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Binding specificity of influenza C-virus to variably *O*-acetylated glycoconjugates and its use for histochemical detection of *N*-acetyl-9-*O*-acetylneuraminic acid in mammalian tissues*

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The specificity of influenza C-virus binding to sialoglycoconjugates was tested with various naturally *O*-acetylated gangliosides or synthetically *O*-acetylated sialic acid thioketosides, which revealed binding to 9-*O*-acetylated *N*-acetylneuraminic acid. Binding was also observed with a sample of Neu5,7Ac_2-GD3, however at a lower degree. Sialic acids with two or three *O*-acetyl groups in the side chain of synthetic sialic acid derivatives are not recognized by the virus. In these experiments, bound viruses were detected with esterase substrates. Influenza C-virus was also used for the histological identification of mono-*O*-acetylated sialic acids in combination with an immunological visualization of the virus bound to thin-sections. The occurrence of these sialic acids was demonstrated in bovine submandibular gland, rat liver, human normal adult and fetal colon and diseased colon, as well as in human sweat gland. Submandibular gland and colon also contain significant amounts of glycoconjugates with two or three acetyl esters in the sialic acid side chain, demonstrating the value of the virus in discriminating between mono- and higher *O*-acetylation at the same site. The patterns of staining showed differences between healthy persons and patients with colon carcinoma, ulcerative colitis or Crohn's disease. Remarkably, some human colon samples did not show *O*-acetyl sialic acid-specific staining. The histochemical observations were controlled by chemical analysis of tissue sialic acids.

Keywords: Influenza C-virus, *N*-acetyl-9-*O*-acetylneuraminic acid, sialate 9-*O*-acetylesterase, haemagglutinin, histochemistry

Abbreviations: BSA, bovine serum albumin, BSM, bovine submandibular gland mucin; HAU, haemagglutination units; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac_2, *N*-acetyl-9-*O*-acetylneuraminic acid; Neu5,7,9Ac_3, *N*-acetyl-7,9-di-*O*-acetylneuraminic acid; Neu5,7,8,9Ac_4, *N*-acetyl-7,8,9-tri-*O*-acetylneuraminic acid; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

Introduction

Sialic acids constitute a family of over 40 different sugars, often found as terminal monosaccharides of animal glycoconjugates. The most frequently observed modification of the parent sugar *N*-acetylneuraminic acid is *O*-acetylation at position 9 to form *N*-acetyl-9-*O*-acetylneuraminic acid [1–3]. During the last few years there has been a growing interest in the occurrence, metabolism and especially function of *O*-acetylated sialic acids of glycoproteins and gangliosides. So far, *O*-acetylated sialic acids have been found to render complex carbohydrates more resistant to sialidase action and are thus believed to extend the lifetime of, for example, serum glycoproteins,

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*Dedicated to Prof. Dr Nathan Sharon on the occasion of his 70th birthday.

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blood cells [4], or colon mucin [5, 6]. They also hinder the invasion of mouse erythrocytes by malaria parasites [7] and inhibit the recognition of sialic acids by sialoadhesin of mouse macrophages [8] or by the lymphocyte receptor CD22 [9]. An antirecognition effect of 9-*O*-acetylated sialic acids is also exerted on the interaction of cells with influenza A and B viruses, which bind only to non-*O*-acetylated sialic acids [10]. *O*-Acetylated sialic acids appear to play significant roles in the function of neuronal tissues, such as in the development of rat brain [11, 12]. There are examples of *O*-acetylated sialic acids as differentiation antigens in rat colon [13] and in chicken erythrocytes [14]. In some diseases, a change of the *O*-acetylation pattern of tissues has been observed, for example in colon carcinoma, involving a reduction of the amount of higher *O*-acetylated sialic acids [15–18]. *O*-Acetylated sialic acids have been found as tumour-associated antigens like Neu5,9Ac_2-GD3 [19, 20] and Neu5,9Ac_2-GD2 [21] in human melanoma. One of the most striking and best defined pathobiochemical roles of Neu5,9Ac_2 is its function as a receptor determinant for viruses such as influenza C-virus and some corona viruses [22–24]. Influenza C-viruses also possess a receptor-destroying enzyme which was characterized as an esterase (EC 3.1.1.53) with high specificity for 9-*O*-acetyl groups of sialic acids as natural substrate [25], whereas the specificity of the viral haemagglutinin has not been studied in detail.

Most commonly used methods for the detection of sialic acids require their previous release from glycosidic linkages by either enzymatic or chemical hydrolysis before analysis by colorimetric or chromatographic methods [26, 27]. Problems generally observed with these methods include incomplete release of sialic acids, de-*O*-acetylation [2] and migration of *O*-acetyl groups [28]. Thus, new methods, which allow the detection of *O*-acetylated sialic acids on glycoconjugates without previous liberation, are of great interest. The receptor determinant for influenza C-virus has been shown to be Neu5,9Ac_2 and the virus has been used to detect glycoconjugates containing this sialic acid in a solid phase assay and overlay techniques [29, 30]. Since a direct demonstration of binding to 7-mono-*O*-acetyl sialic acid and oligo-acetyl sialic acids has not been presented, further studies were carried out to clarify this specificity using gangliosides and synthetic sialyl glycosides.

In histochemical staining procedures various basic dyes and the mild periodic acid Schiff technique (mPAS) were used to detect unsubstituted or variously *O*-acetylated sialic acids [31–33]. These methods, however, lack specificity for detection of mono-*O*-acetylated sialic acids in tissue sections. Accordingly, we report here a new histochemical technique using influenza C-virus to allow the specific detection of *O*-acetylated sialic acids in tissue sections. Very recently another technique employing the same principle, but using chimeras of influenza C-virus haemagglutinin and the Fc portion of IgG antibody was reported [9, 34].

Materials and methods

Virus and antibody

Influenza C-virus (strain Johannesburg/1/66), grown in embryonated chicken eggs, and anti-influenza C-virus rabbit antiserum were obtained from Dr G. Herrler, Institut für Virologie, Universität Marburg, Germany. Viruses were concentrated from allantoic fluid by differential centrifugation and suspended in phosphate-buffered saline, pH 7.2 (PBS; 137 mM NaCl, 3 mM KCl, 8.5 mM $Na_2HPO_4 \times 2\ H_2O$, 1.5 mM KH_2PO_4) containing 1% BSA. Haemagglutination titres were determined at 4 °C as described by Herrler and Klenk [22]. Anti-rabbit IgG fluorescein $F(ab)_2$ fragment from goat was from Boehringer Mannheim, Germany.

Glycoconjugates and sialosides

Neu5,7Ac_2-GD3 from hamster melanoma [35] and a mixture of GD3, Neu5,7Ac_2-GD3 and Neu5,9Ac_2-GD3 as well as Neu5,7,9Ac_3-GD3 from bovine buttermilk [36] were gifts from Professor Dr R.K. Yu and Dr S. Ren, Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Richmond, Virginia, USA. Sialic acid thioketosides [37], the chemical nature of which is shown in Fig. 4a, were provided by Professor Dr H. Faillard and Dr A. Roth, Institut für Biochemie, Universität des Saarlandes, Saarbrücken, Germany. Neu5,9Ac_2-GD3 from cheese whey was a gift from Snow Brand Milk Products Co. Ltd, Tokyo, Japan. BSM was isolated as described earlier [38].

Analysis of sialic acids and stability of O-acetyl groups

For qualitative analysis, sialic acids were released from glycosidic linkages of glycoconjugates and tissue samples by mild acid hydrolysis with 0.5 M formic acid at 70 °C for 1 h. Liberated sialic acids were directly converted to fluorescent derivatives with 1,2-diamino-4,5-methylenedioxybenzene (DMB) [26, 39]. The derivatives were separated isocratically by reversed-phase chromatography on an RP-18 cartridge (25 × 0.4 cm, Merck, Darmstadt, Germany) in acetonitrile:methanol:water (9:7:84, by vol) as mobile phase at a flow rate of 1 ml min^{-1}. Elution was monitored with a 980-fluorescence detector (Applied Biosystems, Weiterstadt, Germany), using an excitation wavelength of 343 nm and a cut-off emission filter of 389 nm.

Sialic acid standards were prepared from bovine submandibular gland mucin [38]. A fraction of higher *O*-acetylated sialic acids obtained from this source by preparative TLC was subjected to the same acidic

treatment in order to estimate the degradation of *O*-acetylated sialic acids under these hydrolytic conditions. Neu5,9Ac$_2$ was treated with formaldehyde as will be described for tissue sections below. The sialic acid nature of the fluorescent peaks was proven by saponification of *O*-acetyl groups and degradation of sialic acids by acylneuraminate-pyruvate lyase in parallel assays [40].

Thin-layer chromatography of gangliosides

Gangliosides were separated on glass-backed HPTLC plates (10 × 10 cm, silica gel 60, Merck) using chloroform:methanol:0.5% aqueous $CaCl_2 \times 2\,H_2O$ (55:45:10, by vol) as solvent system. The gangliosides were visualized by spraying with the orcinol:Fe^{3+}:HCl reagent followed by heating at 95 °C for about 30 min [26, 39].

Alkali treatment of gangliosides

Gangliosides were applied on HPTLC plates and exposed to concentrated ammonia vapour in a chamber at room temperature for approximately 12 h. After thorough drying, the same amounts of the samples and additional controls were applied on separate lanes and the plates developed as described above.

HPTLC-overlay assay

HPTLC-overlay assays were carried out similarly to the procedure described by Zimmer *et al.* [29, 30]. The gangliosides were chromatographed on HPTLC-plates as described above. After development, the plate was first dried in a stream of cold air for 10 min and then in a vacuum desiccator under reduced pressure for 30 min. The plates were dipped for 2 min in a solution of 0.4% polyisobutylmethacrylate (Plexigum P 28, Röhm, Darmstadt, Germany) in diethyl ether at room temperature for fixation, dried first for 15 min in a stream of cold air and then for 45 min in vacuo, followed by immersion in PBS containing 1% BSA for 60 min at room temperature. Then the plates were covered with influenza C-virus suspension in PBS (250 HAU ml^{-1}) containing 0.5% BSA and kept for 1 h at 4 °C. Finally, the plates were washed by dipping three times in PBS containing 0.05% Tween 20, and bound virus was visualized (red colour) with 1 mM α-naphthyl acetate in PBS containing 0.14% 2-amino-5-chlorotoluene × 1/2 $ZnCl_2$ (Fast Red TR-salt, Sigma, Deisenhofen, Germany). After 45 min at room temperature, the reaction was stopped by dipping the plates into H_2O. For Neu5,7Ac$_2$-GD$_3$ the corresponding steps were carried out in PBS, pH 6.8, at 4 °C.

Solid-phase assay on microtitre plates

Similar to the previously described assay [29, 30], gangliosides were dissolved in methanol to give a concentration of up to 2 µg sialic acid per ml; 100 µl of this solution was added to wells of microtitre plates (NUNC Immunomodule Maxisorb F8, Roskilde, Denmark) and the solvent allowed to evaporate for 2 h at room temperature. The plates were kept for 30 min under reduced pressure in a vacuum desiccator. Remaining binding sites on the polystyrene surface were saturated by incubation with 2% BSA in PBS for 1 h at 4 °C. The wells were washed twice with 200 µl PBS containing 0.05% Tween 20. For virus binding each well received 100 µl influenza C-virus suspension in PBS with an activity of 2 HAU. After incubation for 1 h at 4 °C, the wells were washed three times with PBS containing 0.05% Tween 20 at 4 °C, followed by incubation with 100 µl 60 µM methylumbelliferyl acetate in PBS at 37 °C for 45 min. The reactions were stopped by the addition of 100 µl ethanol, the solutions transferred into caps and 800 µl H_2O added. The amount of released 4-methylumbelliferone was assayed in a Perkin Elmer 1000 M fluorimeter at wavelengths of 365 nm for excitation and 450 nm for emission. Negative controls were: (a) incubation of glycoconjugates at 4 °C with PBS instead of virus; (b) saponification, as described above, of glycoconjugates before incubation with influenza C-virus. Non-enzymatic hydrolysis of methylumbelliferyl acetate was monitored by incubation at 37 °C without addition of virus. In the case of 7-*O*-acetylated sialic acids, PBS was adjusted to pH 6.8 instead of 7.2.

Inhibition assay

This assay was performed on microtitre plates as described above. BSM was dissolved in PBS in a concentration of 7.3 µg sialic acid per 100 µl; 90 µl of this solution was given into the well and the plates kept overnight at 4 °C. The solutions were removed and the remaining binding sites in the wells were saturated by incubation with 1% BSA in PBS for 1 h at room temperature.

Synthetic sialosides were tested in triplicate for their potency to inhibit influenza C-virus binding to the immobilized BSM. They were preincubated with influenza C-virus (1 HAU ml^{-1}) in PBS containing 1% BSA in a total volume of 100 µl at 4 °C for 30 min in microtitre plates and then added to the BSM-coated wells. After incubation for 10 min at 4 °C, the wells were washed three times with PBS containing 0.05% Tween 20 to remove unattached virus. Bound influenza C-virus was detected with 4-methylumbelliferyl acetate as described above.

Inhibition of virus binding was compared with the maximum binding obtained without sialoside. Non-specific binding to the microtitre plate was evaluated using saponified bovine submandibular gland mucin.

Origin and preparation of tissue sections

Human colon epithelium was obtained from patients with chronic constipation having histologically normal epithelium (taken as control), from fetal lower colon tissue obtained at sterile abortion from 15 (one sample) and 18

(three samples) weeks of gestation, and from patients with colon carcinoma, ulcerative colitis and Crohn's disease. These tissues were provided by Professor Dr M. Dietel and Dr I. Leuschner (Institut für Pathologie, Universität Kiel, Germany) as well as by Mr P. Durdey, Department of Surgery, Bristol Royal Infirmary and Professor J. Berry, Department of Pathology, St Michael's Hospital, United Bristol Health Trust, University of Bristol, Great Britain. Human skin was provided by Professor Dr E. Christophers and Dr U. Mrowitz, Klinik für Dermatologie, Venerologie und Allergologie, Universität Kiel, Germany. Glandula submandibularis of cow was received from a local slaughter house and liver of Wistar rats (male, 1 year old) from the animal house of Kiel University. Tissue samples were fixed in 10% formaldehyde in PBS for 24 h immediately after surgery or slaughter.

Paraffin-embedding and preparation of thin-sections were carried out as described by Romeis [41]. Serial sections of 4–6 µm were prepared for histological examination and stored at 4 °C in dry atmosphere.

Histochemical detection of side-chain mono-O-acetylated sialic acids

Tissue sections were deparaffinized [41] and incubated with 2% milk powder in PBS for 1 h at room temperature, followed by influenza C-virus suspension (100 HAU ml^{-1}) for 1 h at 4 °C. After washing with PBS containing 0.05% Tween 20 (3 times 5 min, each), bound influenza C-virus was immunologically detected by incubation with rabbit antiserum followed by treatment with the fluorescent anti-rabbit IgG antibody for 1 h at 4 °C, each. Saponified and sialidase-treated preparations were used as controls. Saponification of fixed tissue sections was done with 0.1 M NaOH for 1 h at 4 °C, followed by washing three times with PBS. Sialidase incubation was carried out with *Clostridium perfringens* and *Arthrobacter ureafaciens* sialidase, 1 U ml^{-1}, each, for 16 h at 37 °C in 50 mM acetate buffer, pH 5.0. Controls were obtained by incubation with buffer alone. The slices were inspected using a Zeiss Axiophot photo-microscope.

Results

In order to determine the binding specificity of influenza C-virus haemagglutinin, differently *O*-acetylated gangliosides were compared in the HPTLC overlay assay. Figure 1 shows that binding of influenza C-virus was found with Neu5,9Ac_2-GD3 as well as with Neu5,7Ac_2-GD3. When both gangliosides were immobilized on microtitre plates at different concentrations, Neu5,9Ac_2-GD3 exhibited approximately two-fold stronger receptor activity towards influenza C-virus than the 7-*O*-acetylated compound (Fig. 2). In contrast to gangliosides with sialic acids mono-*O*-acetylated at the side-chain, Neu5,7,9Ac_3-GD3 did not interact with the receptor in the HPTLC overlay assay

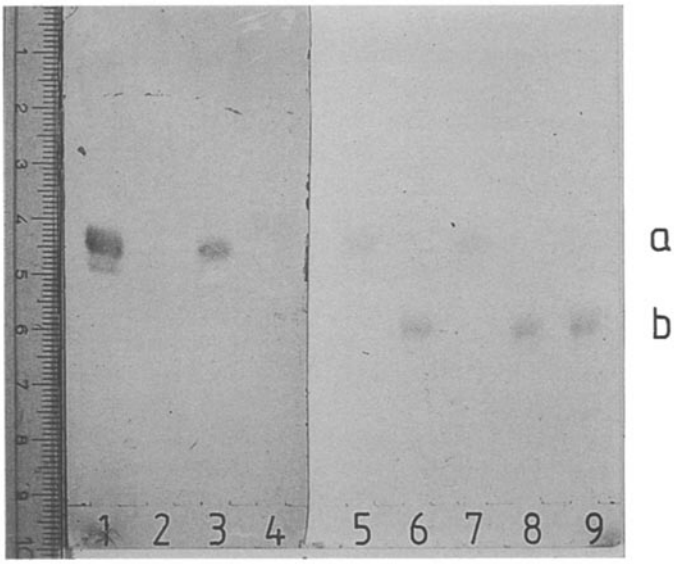


Figure 1. HPTLC-overlay assay of Neu5,7Ac_2-GD3 from hamster melanoma with influenza C-virus. Bound viruses were strained red in nature. Lanes 1–4 were used for virus incubation, lanes 5–9 were sprayed with the orcinol/Fe^{3+}/HCl reagent. Lanes 1 and 5: Neu5,9Ac_2-GD3 standard; lanes 2 and 6: Neu5,9Ac_2-GD3 standard saponified; lanes 3 and 7: Neu5,7Ac_2-GD3; lanes 4 and 8: Neu5,7Ac_2-GD3, saponified; lane 9: GD3 standard. One µg of the compounds was applied to each line. a, Neu5,7Ac_2-GD3 or Neu5,9Ac_2-GD3; b, GD3.

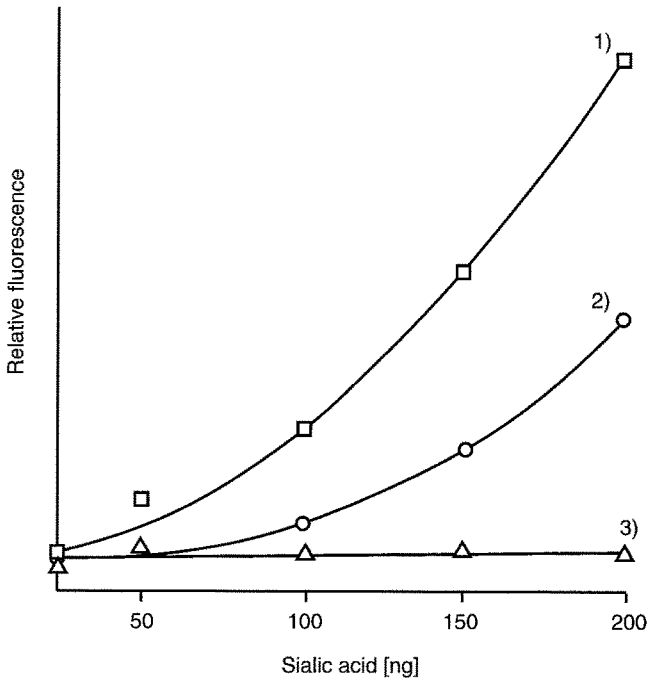


Figure 2. Binding of influenza C-virus to different amounts of *O*-acetylated GD3, expressed as the sialic acid contents, coated on microtitre plates: 1) Neu5,9Ac_2-GD3; 2) Neu5,7Ac_2-GD3; 3) GD3.

(Fig. 3). Alkaline treatment of the gangliosides resulted in loss of receptor activity in the HPTLC overlay assay for all compounds.

For the soluble sialic acid thioketosides (Fig. 4a) that do not bind to the wells of the microtitre plates, a different test had to be developed in which binding of the virus to immobilized BSM was tested after preincubation of virus and soluble sialoside. In this assay, a significant inhibition of binding of influenza C-virus to BSM was found in the presence of Neu5,9Ac$_2$-thioketoside, whereas the Neu5,7,8,9Ac$_4$-thioketoside inhibited binding to a much lower extent, similar to the non-*O*-acetylated Neu5Ac-thioketoside (Fig. 4b).

Before thin-sections of various tissues were analysed for the presence of Neu5,9Ac$_2$-containing sialoglycoconjugates, the stability of sialic acid *O*-acetyl groups was tested. When Neu5,9Ac$_2$ was treated in the same way as thin-sections, i.e. incubation in PBS containing 10% formaldehyde at pH 7.2 for 48 h at room temperature, less than 2% of the *O*-acetyl groups were lost, which is in the same order as the control incubation without formaldehyde.

The sialic acid composition of paraffin-embedded thin-sections was analysed after mild acid hydrolysis by fluorimetric HPLC. An example of sialic acids from human colon mucosa is given in Fig. 5 showing the

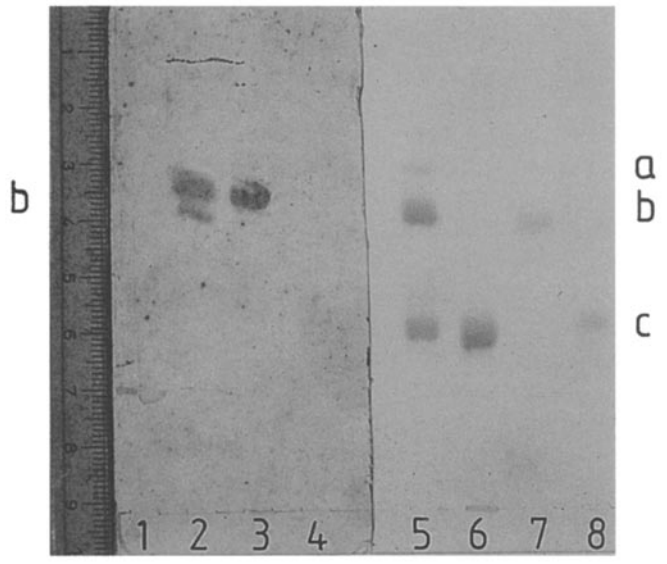
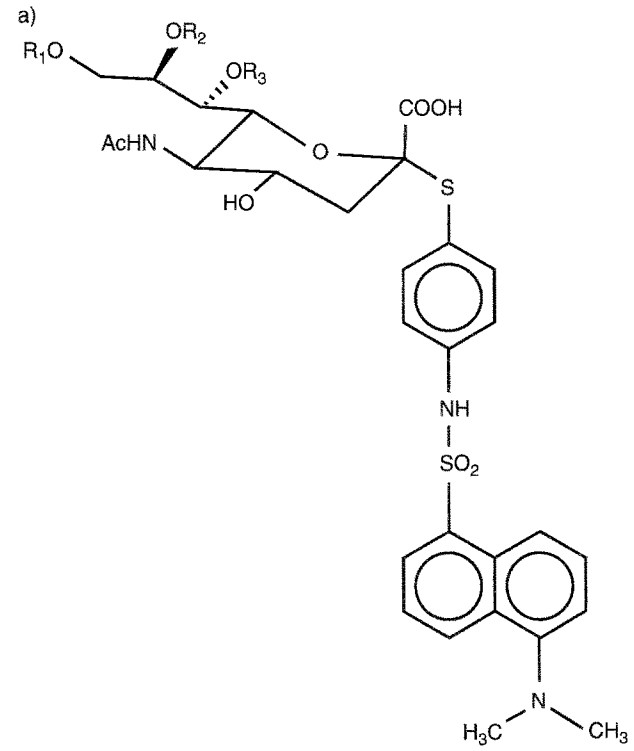


Figure 3. HPTLC-overlay assay of O-acetylated gangliosides from bovine buttermilk with influenza C-virus. Lanes 1–4 were used for virus incubation as described in Materials and methods. Lanes 5–8 were sprayed with orcinol/Fe^{3+}/HCl reagent. Lanes 3 and 5: mixture of GD3, Neu5,9Ac$_2$-GD3/Neu5,7Ac$_2$-GD3, and Neu5,7,9Ac$_3$-GD3 from bovine buttermilk: lanes 1 and 6: as lanes 3 and 5, saponified; lanes 2 and 7: Neu5,9Ac$_2$-GD3-standard; lanes 4 and 8: Neu5,9Ac$_2$-GD3 standard, saponified. a; Neu5,7,9Ac$_3$-GD3; b, Neu5,7Ac$_2$-GD3 and Neu5,9Ac$_2$-GD3; c, GD3.



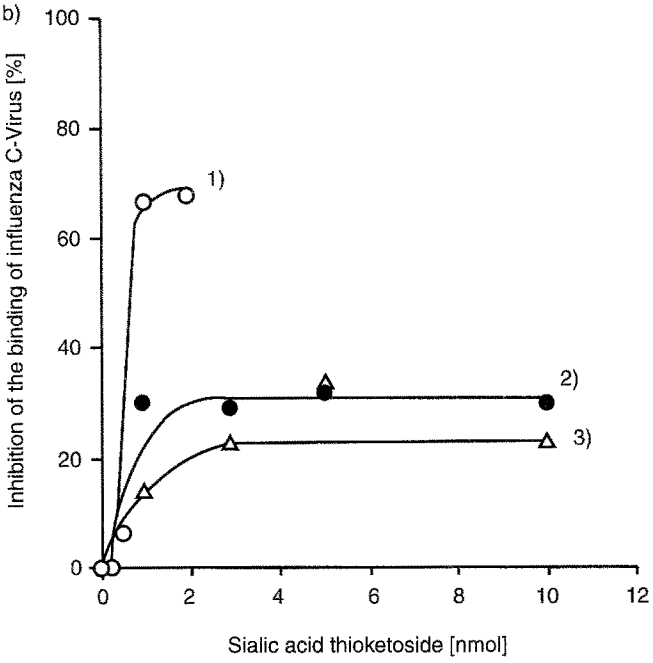


Figure 4. Inhibition of the binding of influenza C-virus to BSM coated on microtitre plates with *O*-acetylated sialic acid thioketosides. a) Structures of the glycosides: 1, 2α-[4-(dansylamino)phenylthio]-9-*O*-acetyl-*N*-acetylneuraminic acid (R_1 = Ac; R_2, R_3 = H); 2, 2α-[4-(dansylamino)phenylthio]-*N*-acetylneuraminic acid (R_1, R_2, R_3 = H); 3, 2α-[4-(dansylamino)phenylthio]-7,8,9-tri-*O*-acetyl-*N*-acetylneuraminic acid (R_1, R_2, R_3 = Ac). b) Inhibition of influenza C-virus binding to BSM immobilized on microtitre plates with the sialic acid thioketosides: 1, Neu5,9Ac$_2$-thioketoside; 2, Neu5Ac-thioketoside; 3, Neu5,7,8,9Ac$_4$-thioketosides.

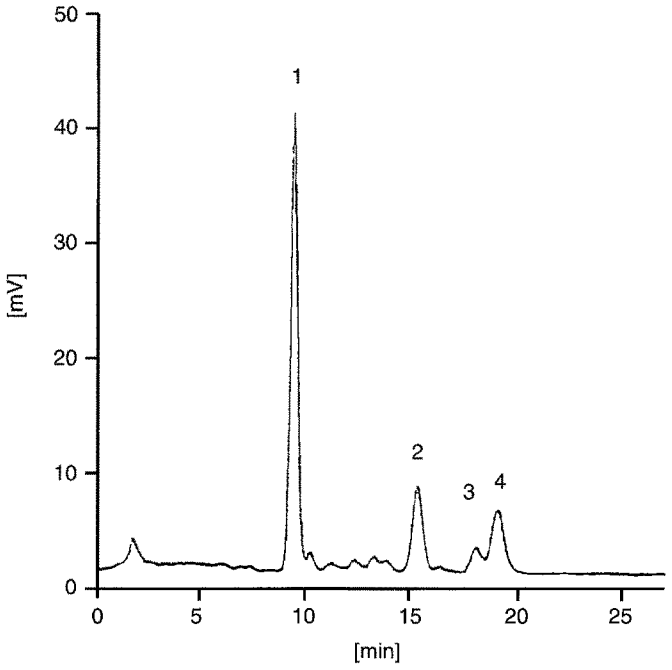


Figure 5. Sialic acids from normal human colon after acid hydrolysis detected by fluorimetric HPLC. 1, Neu5Ac; 2, Neu5,9Ac_2; 3 and 4 higher *O*-acetylated sialic acids. Peaks 2–4 disappeared on mild alkali treatment, with a concomitant increase of the Neu5Ac peak, showing their *O*-acetyl sialic acid nature.

presence of Neu5,9Ac_2 together with two peaks of higher O-acetylated sialic acids.

Although thin-sections usually were stained with the virus immediately after processing of the tissue samples, prolonged storage of the paraffin-embedded sections did not significantly influence the content of *O*-acetyl groups, as was shown for instance with bovine submandibular gland slices stored for 18 months, with both staining and chemical analysis using HPLC.

A well-known tissue with a high content of *O*-acetylated sialic acids as component of the salivary mucin is the bovine submandibular gland. The bright stain of the gland's lobuli, in which mucin is stored, is shown in Fig. 6. It largely disappears after sialidase treatment or saponification of the *O*-acetyl groups (not shown). Another tissue known to contain *O*-acetylated sialic acids is the human colon mucin. Figure 7 shows tissue sections of human colonic mucosa stained with influenza C-virus. The mucin-containing goblet cells and the mucus layer on the luminal surface are intensely stained. This staining disappears after the removal of *O*-acetyl groups by alkali treatment or of the sialic acids by sialidase treatment.

This method was applied to the staining of 10 control colon samples from adults, to four samples from fetuses and to diseased colon tissues, i.e. colon carcinoma ($n = 10$), ulcerative colitis ($n = 6$) and Crohn's disease ($n = 6$). Most control, fetal and diseased tissues showed a positive reaction of variable intensity. Strikingly, however, a few specimens did not bind the virus at all; this was observed in three of the 10 cases of control colon, three of the 10 cases of colon carcinoma and one of the six samples of Crohn's disease.

The staining patterns in diseased colon were much less regular than in control tissues. Irregular staining of the goblet cells was observed in two out of six samples of Crohn's disease and in three of six samples of ulcerative colitis. In the latter disease, an irregular staining of the goblet cells attracted special attention (Fig. 8). In undifferentiated colon carcinoma goblet cells containing *O*-acetylated mucus were absent and the virus bound in some cases to the luminal mucin, giving rather heterogeneous patterns.

The four examples of human fetal colon analysed showed a uniform, strong virus-binding of the goblet cells.

Tissues expressing non-mucin *O*-acetylated sialic acids can also be stained with influenza C-virus. This was shown with rat liver, where the endothelia of the capillaries reacted intensely (Fig. 9). Furthermore, some of the sweat glands of human skin studied reacted in the same way (Fig. 10). In both cases, the interaction with the virus did not occur after saponification or sialidase treatment, showing the sialic acid specificity of this staining reaction.

Discussion

The wide occurrence of *O*-acetylated sialic acids in nature and the increasing understanding of their biological and pathobiochemical significance has stimulated research into their direct demonstration in tissues. As mentioned in the Introduction, earlier PAS staining was applied for this purpose, and saponification of *O*-acetyl groups as well as sialidase treatment used to increase the specificity of this reaction. The lectin from the marine crab *Cancer antennarius* has been used to detect Neu5,9Ac_2-GD3 isolated from melanoma in a haemagglutination assay [42], but its use for the visualization of Neu5,9Ac_2 in tissue sections has not been described. This lectin together with that from the snail *Achatina fulica* [43] has a binding preference for Neu5,9Ac_2. However, both lectins can also bind to oligosaccharide structures without *O*-acetylation of sialic acids, which limits their suitability as specific tools for the analysis of *O*-acetylated sialic acids. An immunoglobulin isolated from human placenta has also been shown to recognize Neu5,9Ac_2 and to a lesser extent also Neu5Ac and Neu4,5Ac_2 [44, 45]. This interaction with Neu5,9Ac_2 was confirmed by NMR-spectroscopic studies (Siebert *et al.*, unpublished). It is not known whether these lectins or the immunoglobulin also recognize Neu5,7Ac_2 or sialic acids with several *O*-acetyl groups in the glycerol

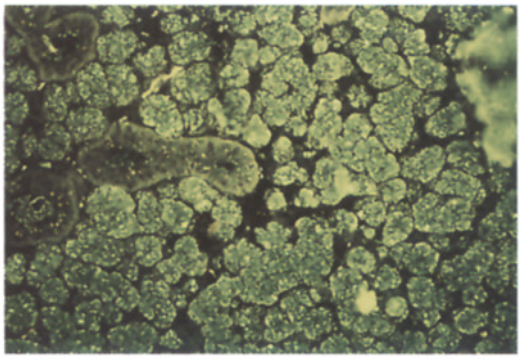


Figure 6. Staining of 9-*O*-acetylated sialic acids with influenza C-virus of the lobes of bovine submandibular gland. Magnification ×200.

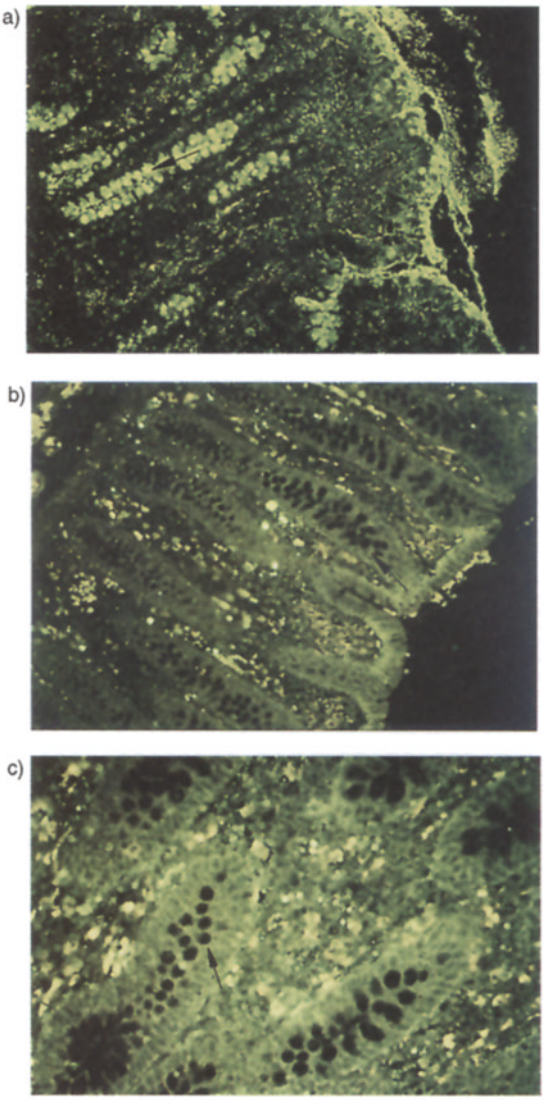


Figure 7. Staining of goblet cells (arrow) and mucus on the luminal surface of normal human colon. a) Native tissue, containing *O*-acetylated sialic acids. The abolition of goblet cell staining is shown in b) saponified tissue and c) sialidase-treated tissue.

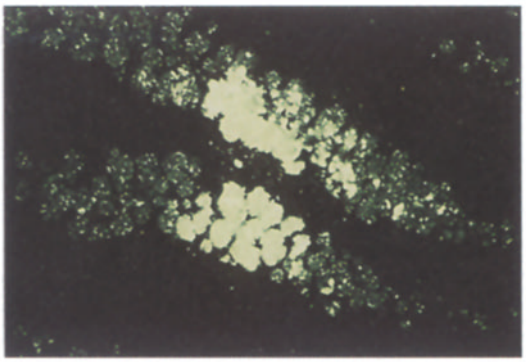


Figure 8. Staining with influenza C-virus of a colonic biopsy from a patient with ulcerative colitis. The irregular staining of the goblet cells is remarkable. Magnification ×400.

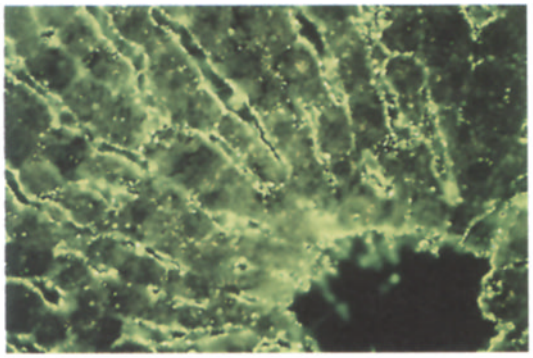


Figure 9. Staining of intralobular sinosides from rat liver with influenza C-virus. Magnification ×400.

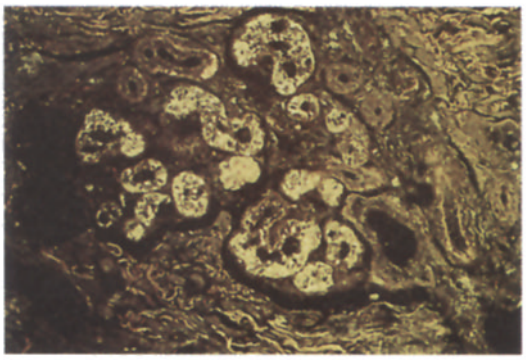


Figure 10. Occurrence of 9-*O*-acetylated sialic acids in sweat glands from human skin, as demonstrated by staining with influenza C-virus. Magnification ×200.

side chain. Although a number of monoclonal antibodies for Neu5,9Ac$_2$-GD3 have been described [19, 20, 46], there is to our knowledge no antibody available for the general and specific detection of Neu5,9Ac$_2$ in glycoproteins.

As shown by Zimmer *et al.* [29, 30], several gangliosides and glycoproteins are able to mediate influenza C-virus attachment if they contain Neu5,9Ac_2. In the present study, the microtitre and HPTLC overlay assays described in this reference [29, 30] were slightly modified, especially in order to reduce background staining. It is further demonstrated that influenza C-virus-binding is relatively independent of the molecule to which Neu5,9Ac_2 is linked, since the virus binds well to synthetic sialosides such as Neu5,9Ac_2-thioketoside but not to the corresponding non-*O*-acetylated compound. A similar observation was also made with a polyacrylamide bearing Neu5,9Ac_2 [47] described earlier. However, additional *O*-acetyl groups in the sialic acid side chain inhibit virus binding as revealed by the Neu5,7,8,9Ac_4-thioketoside and Neu5,7,9Ac_3-GD3 from bovine buttermilk. Interestingly, the virus also binds to the mono-*O*-acetylated Neu5,7Ac_2-GD3 isolated from hamster melanoma, although at a lower degree when compared with the 9-*O*-acetyl isomer. One has to take into consideration that this binding may be due to the migration of some of the *O*-acetyl groups to C-9. However, *O*-acetylation of the original material exclusively in position 7 was shown by ^{1}H-NMR spectroscopy [35] and the stability of this residue in dry state at room temperature confirmed by these authors. It can furthermore be assumed that this position was retained under the experimental conditions, since the overlay assay was carried out at pH 6.8 instead of 7.2 where the rate of migration of the *O*-acetyl group from the 7- to the 9-position is slow [28]. Since 8-*O*-acetylated sialic acids have so far only been detected in trace amounts in sialic acid mixtures [2, 38], possibly as intermediates during the migration of an acetyl group from 7 to 9 [28], corresponding sialoglycoconjugates could not be analysed in the new test.

Based on all these experiments, influenza C-virus can thus be considered as a specific and sensitive probe for sialic acids mono-*O*-acetylated at the side chain. Having this property, it is more selective with regard to the nature of the terminal sugar than the lectins described above. It can be used more generally than antibodies made against highly specific structures, because the reactivity with the viral haemagglutinin is not significantly influenced by the nature of the molecule to which the *O*-acetylated sialic acid is bound. Furthermore, the test enables quantification as shown for e.g. *O*-acetylated gangliosides in the microtitre plate assay. Since influenza C-virus also binds to the synthetic 9-*N*-acetylated Neu5Ac [48], it can be expected that glycoconjugates artificially labelled with this sialic acid and thus bearing a stable acetyl group at position 9 can also be visualized on cells by this virus.

To demonstrate the use of influenza C-virus as a tool for the detection of mono-*O*-acetylated sialic acids in tissues, a histological test was established. Chemical sialic acid analysis proved that the fixation and embedding process did not significantly alter the quantity and quality of sialic acids in the slices under the conditions tested here. It was mentioned above that storage of the embedded tissue-sections did not significantly influence the amount and nature of sialic acids in the sections. Stability of *O*-acetylated sialic acids during longer storage in embedded tissue-sections was also observed by Reid *et al.* [49]. Furthermore, earlier analyses have confirmed the preservation of sialic acids in bovine submandibular glands [31] and human colorectal mucosa [49] during the fixation and embedding process. The possible loss of glycolipids containing *O*-acetylated sialic acids during the deparaffinizing steps was not tested and remains to be assessed. However, the main staining in many tissues studied here is associated with structures, e.g. mucins of intestinal goblet cells, which will not be glycolipid.

Analysis of bound virus by the colorigenic or fluorigenic esterase substrates used in the overlay or microtitre assays is hampered in the histochemical studies, as the diffusion rate of the fluorigenic dye is too high, and tissue esterases must be inhibited by the very toxic diisopropylfluorophosphate (DFP) in order to reduce background staining. Instead, anti-viral antibodies were used to identify bound viruses. With this modified test the specific detection of 9-*O*-acetylated sialic acids was possible, since virus-binding was abolished not only after saponification but also by previous treatment with sialidase. Binding to higher *O*-acetylated sialic acids in this tissue can be excluded on the basis of the specificity studies made with corresponding isolated or synthesized sialic acid-containing substances. It would be conceivable that the haemagglutination-active, soluble HEF-glycoprotein isolated from influenza C-virus [50] may interact more easily with its Neu5,9Ac_2-ligand due to higher mobility and reduced size when compared with the whole virus. However, we failed to stain tissue slices with this glycoprotein and corresponding antibodies. This is in accordance with a report by Formanowsky and Meier-Ewert [51] using chicken erythrocytes, who did not observe binding or haemagglutination with this receptor. These difficulties were overcome by the binding of the esterase-haemagglutinin moiety of HEF to the Fc-portion of IgG. The corresponding chimeric molecule has been used for tissue staining of 9-*O*-acetyl sialic acids [9, 34], although a detailed comparison of the value of this new reagent with influenza C-virus staining has not been reported. Staining with intact virus particles has the advantage of a relatively easy access to this virus and the handling of this test, particularly because it does not require DFP for esterase inhibition.

It has been described that the content of higher *O*-acetylated sialic acids of colon mucin is decreased in the case of colon carcinoma or ulcerative colitis [15–18, 52].

Recently, the importance of sialic acid *O*-acetylation in the interpretation of histochemical staining in colonic tissue has been examined [53, 54]. The presence of *O*-acetylated sialic acids in colonic tissue sections prevents the binding of antibodies recognising sialylated structures and in particular the sialyl-Tn antigen [53]. Mild saponification reveals these sialylated epitopes in normal tissue where sialyl-Tn was previously thought to be absent. The loss of *O*-acetylation observed in colon cancer [15–18] resulted in unhindered binding of anti-sialyl-Tn antibodies and led to the interpretation that this was an exclusively cancer-associated antigen in the colon. Whether this block in binding is due to mono- or higher *O*-acetylation of sialic acids has not been determined.

Binding studies with sections of human colon epithelium from these diseases demonstrate a similar extent of influenza C-virus-binding as for normal tissues – which may be interpreted to mean that the amount of mono-*O*-acetylated sialic acids is not significantly altered in these cases. In consequence, the reduction in sialic acid-*O*-acetylation observed by chemical means in diseased colon seems to affect mainly sialic acids carrying several *O*-acetyl groups in the side chain and not significantly the amount of the mono-*O*-acetylated species. These results agree with those of other authors [13, 15, 17] who also found no difference in the content of Neu5,9Ac$_2$ in the case of colon carcinoma using other detection assays.

A major difference in the regularity of goblet cell staining was found between normal human adult or fetal colon and tissue samples from ulcerative colitis and Crohn's disease, where the staining was much more irregular (Fig. 8). Strikingly, some normal and pathological colon samples could not be stained by the viruses, which corresponds to the observation made by Campbell *et al.* [55] that not all people seem to express *O*-acetylated sialic acids and there exist racial variations in the *O*-acetylation phenotype of human colonic mucosa. Investigation of a larger number of tissue specimens is currently being carried out in order to assess the value of viral haemagglutinin staining in both normal and disease situations.

The staining of cells mainly lining the capillaries approaching the central sinus of a rat liver lobe (Fig. 9) is rather striking. This pattern is similar to that published by Klein *et al.* [34] using the influenza C-virus haemagglutinin-esterase-Fc chimeras for staining of the same tissue. It is tempting to speculate that the frequent occurrence of *O*-acetylated sialic acids on endothelia of blood vessels, which in the latter publication was also observed in other tissues, e.g. kidney glomerula, represents a mechanism to protect the endothelial cells against aggressive agents in the blood stream such as enzymes, antibodies, or white blood cells. This discovery should also be considered in the light of the adhesion molecules, e.g. the selectins [56].

The staining of *O*-acetylated sialic acids in various human tissues, including sweat glands shown here, as well as the wide occurrence of these sialic acids in rat tissues, shown here with rat liver and also by Varki's group [9, 34], stimulates the need to look for this sialic acid in more human and animal tissues. Such studies may contribute to our understanding of the role of sialic acid *O*-acetylation, which is facilitated by the availability and described specificity of the influenza C-virus haemagglutinin. It has to be established by future experiments whether the test with intact virus particles or the soluble chimeric protein is best suited for this.

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