17 Viral Lectins for the Detection of 9-O-Acetylated Sialic Acid on Glycoproteins and Glycolipids

B. Schultze, G. Zimmer, G. Herrler

A number of viruses are able to recognize specific carbohydrate structures and to use these structures for the attachment to the cell surface. Influenza C virus and bovine coronavirus specifically attach to receptors containing N-acetyl-9-O-acetyl-neuraminic acid. Therefore, they can be used as lectins for the detection of glycoconjugates containing this type of sialic acid. These viruses also contain an acetylesterase on their surface which can be exploited for the detection of virions bound to immobilized glycoconjugates (Zimmer et al. 1992). As shown in Fig. 1, in the case of influenza C virus the acetylesterase activity is a function of the surface glycoprotein HEF that also mediates the binding to N-acetyl-9-O-acetylneuraminic acid. In the case of bovine coronavirus the acetylesterase is a function of the HE protein. Though this protein also recognizes 9-O-acetylated sialic acid, the binding of virus is primarily due to a another surface glycoprotein designated S (Schultze et al. 1991). The esterases of both viruses are able to cleave the synthetic substrate α-naphthyl acetate giving rise to naphthol. The latter compound reacts with a diazonium ion such as Fast Red resulting in a coloured insoluble complex, which reveals the presence of bound virus.

Influenza A and B viruses and paramyxoviruses have been used for the detection of gangliosides containing N-acetylneuraminic acid (i.e. sialic acid lacking an O-acetyl group) in defined linkage types. These viruses contain a neuraminidase rather than an acetylesterase. As no commercial detection system is available that makes use of neuraminidases to yield a coloured insoluble complex, binding of these viruses has to be determined by other means, e.g. enzyme-linked immunoreagents.

Here we describe how bovine coronavirus and influenza C virus can be used to detect 9-O-acetylated sialic acid on glycoproteins bound to nitrocellulose as well as on gangliosides bound to thin-layer plates. In addition a microtiter assay is desribed, which is useful for quantitative studies.

17.1 Experimental Part

17.1.1 Detection of Glycoproteins Containing N-Acetyl-9-O-Acetylneuraminic Acid by Bovine Coronavirus

Materials

- Nitrocellulose (0.45 µm, Schleicher & Schuell, Dassel, Germany)
- Phosphate-buffered saline (PBS): 8.0 g NaCl, 0.2 g KCl, 1.15 g $Na_2PO_4 \times 2\,H_2O$, 0.2 g KH_2PO_4 in 1 l H_2O, pH 7.2
- PBS/BSA: 1% bovine serum albumin (Serva, Heidelberg, Germany) in PBS
- Nonfat dry milk: 10% suspension in PBS
- PBS/Tween: 0.1% Tween 20 (Serva, Heidelberg, Germany) in PBS
- Rat serum

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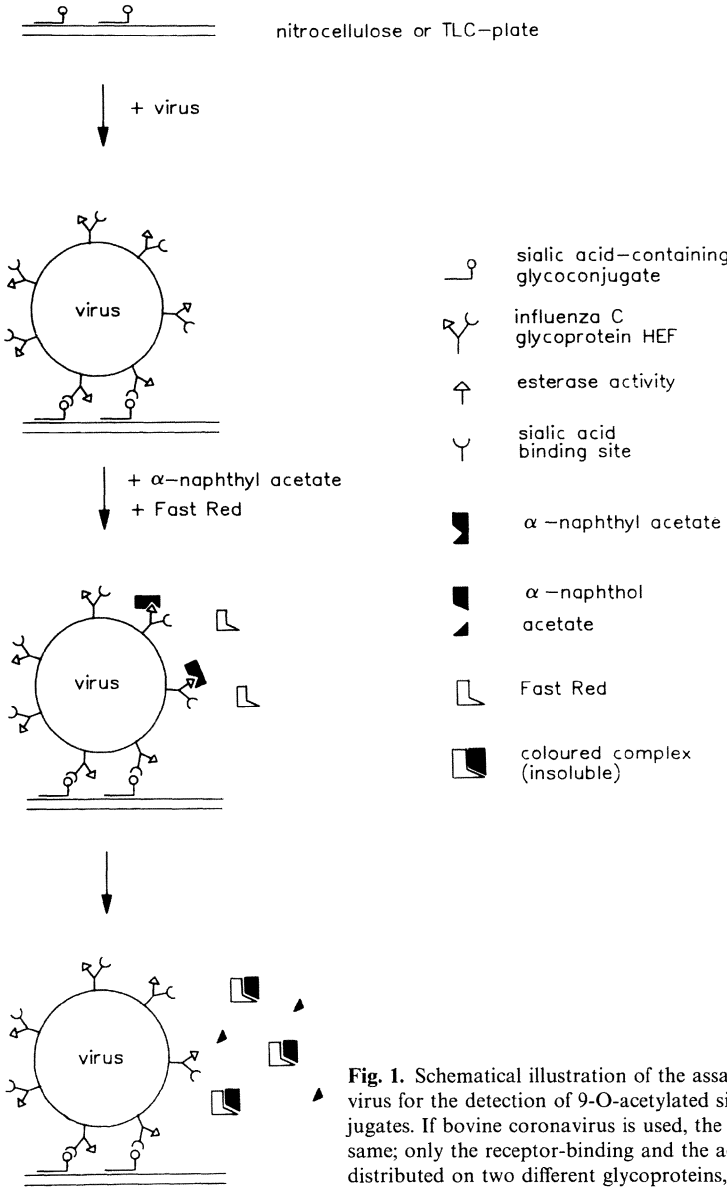

Fig. 1. Schematical illustration of the assay using influenza C virus for the detection of 9-O-acetylated sialic acid on glycoconjugates. If bovine coronavirus is used, the reaction scheme is the same; only the receptor-binding and the acetylesterse activity are distributed on two different glycoproteins, S and HE

- α-Naphthyl acetate (Sigma, Deisenhofen, Germany): 50 mM stock solution in acetone
- Esterase substrate A: 1 mM α-naphthyl acetate in PBS, 0.1% Fast-Red TR-salt (Sigma, Deisenhofen, Germany), filtered (e.g., folded filters, Schleicher & Schuell, Dassel, Germany)
- Biotinylated anti-rabbit donkey immunoglobulin (Amersham, Braunschweig, Germany)

- Streptavidin-biotinylated horseradish peroxidase complex (Amersham, Braunschweig, Germany)
- 4-Chloro-1-naphthol (Sigma, Deisenhofen, Germany): 3 mg/ml in DMSO
- H_2O_2 38%, (Fluka, Neu-Ulm, Germany)
- Bovine coronavirus (BCV) is grown in MDCK I cells (Schultze et al. 1990). After clarification of the supernatant by low speed centrifugation, the virus is used for the binding assay

Procedure

1a. Different dilutions of the glycoproteins to be analyzed are applied to nitrocellulose in a volume of 2 µl and air-dried for about 20 min
1b. Proteins are separated by SDS-polyacrylamide gel electrophoresis and blotted on a nitrocellulose sheet
1c. Control samples were incubated with 0.1 N NaOH for 1 h at room temperature in order to release O-acetyl groups. After three washes with PBS, the blocking reagent was added
2. Nonspecific binding sites are blocked with nonfat milk overnight at 4 °C (alternatively, PBS/BSA may be used)
3. The nitrocellulose strips are washed three times for 5 min with PBS/Tween and incubated for 1 h with BCV (256 HA-units/ml) at 4 °C
4. After washing three times with PBS–Tween at 4 °C, bound BCV is detected by immersing the nitrocellulose in esterase substrate A. After incubation for 5–10 min at room temperature, the reaction can be stopped by replacing the substrate solution with distilled water

Alternative

If the assay is applied to viruses lacking an acetylesterase, a conventional immunological detection system has to be used to detect bound virus.

1–3. As above
4. The following steps (4–6) are performed at 4 °C. After three washes with PBS/Tween, the nitrocellulose strips are incubated for 1 h with a rabbit antiserum directed against BCV (1 : 1000 dilution)
5. The strips are washed three times with PBS/Tween and then incubated for 1 h with a 1 : 1000 dilution of biotinylated anti-rabbit donkey immunoglobulins
6. Following three washes with PBS/Tween, the nitrocellulose is incubated for 1 h with streptavidin-biotinylated horseradish peroxidase complex (1 : 1000) and then washed again
7. Bound BCV is detected by incubation of the strips with PBS, 4-chloro-1-naphthol, H_2O_2 (500 : 100 : 1) for 10–20 min at 20 °C. The color development is stopped by washing with distilled water

17.1.2 Detection of Gangliosides Containing 9-O-Acetylated Sialic Acid by Influenza C Virus

Materials

- PBS, PBS/BSA, PBS/Tween, α-naphthyl acetate, esterase substrate A: see above
- Glass-backed high-performance thin-layer chromatography (HPTLC) plates (10 × 10 cm or 5 × 10 cm), silica gel 60 (Merck, Darmstadt, Germany)
- Polyisobutylmethacrylate (Plexigum P28; Röhm, Darmstadt, Germany)

- Bovine brain gangliosides (BBG), prepared as described (Svennerholm and Fredman 1980)
- Orcinol spray reagent: 40.7 ml 37% HCl, 0.1 g orcinol (Sigma, Deisenhofen, Germany), 1 ml 1% aqueous $FeCl_3$, 25 ml H_2O
- Influenza C virus (strain Johannesburg/1/66) is grown in embryonated chicken eggs as described (Herrler et al. 1979). The allantoic fluid is clarified by low speed centrifugation (4000 *g*, 10 min) and stored in small aliquots at − 80 °C. The virus is quantitated by a hemagglutination assay using 0.5% chicken erythrocytes. The hemagglutination titer (HA-units/ml) indicates the reciprocal value of the maximum dilution that causes agglutination of the erythrocytes.

Procedure

1a. Gangliosides containing 1–5 μg sialic acid are applied to each lane of the glass-backed HPTLC-plates and chromatographed. A suitable solvent system is chloroform/methanol/0.2% aqueous $CaCl_2$ (60:40:9)

1b. Control samples are exposed to ammonia vapors at room temperature for about 12 h in order to release O-acetyl groups. After thorough drying, the plates are chromatographed as described above

1c. For chemical detection of gangliosides after chromatography, a dried control plate is sprayed with the orcinol reagent, covered by a second glass plate and heated at 120 °C for 20 min. Gangliosides are indicated by a characteristic violet color

2. The developed chromatogram is dried thoroughly under a stream of air for at least 10 min at room temperature
3. The plate is dipped for 2 min in diethylether containing 0.5% polyisobutylmethacrylate and dried as above
4. After spraying with PBS, the plate is immersed in PBS/BSA for 60 min at room temperature
5. Some drops of allantoic fluid containing influenza C virus with a hemagglutinating activity of at least 256 HA-units/ml are added and spread over the whole chromatogram by covering it with a piece of parafilm. Virus is allowed to bind for 60 min at 4°C
6. The plate is washed three times with PBS/Tween at 4 °C, 5 min each
7. Bound virus is visualized by immersing the plate in esterase substrate A for about 30 min at room temperature. The reaction is stopped by rinsing the chromatogram with H_2O and drying it as above.

17.1.3 Microtiter Assay for the Detection of 9-O-Acetylated Sialic Acid

Materials

- PBS, PBS/BSA, PBS/Tween, and influenza C virus: see above
- 96-well, flat-bottom polystyrene microtiter plates (Immuno-Module MaxiSorp F8, Nunc, Wiesbaden, Germany)
- Fluorescence spectrophotometer (Perkin-Elmer, Offenbach, Germany) with plate reader
- 4-Methylumbelliferyl acetate: 2 mM stock solution in acetone/H_2O (1:1, by vol)
- *p*-Nitrophenyl acetate (Sigma, Deisenhofen, Germany): 100 mM stock solution in acetone
- Esterase substrate B: 60 μM 4-methylumbelliferyl acetate in PBS
- Esterase substrate C: 1 mM *p*-nitrophenyl acetate in PBS.

Procedure

1a. Glycoproteins to be analyzed are dissolved in PBS (up to 10 µg of bound sialic acid/ml); 100 µl are added per well of the microtiter plate and incubated overnight at 4 °C

1b. Gangliosides are applied in a volume of 100 µl methanol containing up to 1 µg of bound sialic acid. The solvent is allowed to evaporate completely at room temperature. For the analysis of glycolipids the BSA concentration of PBS/BSA is raised to 2% and the wells are washed with PBS rather than with PBS/Tween (see steps 2, 3, and 5)

1c. In control wells, immobilized glycoconjugates are saponified with 0.1 N NaOH at room temperature to release O-acetyl groups. After 30 min, cells are washed three times with PBS

2. Remaining binding sites are blocked by incubation with PBS/BSA (200 µl/well) for 1 h at room temperature (2 h for glycolipids)

3. The wells are washed twice with PBS/Tween (200 µl/well)

4. After dilution with PBS, influenza C virus is added to the wells in a volume of 100 µl containing an acetylesterase activity of about 1–2 mU. Virus is allowed to bind for 1 h at 4 °C

5. The wells are washed three times with PBS/Tween at 4 °C

6. Each well is incubated with 100 µl esterase substrate B for 10–30 min at 37 °C. The reaction is stopped by the addition of 100 µl ethanol. The amount of 4-methylumbelliferone released is determined using a fluorescence spectrophotometer operating at wavelengths 365 nm for excitation and 450 nm for emission. If a fluorimeter is not available, esterase substrate C can be used. In this case the reaction is monitored at 405 nm.

17.2 Results

The detection of glycoproteins containing 9-O-acetylated sialic acid by bovine coronavirus is shown in Fig. 2. Different dilutions of rat serum have been used for a

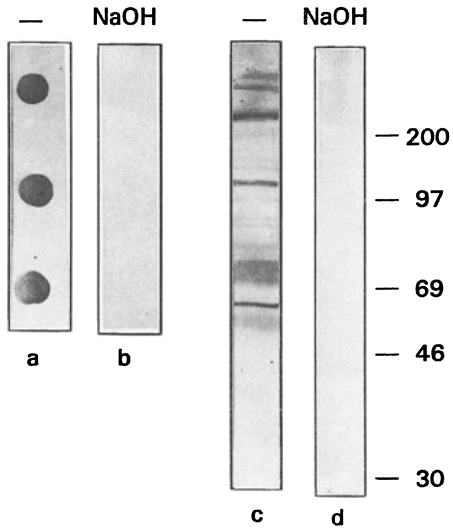

Fig. 2. Detection of O-acetylated sialic acid on rat serum proteins. *Lanes a and b* binding of the BCV to rat serum proteins spotted on nitrocellulose is detected by an enzyme linked immunoassay; *lanes c and d* rat serum was analyzed by SDS-polyacrylamide gel electrophoresis prior to blotting on nitrocellulose; binding of BCV was detected by viral esterase activity. Samples were incubated with PBS (*lanes a and c*) or with sodium hydroxide (*lanes b and d*) before incubation with BCV

spot assay (1:100, 1:500, 1:1000, lanes a and b, from top to bottom) using the immunological reagents to visualize bound virus. In lanes c and d, the serum proteins have been separated by SDS-polyacrylamide gel electrophoresis prior to blotting on nitrocellulose; the viral esterase has been used to visualize bovine coronavirus bound to individual glycoproteins. Virus binding is abolished if the proteins on the nitrocellulose strips are pretreated with sodium hydroxide (lanes b and d).

A typical example for the detection of gangliosides containing 9-O-acetylated sialic acid by influenza C virus is shown in Fig. 3. After thin-layer chromatography of bovine brain gangliosides, several bands are stained by the virus binding assay (lane 3). Alkaline pretreatment of the sample results in the disappearance of the three slower migrating bands (lane 4) indicating that they represent gangliosides containing N-acetyl-9-O-acetylneuraminic acid. The bands at the top of the chromatogram may be due to acid phospholipids and sulfatides, which migrate more rapidly than most gangliosides. These compounds appear to interfere with the polyisobutylmethacrylate coating and are often not well covered by the plastic film, so that nonspecific

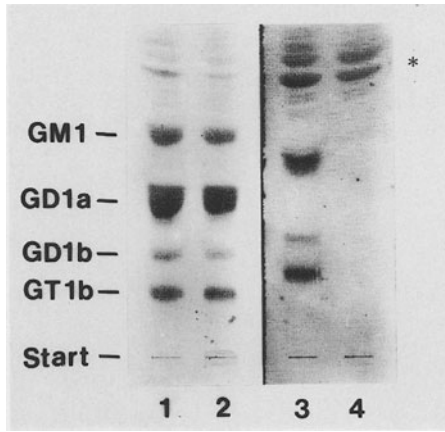

Fig. 3. Detection of 9-O-acetylated sialic acid on gangliosides from bovine brain separated by HPTLC. Prior to development, *lanes 2 and 4* have been exposed to ammonia vapors. *Lanes 1 and 2* were sprayed with the orcinol reagent staining the main gangliosides *GM1, GD1a, GD1b,* and *GT1b. Lanes 3 and 4* were overlayed with influenza C virus for the detection of 9-O-acetylated gangliosides. The *asterisk* indicates nonspecific staining

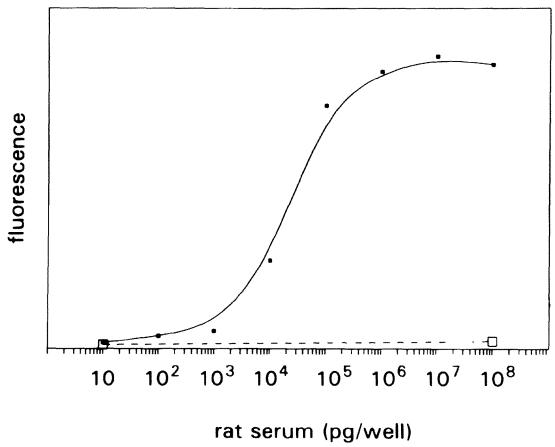

Fig. 4. Binding of influenza C virus to serial dilutions of rat serum immobilized in microtiter wells. Binding of influenza C virus was assayed using 4-methylumbelliferyl acetate as substrate. (——) untreated serum; (----) alkali-treated serum

staining in these areas may be observed. Gangliosides containing 9-O-acetylated sialic acid constitute only a minor fraction. They are not stained by orcinol (detection limit about 0.5 μg sialic acids). This reagent only reveals the four major ganglioside species (lane 1) and is not affected by alkaline pretreatment of the sample (lane 2).

In Fig. 4, a virus binding assay with microtiter plates is shown. This assay is useful for quantitative studies.

17.3 Remarks

Because of the instability of the 9-O-acetyl group of sialic acid under alkaline conditions, care was taken to avoid pH values above 9.0.

The alkaline pH values of the electrophoresis buffers (Laemmli 1970) do not interfere with the binding assay.

For electroblotting, the method described by Kyhse-Andersen (1984) was modified by lowering the pH value of the two buffers at the anode site from 10.4 to 9.0 (facing the anode) and 7.4 (facing the nitrocellulose), respectively.

Treatment with alkaline methanol, which is commonly used in the preparation of gangliosides to destroy contaminating phospholipids, is not practicable, if O-acetylation of gangliosides is to be preserved.

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