skin (8 samples) taken from skin adjacent to the tumours were used. The embedded samples were incubated at 50°C for 2 h. Paraffin was removed and the samples

were treated with 3–5 ml xylol for 2 days at room temperature. The weight of the deparaffinised samples varied from 18 to 2,000 mg. After freezing in liquid nitrogen, the samples were ground in a mortar, homogenised in 5–8 ml cooled (4°C) chloroform/methanol (1 : 2), and then treated with ultrasound for 10 min. After removal of the solvent mixture, this treatment was repeated two times with chloroform/methanol (1 : 2) and three times with chloroform/methanol (2 : 1). The combined extracts were concentrated and applied to a column (0.5 × 5 cm) of Fractogel TSK DEAE 650 (s) (Merck). After removal of the neutral glycolipids by washing with methanol (50 ml), the acidic glycolipids were eluted with 1 M ammonium acetate in methanol (50 ml). The eluted fractions were concentrated to dryness and then desalted by subsequent lyophilisation (*39*). Alternatively, the samples were cut while still embedded with a microtome, followed by the removal of paraffin as described. Then the glycolipids were extracted as described above. Fresh samples were cut and homogenised in water (weight/volume 1:10). The extraction of the glycolipids was carried out as described above.

The presence of *O*-acetylated gangliosides in the extracts was investigated by means of the influenza C virus assays, which were carried out as described for the artificially modified gangliosides. Total gangliosides were stained with the orcinol/Fe^{3+}/HCl spray reagent (see above). Apart from this, the sialic acid content was determined by fluorometric HPLC after the hydrolysis of untreated and lipid-extracted material as described above.

RESULTS

Preparation of O-Acetylated Glycoconjugates—In order to judge the validity of the assay, the sensitivity and specificity for individual glycoconjugates were examined for both the overlay-assay and the solid-phase assay. For these investigations different gangliosides and glycoproteins containing $Neu5,9Ac_2$ were used. Apart from the naturally occurring glycoconjugates carrying this sialic acid, *i.e.* glycoproteins from rat serum or BSM, it is also possible to generate 9-*O*-acetylated compounds with defined structures by chemical means (*37*). Significant differences in the yield of *O*-acetylation were found. For example, GM_1 and GM_3 were acetylated at a significantly slower rate than GD_{1a}. For complete conversion, a 1,000-fold excess of TMOAc had to be applied for both monosialogangliosides, whereas a 500-fold excess was sufficient in the case of GD_{1a} in the course of 16 h (Table I). The yield also varied with the same reaction conditions in the individual assays. Furthermore, the reaction conditions leading to complete conversion yielded small, additional bands migrating faster than the underivatised compounds. When investigating the sialic acid contents of the products by fluorometric HPLC after acidic hydrolysis, two additional sialic acids (RR_T 1.1 and 1.42) were found in smaller amounts (1–8%) apart from residual Neu5Ac (RR_T 1) and the main product $Neu5,9Ac_2$ (RR_T 1.6). Both could be saponified by treatment with NaOH, indicating them to be *O*-acetylated sialic acids, most likely $Neu5,7Ac_2$ and $Neu5,8Ac_2$. Higher *O*-acetylated compounds were not found. In the case of GM_3 it was, however, possible to choose conditions (700-fold molar excess of reagent) where the underivatised ganglioside was still present (70%), but only one product was formed. These mixtures were used for the following binding studies with influenza C viruses without any further purification.

O-Acetylation of the glycoprotein AGP, containing 16 sialic acid residues per molecule, was rather variable (Table I), and the maximum, 70%, was reached with a 1,000-fold molar excess of TMOAc, although the conversion was often lower at this concentration. The acetylation exclusively took place at position 9 of the sialic acid, as shown by fluorometric HPLC analysis of the liberated sialic acids. For the following studies of the sensitivity of the binding assay with influenza C virus, a mixture of AGP and 5% of $Neu5,9Ac_2$ was used.

Sensitivity—1. Solid-phase assay for gangliosides and glycoproteins: The sensitivity of the solid-phase assay was examined for different *O*-acetylated glycoconjugates. Since it has to be considered that only a part of an applied sample might be adsorbed to the surface of the solid support, the immobilisation behaviour of gangliosides and glycoproteins was investigated using radiolabelled compounds. As a standard ganglioside, radiolabelled GM_3 was *O*-acetylated and then used directly for the immobilisation study. At total ganglioside concentrations above 0.1 nmol/100 μl the amount of ganglioside attached varied significantly between individual measurements. Only in the case of lower concentrations was a nearly linear relation between the applied and adsorbed quantities found (Fig. 2a). Under these conditions up to 90–95% of the material was adsorbed. The immobilisation behaviour of non-acetylated and *O*-acetylated gangliosides was similar. The radioactivity in these experiments was determined using a liquid scintillation reagent. Due to the incomplete contact of this reagent with the surface of the wells, a loss of 9% of radiation was observed. In the studies described above these effects were taken into account.

In the case of glycoproteins, *O*-acetylated human α_1-acid glycoprotein (5% $Neu5,9Ac_2$) was used as a standard. Both the non-acetylated and *O*-acetylated glycoproteins were radiolabelled with ^{125}I, and then directly used to examine the adsorption to the solid support. In contrast to gangliosides, less than 5% of both forms of the glycoprotein from solutions with concentrations in the range of 0.13 and 33 pmol/100 μl were immobilised (Fig. 2b). Furthermore, the adsorbed amounts differed significantly between the *O*-

TABLE I. **Chemical *O*-acetylation of various natural and synthetic glycoconjugates using trimethyl orthoacetate (*37*).** The molar excess of the acetylating reagent was calculated on the basis of the sialic acid content of the corresponding molecule. In the case of GD_{1a}, however, the molar excess and the yield were related to only one sialic acid residue. The percent 9-*O*-acetylation of the sialic acid residues is indicated.

Glycoconjugates	Relative yield of *O*-acetylation						
	Molar excess of trimethyl orthoacetate						
	10	50	100	200	500	700/750	1,000
GM_1	0	0	0	0	0	—[a]	100
GD_{1a}	10	20	35	85	100	—	—
GM_3	0	0	0	4	12	30–50	30–100
^{3}H-GM_3	—	—	—	—	—	—	30–50
GSC 23	0	0	0	0	0	0	—
GSC 24	0	0	0	0	5	50	—
GSC 50	0	0	0	5	10	80	—
GSC61	—	—	—	—	—	40	—
AGP	—	—	—	—	15	—	5–70

[a]—, not determined.

acetylated and non-acetylated proteins. Only 2.5% of the non-acetylated compound was adsorbed, whereas 5% of the acetylated AGP was immobilised. At concentrations over 33 pmol/100 µl the relative amount of bound material decreased, but there was still an almost linear correlation between the applied and adsorbed amounts, as was tested up to 140 pmol, which led to about 4,000 fmol binding. On the basis of these results the relative amount of an adsorbed glycoconjugate can be directly determined using the graph in Fig. 2b, when the applied quantity is known.

In the experiment involving the radioactive ganglioside mixture shown in Fig. 2a, the relative amount of the adsorbed *O*-acetylated compound was unknown. Therefore, an influenza C virus assay was carried out using the radiolabelled *O*-acetylated GM_3 containing 30% *O*-acetylated sialic acid, in the range of 0.11 and 14.81 nmol/100 µl. Directly after the whole virus assay procedure the adsorbed radioactivity was determined. The results are shown in Fig. 3a. Though the obtained fluorescence increased with higher ganglioside concentrations, there was no linear correlation between the fluorescence and the amount of the virus counter-receptor. Throughout the whole range of applied ganglioside concentrations the fluorescence of the methylumbelliferone formed was relatively low when compared with the control experiments, in which the corresponding non-acetylated ganglioside was used (relative fluorescence, 20–30).

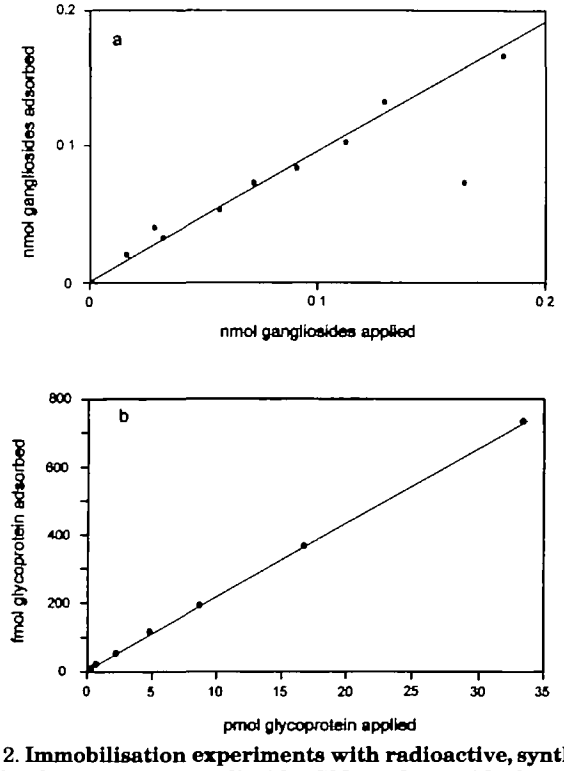

Fig. 2. **Immobilisation experiments with radioactive, synthetically *O*-acetylated ganglioside GM_3 and α_1-acid glycoprotein.** (a) The relative amounts of a mixture of tritium-labelled $Neu5,9Ac_2$-GM_3 (30%) and GM_3 (70%) adsorbed to microtiter plates were determined in the range of 0–0.2 nmol/100 µl by measurement of the radioactivity bound to the wells. (b) [^{125}I]AGP containing 5% $Neu5,9Ac_2$ in its sialic acid fraction was applied in the range of 0–35 pmol to microtiter plates and then the amount of adsorbed glycoprotein was determined.

For investigation of the sensitivity of the virus test for glycoproteins, acetylated AGP at concentrations between 0.27 and 34.4 pmol/100 µl was applied. In this range a functional relation between the applied and adsorbed amounts was found, as described above (Fig. 2b). The results of the binding assay with influenza C viruses are shown in Fig. 3b, demonstrating that about 0.1 pmol of this glycoprotein can be easily detected with this virus. On the basis of 5% *O*-acetylation of the sialic acid content, this amount corresponds to 0.08 pmol of glycosidically linked $Neu5,9Ac_2$ (Table II).

2. Overlay-assay:

2.1. TLC overlay of gangliosides. The gangliosides were separated by TLC before treatment with influenza C virus, thus allowing the investigation of individual gangliosides in a mixture. As examples, GM_3 and its 9-*O*-acetylated derivative are shown in Fig. 4. Since the intensity of the colour, which develops during the assay, is not proportional to the amount of bound virus, it is not possible to exactly quantify the *O*-acetylated gangliosides with this assay. Therefore, the sensitivity of the assay was determined by evaluating the lowest amount of applied ganglioside with which the

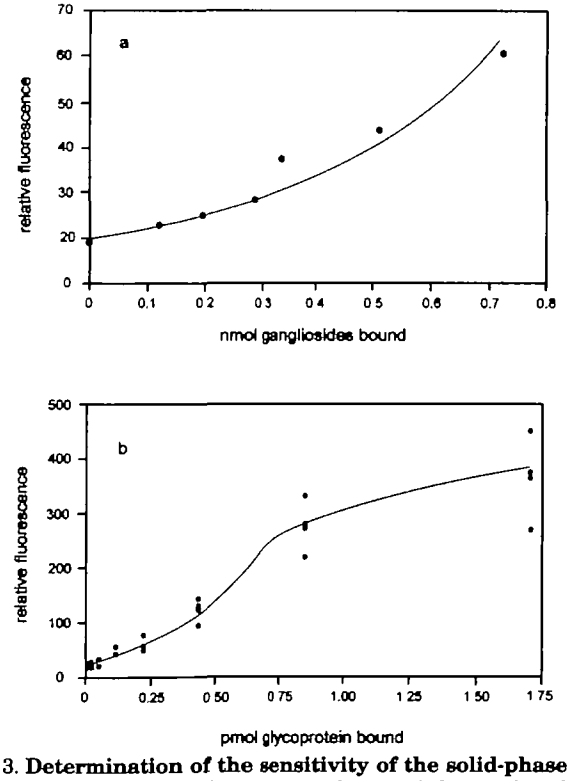

Fig. 3. **Determination of the sensitivity of the solid-phase assay for the detection of $Neu5,9Ac_2$-GM_3 and *O*-acetylated α_1-acid glycoprotein by influenza C virus.** (a) Mixtures of tritium-labelled $Neu5,9Ac_2$-GM_3 and GM_3 containing 30% of *O*-acetylated ganglioside at concentrations between 1.11 and 148.1 µM were examined. After carrying out the solid-phase assay (*33*), the adsorbed amount of ganglioside was determined in each well by radioactivity counting. The relative fluorescence (ordinate) is a measure of virus binding. Non-*O*-acetylated GM_3 served as a control. (b) [^{125}I]AGP containing 5% $Neu5,9Ac_2$ in the sialic acid moiety was applied to mictrotiter plates in the concentration range of 2.7–344 nmol. The amount of adsorbed glycoprotein was determined using data obtained in the immobilisation study shown in Fig. 2b. As a control, non-acetylated AGP (344 nmol) was used.

binding of virus could be visualised. In the case of GM_3, a minimum amount of 800 pmol *O*-acetylated ganglioside could be detected on TLC-plates, whereas 100 pmol was detectable with the solid-phase assay. In comparison, 270 pmol of *O*-acetylated GD_{1a} was still detectable in the overlay assay. For *O*-acetylated GD_3 from buttermilk significantly higher sensitivity was found (Table II).

In some experiments, the detection of specific binding was difficult because of the high background staining. This might be due to unspecific binding of the viruses to the plates, unspecific hydrolysis of the substrate or partial release, and spreading of the bound virus due to the hydrolysis of ligands by the esterase.

2.2. Nitrocellulose membrane overlay of glycoproteins. Rat serum glycoproteins containing 60% *O*-acetylated sialic acids were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Under these conditions binding of influenza C virus to the main glycoprotein was observed at concentrations higher than 2 μg per lane containing about 200 pmol $Neu5,9Ac_2$. This low sensitivity might be due to the conditions for both SDS-PAGE and electroblotting, and especially to the alkaline pHs of the buffers used in these procedures. This was confirmed by the incubation of $Neu5,9Ac_2$ in Tris-buffer of different pH for 1 to 2 h, which led to an appreciable loss of acetyl groups at higher pH values. At pH 8.5, the pH of the buffer used for SDS-PAGE according to Laemmli, saponification of about 20% must be taken into account after 1 h, while at pH 10 this figure was already 42%.

The use of phosphate buffers of pH 7.2 (SDS-PAGE) and 6.5 (electroblotting) during these procedures yielded a threefold increase in sensitivity, corresponding to 71 pmol $Neu5,9Ac_2$ when compared to about 200 pmol under the alkaline conditions (Table II). However, compared to the solid-phase assay, this sensitivity is still significantly lower. Since an amount of 1.52 pmol protein-bound $Neu5,9Ac_2$ could be detected when proteins were directly transferred to the membranes by dot-blotting, a loss of glycoproteins during the electroblotting might be responsible for the low sensitivity.

Specificity—Though influenza C viruses bind to both gangliosides and glycoproteins carrying $Neu5,9Ac_2$, there are differences in recognition between glycoproteins and gangliosides; and also between different gangliosides, indicating further structural requirements for optimal binding of the ligand. As is shown in Fig. 5, there are strong differences in virus binding between individual gangliosides containing $Neu5,9Ac_2$ at a more accessible position, *i.e.* linked to a terminal galactose residue (compare GM_3 with GD_{1a}). The fluorescence obtained with the same amount of *O*-acetylated ganglioside is about twice as high for GD_{1a} when compared to GM_3. Virus binding of GT_{1b} is of intermediary strength.

In order to obtain an insight into the role of the sialic acid molecule itself in this binding, analogues of GM_3 with modified sialic acid residues were examined as ligands for the viruses. In compounds GSC 23 and GSC 24, Neu5Ac was substituted by the corresponding C-7 analogue 5-acetamido-3,5-dideoxy-L-*arabino*-2-heptulopyranosonic acid and the C-8 analogue 5-acetamido-3,5-dideoxy-D-*galacto*-2-octulopyranosonic acid, respectively (Fig. 1). The stereo-

TABLE II. **Sensitivity of the solid-phase and overlay virus assays for different glycoconjugates containing $Neu5,9Ac_2$.** Gangliosides were chemically *O*-acetylated and then the degree of modification was determined on TLC-plates by densitometric measurement. The percentages of $Neu5,9Ac_2$ in GD_{1a} and GD_3 were calculated on the terminal Neu5Ac only. The contents of $Neu5,9Ac_2$ in rat serum glycoproteins and synthetically *O*-acetylated human AGP were determined by HPLC of the released sialic acids. The minimum determinable amount was calculated on the basis of the $Neu5,9Ac_2$ content and is expressed in pmol glycosidically linked $Neu5,9Ac_2$.

Glycoconjugates	Content of $Neu5,9Ac_2$ (%)	Solid-phase assay	Overlay-assay
GM_3-$Neu5,9Ac_2$	30	100	800
GD_{1a}-$Neu5,9Ac_2$	35	12	270
GD_3-$Neu5,9Ac_2$	50	—	32
Human α_1-AGP	5	0.08	—
Rat serum glycoproteins	60	0.5	1.52[a]
			200[b]
			71[c]

—, not determined. [a]Dot-blotting; [b]Western-blotting at pH 8.8; [c]Western-blotting at pH 7.2.

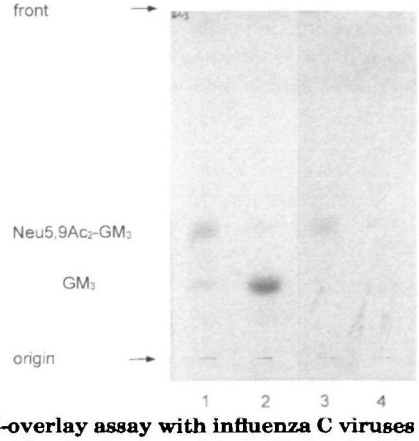

Fig. 4. **TLC-overlay assay with influenza C viruses of *O*-acetylated GM_3.** After development of the TLC-plate in chloroform/methanol/water (65 : 25 : 4), the left half was stained with orcinol reagent (lanes 1 and 2), whereas the right half was treated with influenza C viruses (lanes 3 and 4). Lanes 1 and 3 represent the ganglioside mixture obtained on *O*-acetylation, while lanes 2 and 4 show the saponified sample. The upper band is the *O*-acetylated product, which is stained by the virus.

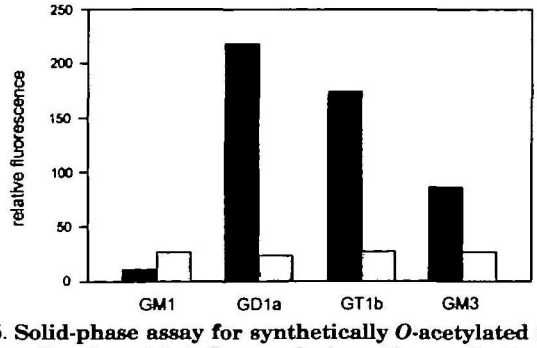

Fig. 5. **Solid-phase assay for synthetically *O*-acetylated natural gangliosides with influenza C virus.** The gangliosides were *O*-acetylated as described under "MATERIALS AND METHODS." The product mixtures were directly used in the assay. For each sample, 1 nmol of the main acetylation product was applied to the microtiter plate (■). As a control the same amount of a saponified sample was used (▨).

chemical arrangement at position 8 of Neu5Ac in GSC 50 has been changed from the *R*- to the *S*-configuration, resulting in 8-epi-Neu5Ac-GM_3, whereas the unmodified sialic acid in compound GSC 61 is linked to position 6 instead of position 3 of the penultimate galactose residue. Apart from these changes, the structure of the sugar chain and the ceramide residue in the synthetic gangliosides is the same as in the naturally occurring ganglioside GM_3. Therefore, the adsorption behaviour of the gangliosides towards the surface of the microtiter plates should be similar.

These compounds were synthetically *O*-acetylated according to (*37*), and the conversion was examined by TLC and densitometric quantification (Table I). Only GSC 23 bearing the C-7 analogue of Neu5Ac was not acetylated under these conditions. Conversions between 40 and 80% were found for the other analogues.

The mixtures obtained after *O*-acetylation were directly used for the solid-phase and overlay assays. The differences in *O*-acetylation of the GM_3 analogues were taken into account. In the solid-phase assay about the same amount of an *O*-acetylated component (2 nmol) was applied to each well. *O*-Acetylated GSC 24 and GSC 50 were not recognised by the viruses (Fig. 6). In contrast, the virus binding to compound GSC 61 containing an α2,6-linked sialic acid was even stronger than that to natural GM_3. GSC 23 was not examined as a receptor for influenza C virus, due to its lack of *O*-acetylation.

Fundamentally, the same results were obtained when these glycolipids were examined after TLC using the overlay-technique, as shown in Fig. 7 for GSC 61, the GM_3 analogue with α2,6-linked Neu5Ac.

Investigation of the O-Acetylation of Gangliosides from Human Melanoma—Since *O*-acetylated GD_3 is a tumour-associated antigen in human melanoma (*17*, *48*, *49*), the kind and variation of *O*-acetylated gangliosides in a larger series of probes were studied using the virus assay. Earlier studies (*35*) have shown that the embedding of samples in paraffin and storage for about 1 year did not affect the *O*-acetylation. Furthermore, no gangliosides were detected in the paraffin-containing xylene layer after treatment of the samples, indicating only a small loss of glycolipids during this procedure. It was therefore possible to investigate a larger series of embedded samples of human normal skin and melanomas at different stages apart from fresh tissue samples.

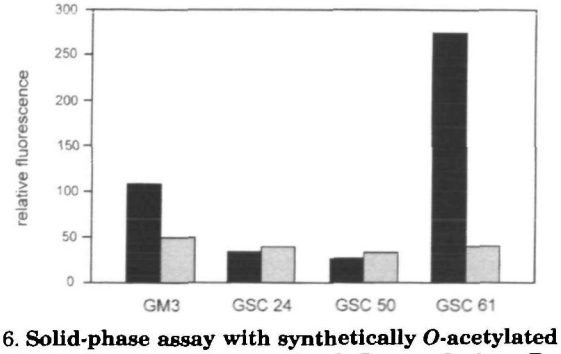

Fig. 6. **Solid-phase assay with synthetically *O*-acetylated GM_3 and its structural analogues using influenza C virus.** For each sample, an amount corresponding to 1 nmol of the main acetylation product was applied to each well (■). As a control, the same amount of a saponified sample was used (▩).

The total sialic acid contents of the individual probes varied between 0.006 and 0.7 mg per g tissue (referred to the weight after the removal of paraffin). After hydrolysis with propionic acid and HPLC analysis, both Neu5Ac and $Neu5,9Ac_2$ were found in most tumour samples. The amount of *O*-acetylated sialic acid occurring in 7 of 11 tumour samples of this analytical series varied between 2–12% of total sialic acids. In contrast, $Neu5,9Ac_2$ (0.5 and 3%, respectively) was found in only 2 out of 6 samples of normal skin. A peak corresponding to Neu5Gc was not detected. When investigating the sialic acid content of the material after removal of the glycolipids, which may belong to glycoproteins, no $Neu5,9Ac_2$ was detectable.

For investigation of the *O*-acetylation with influenza C viruses, both the solid-phase and TLC-overlay assays were used. Since in the first assay the same amount of ganglioside-bound sialic acid was applied to each well of the microtiter plates, the fluorescence observed for normal skin and tumour samples from the same patient can be directly compared. As shown in Fig. 8, in 12 of 14 tumour samples an increase of virus binding was observed in comparison to the surrounding normal skin, thus indicating an increase in *O*-

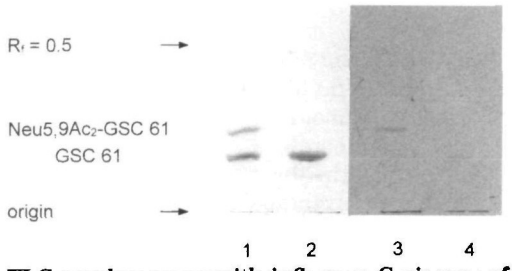

Fig. 7. **TLC-overlay assay with influenza C viruses of the *O*-acetylated GM_3 analogue GSC 61.** After development of the TLC-plate in chloroform/methanol/water (65 : 25 : 4), the left half was stained with the orcinol reagent (lanes 1 and 2), whereas the right half was treated with viruses (lanes 3 and 4). Lanes 1 and 3 represent the ganglioside mixture obtained on *O*-acetylation (the upper band is the *O*-acetylated product, which is stained by the virus), while lanes 2 and 4 show the saponified sample.

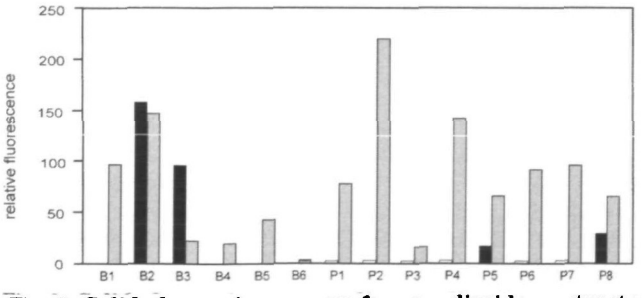

Fig. 8. **Solid-phase virus assay for gangliosides extracted from human melanoma (▩) and samples of normal skin (■).** The fluorescence values obtained for individual samples, the designations of which are indicated, were corrected as to the data for the corresponding saponified samples. For direct comparison, the same amount of ganglioside-bound sialic acid was applied in the cases of melanoma and normal skin tissues from the same patient. The designations represent the following tumour stages: Level II, P4; level III, B3, P3; level IV, P5; level V, B1, P1; metastases, B2, B5, B6, P2, P3, P6, P7. In normal skin surrounding tumours B1, P1, P2, P3, P4, P6 and P7, no *O*-acetylated gangliosides were detected.

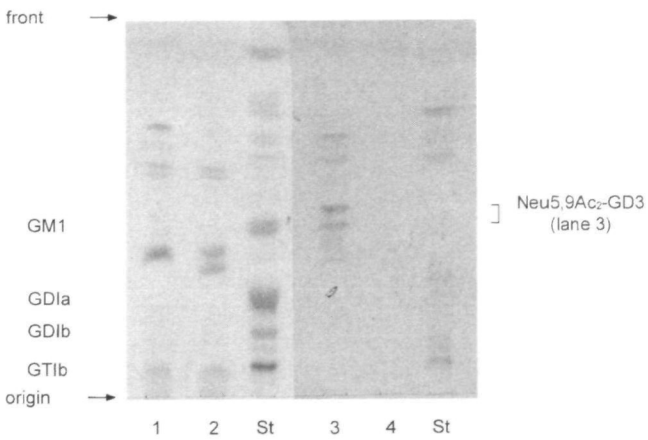

Fig. 9. **TLC-overlay assay with influenza C viruses for gangliosides from a human melanoma sample (P10M).** After development, the right half was treated with influenza C viruses, whereas the left half was stained with the orcinol reagent. In lanes 1 and 3, native gangliosides from tumour probe P10M were applied; lanes 2 and 4 show saponified gangliosides from this tissue. As standards (St), total glycolipids from bovine brain were chromatographed. Neu5,9Ac_2-GD_3 (as a double band?) is the predominant 9-*O*-acetylated ganglioside.

acetylation, whereas only in two cases was the fluorescence obtained for normal skin higher or comparable to the result for the respective tumour sample. These findings correspond to the results obtained with fluorometric HPLC. A relationship between the fluorescence and the stage of melanoma malignancy including metastasis was not seen. However, the fluorescence was highest in the central areas of melanoma tissues.

Using the overlay-technique, for the 11 tumour probes examined up to 5 individual bands of *O*-acetylated gangliosides were detected on virus-binding, although for most samples only one or two compounds were visible, which disappeared on saponification (Fig. 9, lane 4). The upper two bands migrated synchronously with virus-positive spots of bovine brain gangliosides (Fig. 9, St). The *O*-acetylated gangliosides from the tumour samples were not clearly visible after treatment with the less sensitive orcinol-reagent, due to the minute quantities. With this reagent, up to 4 main bands were visible for most tumour samples (Fig. 9, lane 1), which were not stained with the virus. They were not easy to compare with the gangliosides from bovine brain. It should be noted that many bands appeared on staining of bovine brain gangliosides with influenza C virus (Fig. 9, right St). Virus binding was observed for only one probe of normal skin studied.

A direct comparison of the ganglioside patterns of melanoma and normal skin samples was not possible at this stage, since more material is required for structural analysis of the gangliosides observed.

DISCUSSION

The binding properties of influenza C viruses, exclusively recognising 9-*O*-acetylated sialic acids and not other *O*-acetyl isomers, have been frequently used for the detection of Neu5,9Ac_2 in both isolated glycoconjugates and in tissues (*33*, *35*, *50–52*). In contrast to antibodies (*53*) and lectins (*54*, *55*) which also bind to this epitope, influenza C viruses exhibit a more pronounced specificity with regard to the nature of the sialic acid irrespective of the remaining part of the molecule. The studies have shown that several gangliosides and glycoproteins containing Neu5,9Ac_2 are able to mediate influenza C virus attachment. However, there are differences in the affinity depending on the structure and nature of the glycoconjugate.

The *O*-acetylation procedure according to Ogura *et al.* (*37*) allows the generation of glycoconjugates with defined structures containing Neu5,9Ac_2 and one can examine these compounds as receptors of influenza C virus. The variable rates of *O*-acetylation of the gangliosides GM_1, GD_{1a}, GM_3, and the structural analogues of GM_3 as well as the need to use various concentrations of the acetylating reagent TMOAc suggest an influence of the acidity and polarity of these molecules on the reaction. Variations may also be due to the differences in the position of the sialic acid residue(s) within the ganglioside molecule. Acetylation experiments involving the GM_3 analogues showed the influence of the structure of the sialic acid on the reaction. According to the suggested mechanism for this reaction a cyclic acetal is formed between the reagent and two vicinal hydroxyl groups, which is opened to yield the mono-*O*-acetylated product (*37*). The change in the stereochemical arrangement of the hydroxyl group at position 8 in the side chain (GSC 50) seems to facilitate the acetal formation and therefore to lead to a faster reaction. Furthermore, no reaction was found for the analogue GSC 23, which lacks the prerequisitive 1,2-diol structure. These findings confirm the earlier proposed mechanism.

These modified compounds were investigated using both the solid-phase and TLC-overlay assays. Earlier studies (*33*) showed that GM_1 and GM_2 containing Neu5,9Ac_2 as the internal sialic acid were not detected by influenza C viruses, whereas binding was found in the case of GM_3, GD_3, and GD_{1a} having terminal Neu5,9Ac_2. The results obtained in this study show that disialogangliosides carrying Neu5,9Ac_2 at the terminal galactose mediate stronger virus binding than 9-*O*-acetylated GM_3. Furthermore, the binding is stronger for GD_3 than for GD_{1a} (Table II). In these gangliosides Neu5,9Ac_2 is linked to different oligosaccharide chains [Neu5,9Ac_2(α2,3)Gal(β1,4)Glc in GM_3; Neu5,9Ac_2(α2,3)Gal(β1,3)GalNAc in GD_{1a}; Neu5,9Ac_2(α2,8)Neu5Ac(α2,3)Gal(β1,4)Glc in GD_3], representing carbohydrate chains of various lengths with different neutral monosaccharides, types of glycosidic linkages and negative charges. This may result in a different presentation of the binding epitope. It has to be determined whether the virus binding depends on further structural features of the carbohydrate chain or whether it simply serves as a spacer for the epitope. Possible effects on virus binding may also be caused by the way individual gangliosides are adsorbed to the matrix.

Furthermore, the structure of the sialic acid, especially of the side-chain, plays an important role in virus recognition. Truncation of the side-chain as well as a change in the stereochemical arrangement of the hydroxyl groups lead to a complete loss of virus recognition. The nature of the glycosidic linkage of the sialic acid to the penultimate sugar also influences the binding, α2,6-linked Neu5,9Ac_2 mediating stronger virus binding. Further investigations have to be carried out with other structural analogues to elucidate the

role of the other hydroxyl groups and their stereochemical arrangement for virus recognition.

In the present study the sensitivity of both types of influenza C virus assay was thoroughly investigated using radiolabelled GM_3 as a standard ganglioside. For the solid phase assay the immobilisation behaviour was examined to determine the relative amount of a compound which was adsorbed to the matrix. At concentrations higher than 0.1 nmol/100 µl (10^{-6} M) high variation in the adsorbed radioactivity and therefore the ganglioside was observed, whereas an almost linear relation between the adsorbed and applied amounts was found at lower concentrations. Gangliosides form micelles at concentrations higher than the critical micellar concentration (cmc), which is between 10^{-8} and 10^{-10} M in aqueous solutions (*56*). Since methanol is less polar, a higher cmc might be expected. The formation of these micelles and clusters may lead to polymolecular layers resulting in lower accessibility of the receptors and therefore reduced virus recognition. Furthermore, the wrong orientation of the receptor determinant could cause lower sensitivity.

A less sensitive assay for *O*-acetylated gangliosides is TLC-overlay. However, in contrast to the solid-phase assay, individual compounds in a mixture can be investigated as ligands after chromatography. As discussed for the solid-phase assay, the mode of presentation of the epitope $Neu5,9Ac_2$ may have a significant influence on the recognition by the virus. The orientation of the applied glycoconjugate and therefore of the epitope within the silica gel layer is unknown. Furthermore, the fixation procedure may hinder the access of the influenza C viruses. In comparison, immuno assays involving antibodies against special epitopes are known to be more sensitive than the methods described in this study. For example, a fivefold higher sensitivity was found for GD_3-$Neu5,9Ac_2$ on using monoclonal antibodies. However, in contrast to antibodies, influenza C viruses exhibit more pronounced specificity with regard to the nature of the sialic acid irrespective of the remaining part of the macromolecule.

Sensitivity experiments were also performed for glycoproteins. Earlier studies were carried out with BSM containing 4, 7, and 8-*O*-acetylated as well as di-*O*- and tri-*O*-acetylated sialic acids apart from $Neu5,9Ac_2$ (*33*). In the present study *O*-acetylated AGP containing 5% $Neu5,9Ac_2$ as the only *O*-acetylated sialic acid was investigated. Since one molecule of this glycoprotein contains about 16 molecules of sialic acids bound to different *N*-glycosidically linked glycans (*57*, *58*), about one sialic acid moiety per protein molecule was *O*-acetylated. Due to the random *O*-acetylation, a mixture of differently *O*-acetylated molecules has to be expected. In the solid-phase assay, glycoproteins are allowed to adsorb to the surface of the microwells using buffers of pH 7.2 instead of the commonly used buffers of pH 9.5, in order to avoid loss of *O*-acetyl groups. Under these conditions only 2.5–5% of the glycoprotein was bound to the surface, showing a linear relation between the adsorbed and applied amounts of compound within a wide range of concentrations. Apparently, the adsorption of the glycoprotein follows a different mechanism compared to gangliosides. Furthermore, the orientation of the oligosaccharide chains within the glycoprotein seems to facilitate the recognition of the sialic acid by the influenza C viruses. This yielded a thousandfold higher sensitivity of the assay when compared with gangliosides.

Alternatively, individual glycoproteins in a mixture can be investigated using the overlay-technique after electrophoretic separation and transfer to nitrocellulose membranes. The test after this procedure was significantly less sensitive than the solid-phase assay. This could be due to a loss of protein and of *O*-acetyl groups, respectively, during the transfer. It was shown again that the loss of acetylation can be decreased by the use of buffer systems of lower pH. Correspondingly, a threefold increase in sensitivity was found when the SDS-PAGE and electroblotting were carried out at pH 7.2. Nevertheless, the sensitivity is still lower than in the solid-phase method (Table II). A more significant increase in sensitivity was achieved when the proteins were directly transferred to the membranes without previous SDS-PAGE. In this case the sensitivity was comparable with the results of the solid-phase assay.

In the present study gangliosides from human melanoma were investigated and compared with gangliosides isolated from normal skin from the same patient. Since the latter samples were obtained from tissue directly surrounding the tumour, it is not clear whether the metabolism within the cells had already changed or not. However, this material was classified as tumour-free on histological analysis. Material from other unaffected parts of the body was not available. When interpreting the results, it also has to be taken into account that the whole skin was used without any separation of different cell types or skin layers. Thus, the samples contain many different cells apart from a relatively small amount of melanocytes.

The *O*-acetylation of the extracted gangliosides was investigated with both influenza C virus assays. To enable direct comparison of the fluorescence obtained in the solid-phase assay, the same amount of ganglioside-bound sialic acid was applied to each well. Comparison on the basis of equal amounts of gangliosides was not possible due to the fact that the ratio of mono- and disialogangliosides was not known and may differ between individual samples. Furthermore, the sensitivity of the assay varies for different gangliosides, as was shown in this study. According to Portoukalian *et al.* (*59*), mainly the gangliosides GD_3, GM_3, GM_2, and to a smaller extent GD_2 in varying amounts and ratios can be expected in human melanoma and skin.

Using the solid-phase assay, in most tumour samples an increase in influenza C virus binding to the gangliosides was found compared to normal skin, but the results differed between individual samples of the same developmental stage and severity. In several cases no or only little virus binding was observed in both the melanoma and normal skin samples from the same patient, although $Neu5,9Ac_2$ was found in these samples on HPLC after the acid release of sialic acids. Since *O*-acetylation of the total sialic acid content in the tissue probes was in the range of only 2 and 12%, the amount of applied ligands in some cases was probably not sufficient for virus recognition when investigating the glycolipids. Furthermore, the previously discussed effects may influence recognition by the virus.

Using the TLC overlay assay, virus binding to a variable number of ganglioside bands, an example of which is shown in Fig. 9, was found for all individual probes. Whether these findings are due to the heterogeneity of the ceramide moiety or the occurrence of different gangliosides carrying *O*-acetylated sialic acids has to be determined by using

suitable reference gangliosides and specific antibodies. Also new techniques like mass spectrometry for the analysis of minute amounts of glycolipids should be applied in future studies. In addition, it is necessary to collect more tumour samples in order to isolate more glycolipid material for analysis.

However, the results demonstrate that the sialic acid *O*-acetylation of gangliosides in melanoma is more complex than shown in former studies involving monoclonal antibodies against *O*-acetylated GD_3 (*17*). Interestingly, the *O*-acetylation of gangliosides, mainly of GD_3, is also increased in basalioma, another type of skin tumour (*18*, *19*, *60*, and unpublished data), as well as in human squamous cell carcinoma (*60*). Furthermore, the observation of higher sialic acid *O*-acetylation of gangliosides, mainly of GD_3, in murine neuroblastoma cells (*61*) and human breast cancer (*62*) adds to the hypothesis that the corresponding *O*-acetyltransferase is more active in tumours of neuroectodermal origin (*61*). Evidence is accumulating that the production of higher quantities of *O*-acetylated gangliosides, especially of *O*-acetyl GD_3, is related to rapid growth, both under physiological and pathological conditions (*63*). In contrast to neuroectodermal tumours, sialic acid *O*-acetylation of mucin glycoproteins significantly decreases on malignant transformation of human colon epithelial cells (*64*).

It can be expected that this sensitive test involving influenza C virus will be widely applied to the screening of tissue and tumour samples with regard to the occurrence of *O*-acetylated sialoglycoconjugates as, for example, differentiation- and tumour-associated antigens. The assay is, furthermore, very useful for routine assays during the isolation of and substrate specificity studies on sialate-*O*-acetyltransferases and related enzymes (*3*, *4*).

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