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# Analytical Detection of 9(4)-O-Acetylated Sialoglycoproteins and Gangliosides Using Influenza C Virus

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The unique glycoprotein of influenza C virus, designated hemagglutinin (HEF), exhibits three functions: hemagglutination, esterase activity, and fusion factor. As the virus uses 9-O-acetylated sialic acid as a high-affinity receptor determinant for attachment to cells, its binding activity was used to reveal O-acetylated sialic acid residues after polyacrylamide gel electrophoresis and transfer onto nitrocellulose sheets of proteins and thin-layer chromatography of lipids. The specificity of the binding for O-acetylated sialoglycoconjugates was investigated. Our results showed that influenza C virus could detect the different forms of the two murine glycoporphins which are known to be O-acetylated sialoglycoconjugates. The virus also bound to O-acetylated gangliosides isolated from embryonic chicken brain such as purified O-acetylated NeuAca(2-8)NeuAca(2-8)NeuAca(2-3)Galβ(1-4)Glcβ(1-1)ceramide (GT3). The esterase activity of the HEF protein of influenza C virus was used to unmask the sialic acid. After its deacetylation by the virus enzyme, the O-acetylated GT3 was recognized by a monoclonal antibody which binds only to the nonacetylated derivative. The results presented here show that influenza C virus is a discriminating analytical probe for identifying O-acetylated sialoglycoconjugates directly after Western blotting of proteins and thin-layer chromatography of lipids, thus providing a new analytical tool. © 1991 Academic Press, Inc.

Influenza C virus, whose surface glycoprotein (HEF)<sup>2</sup> exhibits receptor-binding, receptor-destroying, and fu-

sion activities (1,2), uses 9-O-acetyl-N-acetylneuraminic acid (Neu5,9Ac<sub>2</sub>) as a high-affinity receptor determinant for attachment to cells (3). The specificity of influenza C virus (INF-C) for Neu5,9Ac<sub>2</sub> has been studied mostly using either hemagglutination assays or binding of radioactively labeled virus. The virus binds to any Neu5,9Ac<sub>2</sub> regardless of the α(2-3) or (2-6) linkage, in Galβ(1-3/4)GalNAc or Galβ(1-3/4)GlcNAc complexes (3). There is no convincing report, dealing directly with the binding activity, which gives evidence of any other carbohydrate structure involved in the specificity of this activity.

A major determinant of the cell tropism of INF-C is the expression of appropriate cell surface receptors (4). Using chicken erythrocytes, this determinant, which contains Neu5,9Ac<sub>2</sub>, was shown to be a differentiation marker, whose expression increased after birth (5).

The HEF protein (6) was isolated and characterized as a neuraminate 9-(4)-O-acetylestase (7). The enzyme seems to be highly specific for naturally occurring large compounds, whereas its activity is much less spe-

dicular gland mucin; DFP, diisopropyl fluorophosphate; DTT, dithiothreitol, EGP, erythrocytic ghost proteins; Fuc, fucose; Gc, glycolyl; Gal, galactose; GalNAc, N-acetyl galactosamin; GlcNAc, N-acetyl glucosamine; GT3, NeuAca(2-8)NeuAca(2-8)NeuAca(2-3)Galβ(1-4)Glcβ(1-1)ceramide; HA, hemagglutination; HEF, hemagglutinin, esterase, fusion factor of influenza C virus; HPTLC, high-performance thin-layer chromatography; INF-C, influenza C virus; IPA, isopropyl alcohol; MAA, *Maackia amurensis* agglutinin; MAb, monoclonal antibody; NBT, nitro blue tetrazolium; Neu5Ac, N-acetylneuraminic acid (sialic acid); Neu5,7Ac<sub>2</sub>, 7-O-acetylated sialic acid; Neu5,9Ac<sub>2</sub>, 9-O-acetylated sialic acid; Neu5Gc, N-glycolylneuraminic; OSM, ovine submaxillary gland mucin; PAS, periodic acid Schiff; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PT, PBS-Tween 20; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TLC, thin-layer chromatography.

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<sup>2</sup> Abbreviations used: BSA, bovine serum albumin; BCIP, 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt; BSM, bovine subman-

cific for small and artificial substrates; in addition, this enzyme can hydrolyze *p*-nitrophenyl acetate as well as  $\alpha$ -naphthyl acetate. The enzymatic activity of the virus is thus a useful tool for studying natural compounds containing sialic acids or their O-acetylated derivatives.

In the present study, INF-C is used for detecting O-acetylated sialoglycoconjugates. After SDS-PAGE and semidry blotting for proteins and TLC for lipids, the binding activity of INF-C virus was studied. The viral esterase activity was used to unmask antigen for monoclonal antibody 18B8 specific for the ganglioside GT3. Specificity of the binding was demonstrated using alkali treatment of glycoconjugates, sialidase treatment, selective inhibitors, and by comparing binding of influenza C virus to that of *Maackia amurensis* agglutinin, a lectin of defined specificity (8).

## MATERIALS AND METHODS

### Materials

#### *Purified Viruses and Monoclonal Antibodies*

The C/Johannesburg/1/66 strain of INF-C was provided by Dr. G. Herrler (Germany). The monoclonal antibody J14 (IgG) was kindly provided by Dr. Sagawara (Japan) (9). Monoclonal antibodies 18B8 (IgM) (10) and A2B5 (IgM) (11) were provided by Dr. Nirenberg (U.S.A.).

#### *Phytolectin and Enzyme*

The digoxigenin-labeled phytolectin, extracted from *M. amurensis* (MAA), was obtained from Boehringer-Mannheim (Germany).

The sialidase extracted from *Vibrio cholerae* was purchased from Calbiochem (U.S.A.).

#### *Purified Mucoproteins*

Bovine submandibular gland mucin (BSM) and ovine submaxillary gland mucin (OSM) were purchased from Bio Carb (Sweden).

### Methods

#### *Virus Purification and Iodination*

The INF-C strain was selected for good growth capacity in chicken embryos and was used throughout this work. The virus was grown in the allantoic cavity of 8-day-old embryonated eggs at 32°C and fluids were harvested 3 days later.

Hemagglutination experiments were carried out as described previously at 4°C to prevent elution (12).

For purification of INF-C, the infected fluids were clarified by centrifugation at 5000g for 30 min at 4°C. Supernatant was then centrifuged at 25,000g in an angular rotor (Beckman J14) for 150 min at 4°C. For an initial volume of infected fluid of 250 ml, pellets were

resuspended in 0.1 ml 10 mM Tris-HCl/1 mM EDTA, pH 7.4 (TE) buffer and left at 4°C overnight. The pelleted particles were dispersed in 0.3 ml of TE by sonication (30 s, 100 W, 20 Hz). The sonicated suspension was layered onto a continuous potassium tartrate gradient (10 to 50%, w/v) in TE buffer and centrifuged at 200,000g in a swinging bucket rotor (Kontron TST41) for 300 min at 4°C. The purified viral suspension was dialyzed against phosphate-buffered saline (NaCl, 100 mM; KCl, 5.5 mM; Na<sub>2</sub>HPO<sub>4</sub>, 20 mM; KH<sub>2</sub>PO<sub>4</sub>, 3 mM; pH 7.2) (PBS) overnight (4°C) and used for experiments. The protein content was determined by Bradford's method, using bovine serum albumin (BSA) as standard protein, with the Bio-Rad protein assay (13). Stock virus used in these experiments contained 516  $\mu$ g of protein/ml.

Purified INF-C was iodinated as described previously for paramyxoviruses using iodogen method (Pierce, The Netherlands) (14,15): briefly, 70  $\mu$ l of purified virus was put in contact for 10 min with 100  $\mu$ Ci of <sup>125</sup>I in a vial. The suspension was then deposited in another vial and 200  $\mu$ l of PBS, used for rinsing the former vial, was added as well as NaI in PBS (250 mM final concentration). To separate free from incorporated <sup>125</sup>I, the virus was passed through a Sephadex G25 PD10 column (Pharmacia, Sweden) preequilibrated with 10 mM Tris-BSA (1%) pH 7.4, and eluted with PBS. The specific activity of the recovered purified <sup>125</sup>I-labeled virus was  $19 \times 10^5$  cpm/ $\mu$ g.

#### *Detection of INF-C Receptor-like Glycoproteins*

*Protein preparation.* Mouse erythrocytes were harvested from the ocular vein of 3-month-old Balb/c males with a Pasteur pipet using Alsever's medium containing neomycin and chloramphenicol.

Ovine erythrocytes were purchased from Diagnostic Pasteur (France).

Hemoglobin-free ghosts were prepared by lysis of the pelleted erythrocytes in 20-fold excess (v/v) of Soeren buffer (Na<sub>2</sub>HPO<sub>4</sub>, 95 mM; NaH<sub>2</sub>PO<sub>4</sub>, 5 mM; pH 8) with 0.3 mM of phenylmethanesulfonyl fluoride (PMSF, Fluka, Switzerland) under continuous stirring at 4°C for 10 min and then washed and centrifuged (30,000g, 30 min) until the obtained pellet was free of red coloration. The pelleted ghosts were stored at -30°C. A protein assay by Bradford's method was performed as described previously (13) to measure the protein content. The proteins were directly solubilized in SDS-PAGE sample buffer before use.

*Gel electrophoresis and protein transfer.* Protein preparations were separated on 10% SDS-polyacrylamide gel, according to Laemmli's method (16), under reducing conditions (using dithiothreitol (DTT), Bio-Rad, U.S.A.) in sample buffer containing 5% SDS and then blotted onto a nitrocellulose sheet (Schleicher &

Schuell, Germany). Transfer was carried out for 60 min at constant current of 1 mA/cm<sup>2</sup> with a Pharmacia/LKB (Sweden) semidry blotter, as described previously (17), except that the transfer buffers were less alkaline, especially the anode 2 buffer which is in direct contact with the nitrocellulose sheet (anode 1 buffer, pH 9.6; anode 2 buffer, pH 8.5; and cathode buffer, pH 9.1).

The staining of the total proteins was made using colloidal gold from Bio Cell (Great Britain) and the amount of proteins loaded is indicated in the figure legends.

*Detection of 9(4)-O-acetylated sialoglycoproteins.* After transfer of proteins, the blots were incubated in the blocking reagent provided by Boehringer-Mannheim in the Dig-Glycan differentiation kit for at least 1 h at room temperature (RT). The blots were then incubated in a suspension of purified virus diluted in PBS-Tween 20 (0.1%, v/v) (PT)-isopropyl alcohol (1%, v/v) with a final concentration of viral protein of 1 µg/ml. The viral esterase activity was inhibited by incubating the virus in the buffer containing diisopropyl fluorophosphate (DFP, Sigma, U.S.A.) (1 mM) for 30 min at RT. After incubation 1 h at RT, or overnight at 4°C, bound virus was revealed by incubating nitrocellulose sheets with MAb J14, specific for HEF protein of INF-C, then with alkaline phosphatase-labeled anti-mouse IgG. The following steps were performed using PT: the blots were first put in contact (1 h RT) with a 1/2000 solution of the MAb J14 then with a 1/1000 solution of the alkaline phosphatase-labeled anti-IgG. Each step of the reaction was followed by three washings, each lasting 10 min, with PBS.

Sialoglycoproteins were revealed with 0.35 mM nitroblue tetrazolium grade III crystalline (NBT) (Sigma) and 0.35 mM 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (BCIP) (Fluka) in 0.1 M Tris-HCl, 0.05 M MgCl<sub>2</sub>, 0.1 M NaCl, pH 9.5, for at least 20 min, at RT.

For competitive inhibition test, DFP-treated INF-C was preincubated with OSM or BSM (100 µg/ml in PT) for 30 min at RT.

#### *Detection of Glycoproteins with M. amurensis Phytoagglutinin*

After transfer of proteins, the blots were incubated, at 4°C overnight, with the digoxigenin-labeled agglutinin MAA (5 µg/ml) in a 0.05 M Tris-HCl, 0.15 M NaCl, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, pH 7.5, buffer. The second step consisted of an incubation with the antibody directed against digoxigenin labeled with alkaline phosphatase (Boehringer-Mannheim). Each step of the reaction was followed by three washings, each lasting 10 min, with 0.05 M Tris-HCl, 0.15 M NaCl (TBS), pH 7.5. The detection of glycoproteins was similar to that described before using BCIP and NBT.

#### *Detection of INF-C Receptor-like Glycolipids*

*Isolation and chromatography of the ganglioside antigens.* Lipids were extracted with chloroform:methanol:H<sub>2</sub>O (4:8:3, by volume) as described previously (10,18). Total lipid extract was submitted to a Folch partition (19). The combined upper phases were desalted by passage through a column of Sephadex LH-20 (Pharmacia) equilibrated with chloroform:methanol:H<sub>2</sub>O (5:5:1, by volume). Lipids were eluted with the same solvent. They were separated into neutral and acidic fractions by ion exchange chromatography on a column of DEAE-Sephadex A-25 (Pharmacia) in the acetate form equilibrated with chloroform:methanol:H<sub>2</sub>O (30:60:8, by volume). Neutral glycolipids were eluted with 10 column vol of methanol. Monosialo and polysialogangliosides were eluted with increasing concentrations of ammonium acetate in methanol (0.02 to 0.5 M). The polysialoganglioside fraction eluted by 0.2–0.5 M ammonium acetate was analyzed in this study.

Glycolipids were then chromatographed on high-performance thin-layer chromatography plates (HPTLC, silica gel 60, Merck, Germany) in chloroform:methanol:0.25% KCl in H<sub>2</sub>O (5:4:1, by volume). Gangliosides chromatographed on glass HPTLC plates were visualized with resorcinol reagent (18).

*Detection of 9(4)-O-acetylated gangliosides.* Gangliosides were chromatographed as described above on aluminium-backed HPTLC plates. Chromatograms were treated as described previously (20) for immunostaining of glycolipid antigens and then overlaid with INF-C, as described before for Sendai virus, a paramyxovirus (21). The dried chromatograms were soaked for 2 min in a 0.1% solution of polyisobutylmethacrylate (Polysciences, Inc., U.S.A.) in hexane. After drying in air, the chromatograms were sprayed with TBS, pH 7.8, with 1% bovine serum albumin and 0.1% sodium azide and soaked in the same buffer until all the silica gel was wet. The plate was then overlaid with a suspension of purified virus, whose viral esterase activity was inhibited by preincubating the virus with DFP (1 mM). The virus was diluted as described for detection of sialoglycoproteins. After 1 hr incubation at 4°C, the plates were washed three times with TBS-BSA, pH 7.8, then immunostained with antibody MAb J14 as described before.

In some experiments, the detection of O-acetylated gangliosides was made directly using INF-C labeled with <sup>125</sup>I by the iodogen method and treated with DFP, as described before; the chromatograms were then washed five times in cold PBS, dried, and exposed to XAR-5 X-ray film (Eastman Kodak, U.S.A.).

#### *Immunostaining of Glycolipid Antigens*

Glycolipid antigens were detected on thin-layer chromatography by autoradiography as previously described

(20). Chromatograms were treated as described before for detection of O-acetylated gangliosides. Instead of viral solution, the plates were overlaid with monoclonal antibody solution diluted 1:20 in TBS-BSA, (pH 7.8, 60  $\mu\text{l}/\text{cm}^2$ ) and incubated for 1 h at RT or overnight at 4°C. The chromatograms were washed four times in TBS-BSA, pH 7.8, and overlaid with TBS-BSA, pH 7.8, containing  $2 \times 10^6$  cpm/ml  $^{125}\text{I}$ -labeled goat anti-mouse IgM antibodies. After 1 h at RT, the chromatograms were washed as before in cold PBS, dried, and exposed to XAR-5 X-ray film (Eastman Kodak).

#### *Alkali Treatment of Chromatograms and Blots*

*Alkali treatment of chromatograms with ammonia vapors.* After chromatography of gangliosides, the chromatograms were dried and placed for 5 h in a tank saturated with ammonia (22). The plates were then removed and put in an airstream overnight to eliminate ammonia. The chromatograms were immunostained as described before.

*Alkali treatment of blots.* The blots were incubated with (0.1 N) NaOH at RT for 30 min and washed with distilled water then with PBS or TBS according to the following treatment.

#### *Sialidase Treatment of Blots*

The blots were incubated with sialidase purified from *V. cholerae* under conditions described previously for treatment of native erythrocytes (4). The blots were then washed as described above for alkali treatment.

#### *Effects of INF-C Esterase Activity on Gangliosides and Glycoproteins*

After chromatography of glycolipids, the chromatograms were overlaid with INF-C virus, whose receptor-destroying enzyme is known to be a 9(4)-O-acetylneuraminidase esterase (3-7). This enzyme is irreversibly inhibited by DFP 1 mM in PBS-isopropyl alcohol (IPA) (1%, v/v). The final protein concentration of purified virus was 1  $\mu\text{g}/\text{ml}$ . Briefly, the chromatograms were treated with polyisobutylmethacrylate and soaked in TBS-BSA, pH 7.8. They were washed twice with PBS and overlaid with treated or untreated virus. After 2 h of incubation at 37°C, the plates were washed three times with TBS-BSA, pH 7.8, and immunostained with 18B8 antibody as described.

After transfer of proteins and quenching, the blots were treated with DFP-treated or untreated virus for 2 h at 37°C. As for glycolipids, the concentration of viral proteins was 1  $\mu\text{g}/\text{ml}$  but 0.1% (v/v) Tween 20 was added to PBS-IPA. The enzymatic activity ceases when the virus is removed and the blots are washed with PBS. The blots were then viro-stained with DFP-treated

virus as described before for detection of sialoglycoproteins.

#### *Comparative Densitometric Analysis of Detected Bands*

The aim of the computer-assisted image analysis was to provide an evaluation of virus binding for comparative purposes. Image analysis was carried out on blots, which had been processed identically, using Visilog software (NOESIS, U.S.A.). A densitometric curve was determined for each analyzed band and the surface under the curve was calculated by integration with Project and Pati software. The percentage of inhibition was determined as follows ( $S_r$  = reference surface (no inhibitor),  $S_i$  = surface with tested inhibitor):  $[(S_r - S_i)/S_r] \times 100$ .

## RESULTS

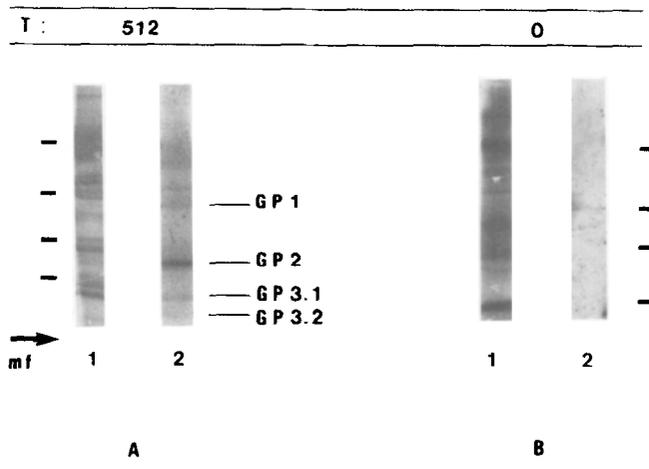
### *Influenza C Virus Binds to Its Receptor-like Glycoproteins on Electrophoregrams*

Binding of INF-C to transferred proteins on nitrocellulose sheets was studied using purified INF-C revealed with a monoclonal antibody directed against the A-1 site of HEF proteins of INF-C.

After electrophoresis and semidry blotting of proteins, extracted from murine and ovine ghost erythrocytes, a variety of procedures were compared for their ability to saturate nonspecific binding sites on nitrocellulose. Saturation with PT-1% BSA, even pretreated with periodate, as described previously (23), was of poor efficacy, probably because of the high nonspecific binding of INF-C to nitrocellulose sheets. We finally used the blocking reagent purchased from Boehringer-Mannheim for saturation.

Nitrocellulose sheets were then incubated with INF-C pretreated with DFP, which is an irreversible inhibitor of serine esterase. The bound virus was revealed with MAb J14, specific for HEF proteins of INF-C.

In the case of murine EGP, as shown in Fig. 1A, lane 2, INF-C recognized one major band on the migration pattern identified as one of the two murine sialoglycoproteins or glycoporphins, designated as GP2. As previously described (24,25), glycoporphins strongly aggregate in aqueous solution. The dissociation of aggregates is not complete in the presence of detergent and this explains why glycoporphins exist in the form of dimers. This is the reason why the number of PAS-positive bands does not reflect the number of glycoporphins following SDS-PAGE analysis. In addition, some bands are due not only to homo- and heterodimers but also to higher oligomers (26). As shown in Fig. 1A, lane 2, four bands detected by INF-C can be identified as GP1, GP2, GP3.1, and GP3.2. GP2 and GP3 were demonstrated to be the two murine glycoporphins, and GP1 was described as the heterodimer of GP2 and GP3 (27-29). GP3.1 was described as the mature complete form of the glyco-



**FIG. 1.** Influenza C virus binds to murine but not to ovine erythrocytic ghost proteins (EGP). After SDS-PAGE of proteins and transfer onto nitrocellulose, transferred proteins were incubated with INF-C as described under Materials and Methods. (A) Murine erythrocytic ghost proteins. Lane 1: 3  $\mu$ g of mouse EGP was stained with colloidal gold. Lane 2: 20  $\mu$ g of mouse EGP was probed by INF-C pretreated with DFP. The oligomeric forms are not identified. (B) Ovine erythrocytic ghost proteins. Lane 1: 3  $\mu$ g of ovine EGP was stained with colloidal gold. Lane 2: 20  $\mu$ g of ovine EGP was probed by INF-C pretreated with DFP. No binding could be detected. "T" indicates the hemagglutination titers; the molecular weights indicated are 94,000, 67,000, 43,000, 30,000  $M_r$ ; mf, migration front.

phorin GP3, and GP3.2 as an incomplete form of it (30). As shown Fig. 1A, lane 2, staining of GP2, and GP1 to a lesser extent, was much stronger than that of GP3. These data are consistent with the previous reports from Sarris and Palade cited above, showing that the majority of the O-acetylated sialyl residues were carried by GP2 and GP1. As shown in Fig. 1A, lane 1, colloidal gold staining of proteins seemed to be the inverse of "viro-staining," since GP3, which carries a small amount of O-acetylated sialic acid, was clearly stained by colloidal gold, whereas the major band recognized by INF-C was not stained at all. For molecular weights higher than 67,000  $M_r$ , the identification is not possible because of aggregates as discussed above. According to the reports cited above, it could be estimated that the total amount of murine O-acetylated glycoproteins put in a lane for detection with INF-C was less than 1.7  $\mu$ g for the results shown GP2 and GP1 could be detected by this technique when only 0.4  $\mu$ g of O-acetylated glycoproteins was put in the lane (data not shown). Thus the detection is sensitive.

In the case of ovine EGP, the virus did not bind to any band, as shown Fig. 1B, lane 2.

These results are an expected illustration of the different affinities of INF-C for murine and ovine erythrocytes, as previously observed with hemagglutination experiments. In the present study, hemagglutination experiments were carried out with the same batches of

erythrocytes used for SDS-PAGE and blotting; the hemagglutination titers of a harvested allantoic fluid infected with INF-C were 512 and 0 HA units with murine and ovine erythrocytes, respectively, as indicated in Fig. 1. These data show that the binding of INF-C is probably due to interaction with sialic acid rather than non-specific interactions.

#### *INF-C Virus Specifically Binds to O-Acetylated Sialic Acid Containing Compounds on Nitrocellulose*

Using mouse erythrocytes, we next compared binding of INF-C with that of *M. amurensis* lectin, which recognizes specifically terminal  $\alpha(2-3)$ -linked sialic acids (8).

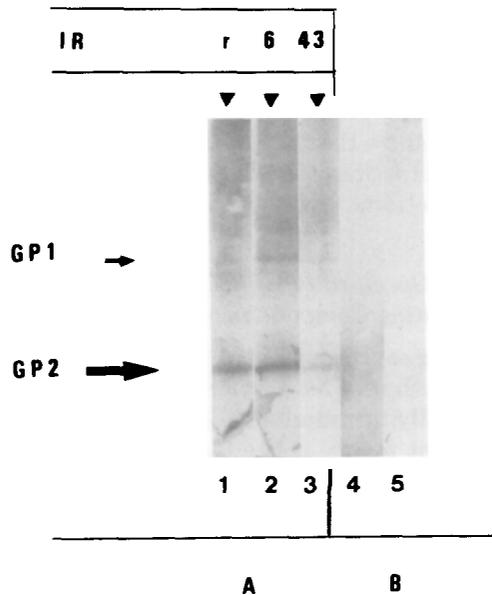
The different forms of the two murine glycoproteins recognized by INF-C, described above, were also detected by MAA. It was noted that GP2 was recognized by MAA as two bands, identified as GP2.1 and GP2.2, according to the previous descriptions of Sarris and Palade (30). The treatment of the nitrocellulose sheet with NaOH, as described under Materials and Methods, to remove the acetyl groups involved in ester linkages, did not modify the binding of MAA (data not shown), suggesting that MAA is specific for sialic acids, O-acetylated or not.

As previously described, INF-C binds to Neu5,9Ac<sub>2</sub> but not to Neu5Ac on erythrocytes and cultured cells (3,5). As shown in Fig. 1, the type of sialoglycoproteins recognized by INF-C exist on murine, which contain high amounts of Neu5,9Ac<sub>2</sub> (27,31), but not on ovine erythrocytes, which lack Neu5,9Ac<sub>2</sub> (32). These results suggest that INF-C bound to Neu5,9Ac<sub>2</sub> on electrophoregrams. In order to verify this specificity, removal of acetyl groups was carried out using alkali treatment or viral O-acetyl esterase activity, and competitive inhibition tests were made with characterized sialoglycoproteins OSM and BSM.

Following treatment of the nitrocellulose sheet with NaOH, as described under Materials and Methods, binding of INF-C could no longer be detected (Fig. 2B, lane 4). This shows that the binding is alkali labile, suggesting that the structure involved in attachment of virus to proteins contains ester linkages, probably in the form of O-acetyl residues. Moreover, before incubation with INF-C, pretreated with DFP, the nitrocellulose sheet was also submitted to untreated virus O-acetyl esterase activity. As shown in Fig. 2B, lane 5, INF-C no longer bound to any glycoprotein. This shows that INF-C recognizes specifically O-acetylated compounds.

Following treatment of the nitrocellulose sheet with the sialidase of *V. cholerae*, INF-C no longer bound to any glycoprotein, showing that INF-C binds to sialoglycoproteins.

OSM was prepared as described previously (33). The following carbohydrate chains have been found on this mucin (34,35): Neu5Ac $\alpha(2-6)$ GalNac, 94%; Gal $\beta(1-3)$ -



**FIG. 2.** The binding of influenza C virus to murine erythrocytic ghost proteins is alkali labile, sensitive to the viral *O*-acetyl-esterase, inhibited by BSM, but not by OSM. (A) Transferred proteins were incubated with INF-C, which was submitted to various pretreatments. Lane 1: INF-C was pretreated with DFP. Lane 2: DFP-pretreated INF-C was preincubated with OSM (100  $\mu$ g/ml). Lane 3: DFP-pretreated INF-C was preincubated with BSM (100  $\mu$ g/ml). IR, Index relative for inhibition percentage comparisons. (B) Transferred proteins were submitted to various treatments before incubation with DFP-pretreated INF-C. Lane 4: nitrocellulose sheet was submitted to NaOH treatment, before staining with INF-C pretreated with DFP. Lane 5: nitrocellulose sheet was submitted to native INF-C, with esterase activity, before staining with INF-C pretreated with DFP. In all lanes, 20  $\mu$ g of mouse EGP was loaded.

GalNac, 4%; Fuc $\alpha$ (1-2)Gal $\beta$ (1-3)GalNac, 2%; and Gal $\beta$ (1-3)[GlcNAc $\beta$ (1-6)]GalNac, 0.3%. BSM contains predominantly Neu5Gc, Neu5Ac, Neu9Ac5Gc, Neu5,9Ac<sub>2</sub>, Neu5,7Ac<sub>2</sub>, and di-*O*-acetylated sialic acids (36,37). As shown in Fig. 2A, OSM did not inhibit INF-C binding to murine erythrocytic ghost proteins (lane 2), whereas BSM did (lane 3). Band GP3 was on the migration front. Densitometric analysis of the binding to GP2 was carried out as described under Materials and Methods. OSM inhibition was very low (6%), whereas BSM inhibited by 43%. These inhibition experiments show that BSM, but not OSM, carry carbohydrate structures detected by INF-C. This result provides further evidence that INF-C recognizes *O*-acetylated sialic acid on transferred proteins.

Taken together, these data show clearly that INF-C specifically recognizes *O*-acetylated sialoglycoproteins (containing predominantly Neu5,9Ac<sub>2</sub>) following transfer onto nitrocellulose.

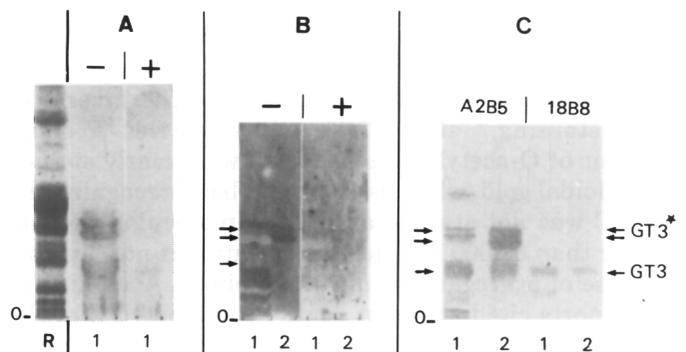
#### *INF-C Also Binds to O-Acetylated Gangliosides: The Binding Is Alkali Labile*

Binding of INF-C to gangliosides was investigated since these molecules are lipids which contain sialic

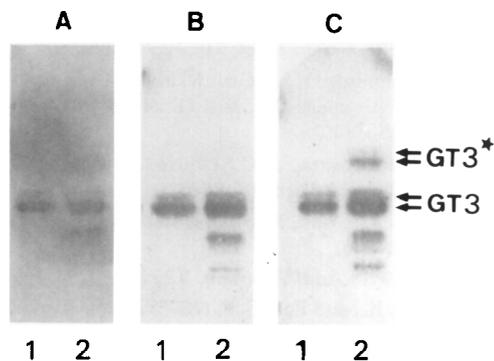
acids, either *O*-acetylated or not. After purification of gangliosides from embryonic chicken brain and separation by TLC, the chromatograms were incubated with INF-C pretreated with DFP. The virus was then revealed as described under Materials and Methods.

As shown in Fig. 3A, the iodinated virus INF-C bound to specific gangliosides, suggesting that some gangliosides of the plasma membrane of embryonic nervous cells can be receptors for the virus. When chromatographed gangliosides were exposed to ammonia vapors, in order to hydrolyze ester linkages, and then incubated with INF-C, no more binding of virus to any gangliosides was observed. The same results were obtained when virus bound to gangliosides was revealed with monoclonal antibody J14 (Fig. 3B, lane 1). The binding of INF-C to the *O*-acetylated trisialogangliosides (9-*O*-acetylated GT3) purified from 10-day embryonic chicken brain (11), Fig. 3B, lane 2, confirms these data. This doublet, which probably differs in the ceramides, is present in the chicken brain extract. It is no longer recognized by the virus after treatment by ammonia vapors.

*O*-acetylated GT3 is also recognized by the monoclonal antibody A2B5, specific for *O*-acetylated and non-*O*-acetylated trisialogangliosides (11) (Fig. 3C), but it is not recognized by the monoclonal antibody 18B8, specific for non-*O*-acetylated trisialogangliosides (10) (Fig. 3C). This non-*O*-acetylated GT3 present in chicken brain extracts and as a contaminant of the preparation



**FIG. 3.** Influenza C virus binds to alkali-labile gangliosides such as 9-*O*-acetylated GT3. After thin-layer chromatography of gangliosides, the chromatograms were either not submitted (-) or submitted (+) to ammonia vapors before incubation with the virus or directly immunostained with the antibodies, as described under Materials and Methods. Receptor-like glycolipids are visualized by viro-staining with <sup>125</sup>I-INF-C, pretreated with DFP (A), or with pretreated virus revealed with the monoclonal antibody (MAb) J14 (B). (C) Immunostaining with the monoclonal antibodies anti-gangliosides. Lane R: gangliosides isolated from 20 mg (wet weight) of 10-day embryonic chicken brain were visualized with resorcinol reagent. Lane 1: gangliosides as in lane R. Lane 2: 15 ng of purified 9-*O*-acetylated GT3. The migration of purified gangliosides is indicated at the margin with arrows. GT3\*, 9-*O*-acetylated GT3.



**FIG. 4.** O-Acetylated gangliosides are unmasked by the esterase activity of HEF protein of INF-C and recognized by MAb 18B8. After thin-layer chromatography of gangliosides, the chromatograms were incubated in allantoic fluid without the virus, as a control (A), or with the virus which was previously incubated with DFP (B), or with the native virus (C). The gangliosides antigens were visualized by immunostaining with MAb 18B8, which binds only to GT3 but not to its O-acetylated derivative. Lane 1: 15 ng of purified GT3. Lane 2: gangliosides from 15 mg (wet weight) of 10-day embryonic chicken brain. The migration of purified gangliosides is indicated at the margin with arrows. GT3\*, 9-O-acetylated GT3.

of the O-acetylated derivative is not recognized by INF-C (Fig. 3B, lanes 1 and 2).

This result suggests that the gangliosides, which are receptors for the virus, are alkali labile and probably contain O-acetylated sialic acid residues.

#### *INF-C Virus Esterase Activity Unmasks Epitopes for Monoclonal Antibodies*

In addition to analyzing binding of INF-C to sialoglycoconjugates, we also took advantage of its esterase activity specific for O-acetylated sialic acids to reveal cryptic antigens detected by monoclonal antibody specific for GT3. This makes the viral protein a tool for analyzing the effects of acetylation in recognition phenomena of sialoglycoconjugates. Incubations of glycolipids were carried out with virus whose enzymatic activity had not been inhibited (no preincubation with DFP). The deacetylated sialic acid residues were then revealed by incubation of gangliosides with the monoclonal antibody 18B8, which is specific for nonacetylated trisialo-gangliosides (10,11).

The incubation of chromatographed gangliosides with the virus revealed a cryptic antigen recognized by the monoclonal antibody 18B8, specific for the trisialo-ganglioside GT3 (Fig. 4C, lane 2), which was not detected with untreated gangliosides (Fig. 4A). Since viral treatment had been performed after chromatographic migration, the unmasked GT3 detected by 18B8 appeared where O-acetylated GT3 had previously migrated. In addition, inhibition of viral esterase activity with DFP inhibited the appearance of the antigen recognized by MAb 18B8 (Fig. 4B). Since monoclonal anti-

body recognized specifically the non-O-acetylated trisialyl structure found in the ganglioside GT3, this suggests that active viral esterase has unmasked the sialyl group, now recognized by the antibody.

#### DISCUSSION

In this report, in order to demonstrate that INF-C virus can be used as a probe to identify O-acetylated sialoglycoconjugates, we used cellular material known to contain high amounts of O-acetylated sialic acid. Mouse erythrocytes, whose sialic acid is 50–60% O-acetylated, provided receptor-like glycoproteins. Embryo chicken brain provided a receptor-like glycolipid mixture, rich in O-acetylated gangliosides. As proposed by Muchmore and Varki (38), who suggested that hemagglutination and receptor-destroying enzyme activities do not share the same binding site of HEF, we used selective inactivation of the viral esterase activity by DFP to prevent virus detachment.

Using "viro-blotting," we could demonstrate that INF-C bound to glycoproteins of murine erythrocytes but not to those of ovine red blood cells. The former are known to contain 9-O-acetylated sialic acid. The failure to recognize ovine sialoglycoproteins was consistent with the lack of hemagglutination by INF-C. In order to verify that the specificity of binding of INF-C was preserved on electrophoregrams, it was shown that INF-C bound to some but not all proteins. The major glycoprotein recognized by INF-C in mouse EGP was identified as the more O-acetylated murine glycophorin (27). This protein was also detected by MAA, which is specific for sialic acids. Alkali, O-acetyl esterase, sialidase treatments, and inhibition experiments supported the expected specificity of INF-C for O-acetylated sialic acids.

INF-C also binds to gangliosides purified from embryonic chicken brain. Viro-staining experiments show that INF-C binds to specific gangliosides which contain alkali-labile structures. The virus binds to the purified O-acetylated GT3, but not to GT3, as expected. These experiments suggest that O-acetylated gangliosides of the cell membrane can be receptors for INF-C, as well as substrates for its esterase which transformed O-acetylated GT3 into GT3.

The technique using INF-C described in this report appears to be a sensitive test for the detection of O-acetylated sialic acid. INF-C is not just a probe, it also provides a biochemical tool for studying the acetylation of sialic acid. Such a tool, with two different and specific activities, can be valuable in studying the occurrence and the localization of O-acetylated sialic acids on glycoconjugate mixtures after their electrophoretic and chromatographic separation. INF-C can also help to study the carbohydrate specificity of monoclonal antibodies.

In addition to qualitative data, the technique using staining with INF-C and assisted by computed image analysis can provide a semiquantitative tool for comparison of content of O-acetylated sialoglycoconjugates in various cells extracts.

The technique reported here could be further extended in order to use INF-C as in *in situ* probe on histological slides. The applications of such techniques in our laboratories for studying the occurrence of O-acetylated sialic acids in various cell lines and in some tissues, such as chicken cerebellum, are in progress, since preliminary experiments showed that INF-C might be neurotropic in experimentally infected chicken embryos.

The biological importance of acetylation of sialic acids is poorly understood. The occurrence of O-acetylated sialic acids on red blood cells can vary not only with the animal species but also with the age of the animal, as was demonstrated for chicken erythrocytes (5). O-acetylation of disialoganglioside GD3 by human melanoma cells was shown to provide a potential tumor-specific antigen (39). INF-C has been used in the past for studying virus receptors: the major receptor for human coronaviruses OC43 and E229 was shown to be O-acetylated sialic acids by comparison with those of INF-C (40). The acetylation of sialoglycoconjugates should interfere with different processes, such as cell maturation; cell recognition, probably with immunological implications; cell differentiation and dedifferentiation; and virus-cell interactions. The use of both binding and esterase activity could provide a better understanding of the biological roles played by O-acetylated sialic acids.

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