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9-O-Acetylated sialic acids have widespread but selective expression: Analysis using a chimeric dual-function probe derived from influenza C hemagglutinin–esterase

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ABSTRACT **While 9-O-acetylation of sialic acids has been reported in some mammalian tissues, the distribution of this modification on specific cell types and molecules is largely unknown. The influenza C virus hemagglutinin–esterase is a membrane-bound glycoprotein that binds specifically to 9-O-acetylated sialic acids (hemagglutinin activity) and then hydrolyzes the O-acetyl group (receptor-destroying activity). A recombinant soluble form of influenza C virus hemagglutinin–esterase wherein the C-terminal transmembrane and cytoplasmic domains are replaced by the Fc portion of human IgG retains both its recognition and enzymatic functions. The latter activity can selectively remove 9-O-acetyl groups from bound or free sialic acids and, under specific conditions, 7-O-acetyl groups as well. Irreversible inactivation of the esterase unmasks stable recognition activity, giving a molecule that binds specifically to 9-O-acetylated sialic acids. These probes demonstrate widespread but selective expression of 9-O-acetylated sialic acids in certain cell types of rat tissues. Patterns of polarized or gradient expression further demonstrate the regulated nature of this modification. Direct probing of blots and thin-layer plates shows selective expression of 9-O-acetylation on certain glycoproteins and glycolipids in such tissues. Thus, 9-O-acetylation is more widespread than previously thought and occurs on specific molecules and cell types.**

Of the variety of modified sialic acids in nature, many arise from O-acetylation at the 4, 7, 8, and 9 positions (1–3). These modifications have been implicated in embryonic development, protection against microbial sialidases, the binding of viruses, and the modulation of complement activation (1, 2). In mammalian tissues, 9-O-acetylation is usually considered an unusual feature of certain neural gangliosides, gut mucins, and erythrocytes (1, 2). While recent improvements in purification and analysis indicate that they may be more widespread (4, 5), direct demonstration of their presence and distribution in intact cells and tissues or on specific molecules remains difficult. Although some monoclonal antibodies against O-acetylated gangliosides exist, these are highly specific, recognizing the O-acetyl esters only in the context of the underlying sugar chain (2). To explore the expression and biology of 9-O-acetylated sialic acids, a specific and sensitive probe is needed that will detect these molecules regardless of underlying structure. The influenza C virus (InfC) has a surface hemagglutinin that specifically recognizes 9-O-acetylated sialic acids (6, 7). The molecule has two other activities, a "receptor-destroying enzyme," which is a 9-O-acetyl esterase (7–9), and a fusion activity at low pH (10). It is therefore called an InfC hemagglutinin-esterase-fusion protein (InfCHEF). At ambient temperatures and neutral pH, the esterase is dominant, making detection of the hemagglutinin activity difficult. However, at low temperatures, or after abrogation of the serine active site of the esterase with diisopropyl fluorophosphate (DFP), the hemagglutinin activity becomes evident (9). InfC particles have been used as probes for 9-O-acetylated sialic acids, with or without DFP treatment (4, 9, 11). However, difficulties with using the whole virion as a probe include the purity and stability of preparations, poor reproducibility, high nonspecific background, and lack of linearity in response. To circumvent these problems, we designed a soluble chimeric molecule with the properties of InfCHEF (minus the fusion activity), termed InfCHE, as well as those of the Fc portion of IgG, termed CHE-Fc.

MATERIALS AND METHODS

Materials. Most chemical and biological reagents were from Sigma. The following were from the sources indicated: restriction enzymes, T4 DNA ligase, Stratagene; AmpliTaq DNA polymerase, Perkin–Elmer; Dulbecco's modified Eagle's medium, GIBCO/BRL; fetal calf serum, HyClone; and 293 human embryonic kidney cells, ATCC. DFP from Aldrich was prepared as a 1 M stock solution in isopropyl alcohol and stored at −20°C.

Chimeric CHE-Fc. The coding sequence of the InfCHEF from InfC/Cal/78 (7, 12) was first modified to eliminate the fusogenic potential of the protein thus creating InfCHE. The codon ATT at position 1336 was converted to GAT (Ile-446 → Asp) by site-directed mutagenesis using PCR and the mutation was confirmed by sequencing. Sequences encoding the C-terminal transmembrane and cytoplasmic domains were replaced by a human IgG1 Fc region sequence fused in the "hinge region"—the latter were derived from the CD5-IgG1 vector (13). An *Xho* I–*Cla* I fragment containing the first 1346 bp of the modified InfCHE cDNA was prepared. A *Cla* I–*Bam*HI fragment corresponding to the next 553 bp of the InfCHE cDNA was obtained by PCR. A primer encoding the sequences starting at bp 1346 and including the *Cla* I site was synthesized with the sequence 5′-ATTTTTGGAATCGATGACCTTATT-3′. Reverse primers containing a *Bam*HI restriction site were synthesized with the sequence 5′-TGCTGGATCCAAGCTGCTTCCCCA-3′. The PCR product was digested with *Cla* I and *Bam*HI. The two fragments were simultaneously ligated into the CD5-IgG1 vector (cut with *Xho* I and *Bam*HI) to give plasmid pCHE-Fc, which was stably cotransfected into 293 cells along with one encoding Geneticin (G418) resistance by using a calcium phosphate method. Supernatants from stably transfected cells selected in G418 and expressing the CHE-Fc protein were clarified by

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Abbreviations: DFP, diisopropyl fluorophosphate; InfC, influenza C virus; HEF, hemagglutinin–esterase-fusion protein; HE, HEF with fusion peptide eliminated; CHE-Fc, chimeric protein of InfCHE and Fc portion of human IgG1; CHE-FcD, DFP-treated CHE-Fc (esterase inactivated); BSM, bovine submaxillary mucin; BSA, bovine serum albumin. Sialic acids are designated using combinations of Neu (neuraminic acid), Ac (acetyl), and Gc (glycolyl) (1, 2).

centrifugation, passed over a protein A-Sepharose column, and eluted with a pH step gradient of 0.1 M phosphate/citrate buffer (pH 5.5 to 3.5). A portion of the CHE-Fc was treated with 1 mM DFP to irreversibly inactivate the esterase activity, and the resulting protein was designated CHE-FcD.

Assay of Esterase and Hemagglutinin Activity. 9-O-[*acetyl*-^{3}H]Acetyl-*N*-acetylneuraminic acid was used as an esterase substrate (14). Breakdown of 4-nitrophenylacetate into 4-nitrophenol was monitored at 400 nm, the release of 1-naphthol from α-naphthylacetate was measured at 321 nm; and the relative fluorescence of 4-methylumbelliferone released from 4-methylumbelliferone acetate was measured as described (15). A mixture of sialic acids from bovine submaxillary mucin (BSM) (16) was treated with the CHE-Fc at pH 6.45, 7.1, or 7.5, or with the inactive CHE-FcD as control, for 1 and 3 h at 37°C. Products were analyzed as their 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride derivatives by reverse-phase HPLC (16). For hemagglutinin activity, serial dilutions of CHE-FcD or CHE-Fc (25 μl, 1 μg/μl) were made in PBS (pH 7.2) with 3% (wt/vol) bovine serum albumin (BSA; 25 μl). Washed mouse erythrocytes (25 μl, 2% packed cell volume) were added to each well; the plates were incubated at 37°C or at 4°C for 1 h, examined for hemagglutination, left at room temperature for 1 h, and reexamined.

Tissue Sources and Preparation. Adult Sprague–Dawley rats were euthanized and exsanguinated by decapitation; tissues were rapidly removed and either embedded in OCT compound and frozen in liquid nitrogen or fixed in 4% (wt/vol) paraformaldehyde for processing as paraffin-embedded sections. Rat blood (16 ml) was collected into 2 ml of 3.85% (wt/vol) sodium citrate, DFP was added to 1 mM, the mixture was incubated on ice for 30 min and then centrifuged, and the plasma was stored frozen. Human erythrocyte ghosts were prepared as described (17). Tissues were homogenized with a Polytron in 4 ml of 20 mM sodium phosphate (pH 6.5) and treated with 1 mM DFP as above. The suspension was centrifuged at 650 × *g* for 15 min, the post-nuclear supernate was collected and then centrifuged for 30 min at 100,000 × *g*. The pelleted membranes and the supernatant were stored frozen.

SDS/PAGE and Western Blot Analysis. Proteins were separated by SDS/PAGE in 7.5% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore), and blots were incubated overnight at 4°C with the CHE-Fc or CHE-FcD (10 μg/ml) in 20 mM Tris/140 mM NaCl/1% BSA. Glycoproteins reacting with the chimera were colorimetrically detected with a goat anti-human IgG (heavy and light chain) antibody conjugated with alkaline phosphatase.

TLC Overlays for Analysis of Lipids. Total ganglioside fractions were prepared from tissues as described (18, 19), spotted on aluminum-backed Silica Gel 60 HPTLC plates, and developed in chloroform/methanol/0.02% $CaCl_2$, 60:40:9 (vol/vol), with or without prior de-O-acetylation with ammonia vapors (19). Plates were impregnated with poly(isobutyl methacrylate) followed by a 15-min incubation in 1% BSA/PBS, and an overnight incubation at 4°C with CHE-FcD or control CHE-Fc, each diluted to 10 μg/ml in 1% BSA/PBS. O-acetylated gangliosides reacting with CHE-FcD were detected with a goat anti-human IgG antibody conjugated with biotin (Vector Laboratories) and a streptavidin-horseradish peroxidase conjugate. As a positive control, melanoma gangliosides were analyzed with monoclonal antibody D1.1, directed against 9-O-acetyl-G_{D3}.

Immunocytochemistry. Tissue cryosections were fixed for 10 min in acetone at −20°C, washed with PBS, incubated in 10% (vol/vol) goat serum/1% BSA/PBS blocking buffer, and then in CHE-Fc D (or CHE-Fc control) diluted in blocking buffer. After detection with alkaline phosphatase-conjugated goat anti-human IgG, sections were counterstained with hematoxylin.

RESULTS

Construction and Expression of a Functional Soluble CHE-Fc Chimera. A cDNA encoding InfCHEF was modified to eliminate the potential membrane-fusion-generating polypeptide sequence, and the region encoding the C-terminal transmembrane domain and the cytoplasmic tail of this modified cDNA (InfCHE) was replaced with that encoding the Fc portion of human IgG1 (13). A three-fragment ligation was necessary because of a *Bam*HI site within the cDNA of InfCHE. Stably transfected 293 cells secreted the chimeric protein (CHE-Fc) that was purified (1–2 mg/liter of spent medium) on protein A-Sepharose. It migrated in nonreducing SDS/PAGE gels as a single species of ≈240 kDa and reduction gave a band of ≈120 kDa (data not shown).

Esterase and Hemagglutinin Activity of the CHE-Fc Chimeric Protein. The release of [^{3}H]acetate from 9-O-[*acetyl*-^{3}H]acetyl-*N*-acetylneuraminic acid was used to screen for transfected clones and for monitoring elution from protein A-Sepharose. Activity is abolished by DFP treatment, indi-

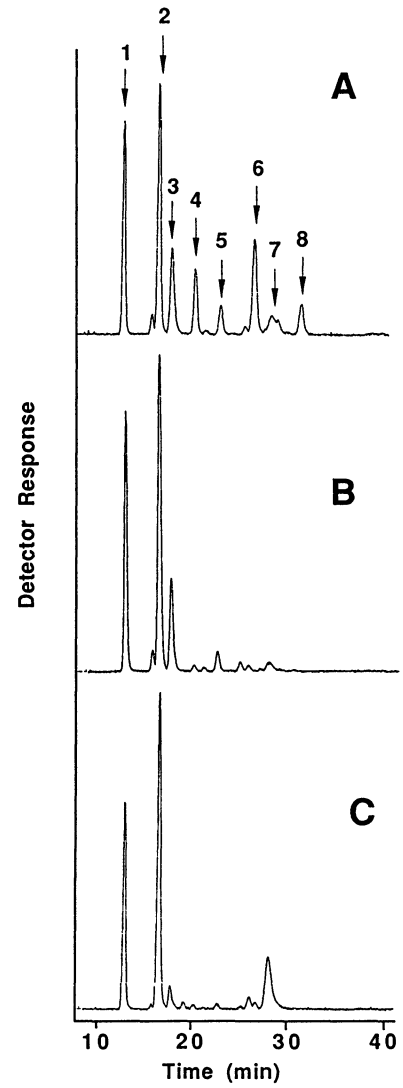

FIG. 1. Examples of HPLC analysis of BSM sialic acids treated with chimeric proteins. Aliquots of the BSM sialic acid mixture were treated as follows. (*A*) CHE-FcD at pH 6.45 for 3 h (negative control). (*B*) CHE-Fc at pH 6.45 for 3 h. (*C*) CHE-Fc at pH 7.5 for 3 h. Peaks corresponding to 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride-derivatized sialic acids are as follows: 1, Neu5Gc (Gc is glycolyl); 2, Neu5Ac; 3, Neu5,7Ac_2; 4, Neu5Gc9Ac; 5, unknown; 6, Neu5,9Ac_2; 7, reagent peak; 8, Neu5,7,9Ac_3.

Table 1. Properties of the InfC molecules studied

Molecule (source)	Esterase activity RT	Esterase activity 4°C	Hemagglutinin RT	Hemagglutinin 4°C	Fusion activity RT	Fusion activity 4°C
InfCHEF (native viral)	+++	+	±	++	++	+
CHE-Fc (recombinant)	+++	+	±	++	−	−
CHE-FcD (recombinant)	−	−	+++	+++	−	−

Fusion activity requires a specific tryptic cleavage site that is eliminated in the recombinant CHE-Fc and CHE-FcD. The esterase activity cleaves 9-O-acetyl esters from sialic acids, and the hemagglutinin activity recognizes 9-O-acetylated sialic acids in any linkage. RT, room temperature. Activity is indicated on a relative scale of − is none to +++ is maximum.

cating conservation of the serine active site. The K_m values for α-naphthylacetate (0.93 ± 0.2 mM) and for *p*-nitrophenylacetate (0.69 ± 0.2 mM) are in a similar range, but somewhat lower than for whole virus particles (15). The pH optimum measured with *p*-nitrophenylacetate was 7.8, again similar to reported values for the native protein. With a mixture of side-chain O-acetylated sialic acids from BSM, the enzyme showed specificity for 9-O-acetyl groups and a relative preference for Neu5,9Ac_2 vs. Neu5Gc9Ac (where Neu is neuraminic acid), again similar to the native molecule (see example in Fig. 1). Since a 7-O-acetyl group can migrate to position 9 at neutral or slightly alkaline pH values (20, 21), the specificity of the enzyme should be affected by pH. Indeed, at pH 7.5, deesterification of 7-O-acetylated, 9-O-acetylated, and 7,9-O-diacetylated sialic acids was seen (Fig. 1), with complete de-O-acetylation after prolonged incubation (data not shown). At pH 6.5, the hydrolysis of 9-O-acetyl groups is slower due to the nonoptimal pH, but no cleavage of the 7-O-acetylated sialic acids occurs even after 3 h (Fig. 1). In fact, a small increase of Neu5,7Ac_2 occurs with longer incubation, due to the action of the esterase on Neu5,7,9,Ac_3. Thus, the soluble esterase can be used to selectively remove 9-O-acetyl esters only (at pH 6.5) or both 7- and 9-O-acetyl esters (at pH 7.5).

Although mouse erythrocytes have high levels of 9-O-acetylated sialic acids (2, 22), the dominance of the esterase activity makes it difficult to detect the binding activity at ambient temperature. Indeed, the CHE-Fc hemagglutinates these cells only at 4°C (dilution, 8 μg/ml), and this disappears when the plate is transferred to room temperature (data not shown). In contrast, the esterase-inactivated CHE-FcD hemagglutinates the erythrocytes irreversibly at both 4°C and 37°C (dilution, 4 μg/ml). Thus, the latter should behave as a specific probe for 9-O-acetylated sialic acids. The properties of CHE-Fc and CHE-FcD are summarized in Table 1.

Exploration of 9-O-Acetylated Sialic Acid Expression in Rat Tissues. We used CHE-FcD to stain various rat tissues for 9-O-acetylated sialic acids, with the negative control of CHE-Fc. Distinctive staining patterns were consistently ob-

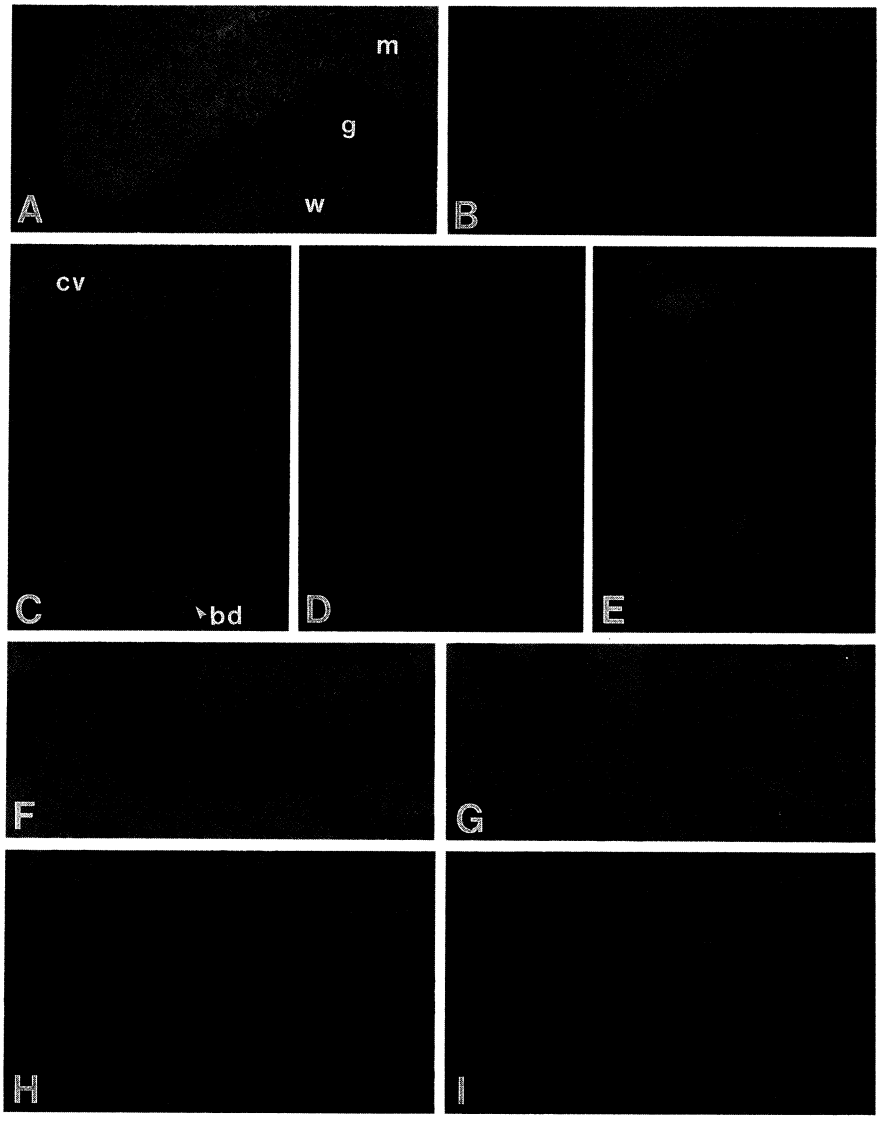

FIG. 2. Examples of tissue staining with the CHE-FcD. Frozen or paraffin-embedded sections of freshly isolated rat tissues were stained with CHE-FcD. CHE-Fc was used as a negative control (shown only in some examples). (*A*) Cerebellum, frozen section with CHE-FcD. (×55.) m, Molecular layer; g, granular layer; w, white matter. (*B*) Cerebellum, control with CHE-Fc. (×55.) (*C*) Liver, frozen section with CHE-FcD. (×220.) cv, Central vein; bd, bile duct. (*D*) Liver, control with CHE-Fc. (×220.) (*E*) Liver, paraffin-embedded section with CHE-FcD. (×220.) (*F*) Colon with CHE-FcD. (Oil immersion, ×550.) (*G*) Colon control with CHE-Fc. (Oil immersion, ×550.) (*H*) Kidney glomerulus with CHE-FcD. (Oil, ×550.) (*I*) Submaxillary glands with CHE-FcD. (×220.)

served (for examples, see Fig. 2). Strong but variable staining of the capillary, venular, and arterial endothelium was seen in most tissues. In the brain, widespread but variable staining was seen in different regions and cell types (detailed data not shown). In general, there was a brighter staining of grey matter than of white matter. In the cerebellum (Fig. 2 *A* and *B*), both the molecular and granular layers were stained. Liver sections (Fig. 2 *C* and *D*) showed intense staining at the peripheral edges of the parenchymal cells and on the endothelium of the central vein. In the portal triad, the endothelia of the hepatic artery and of the portal vein were positive, while the bile duct epithelium was negative. Within the hepatic lobules, the hepatocytes showed a centrilobular gradient of staining, decreasing from the central vein to the portal triad; this was more prominent in paraffin-embedded sections (Fig. 2*E*). The luminal face of the colonic mucosal epithelium was selectively stained (Fig. 2*F*—the lumen content is nonspecifically stained because of alkaline phosphatase in bacteria, see control with CHE-Fc in Fig. 2*G*). In the kidney, the glomerulii were strongly positive (Fig. 2*H*), whereas the tubular epithelium was negative. The medulla of the adrenal gland was intensely stained (data not shown). In the pancreas, the islets of Langerhans were selectively stained, although it is unclear whether this results from staining of the capillary network alone or the islet cells as well (data not shown). The retina was stained throughout, but with variable intensity in the different layers, the outer and the inner plexiform layers being the most positive (data not shown). In contrast to these tissues, parenchymal cells in several other tissues such as the submaxillary gland, pancreas, heart, testis, and skeletal muscle were not stained, although the vascular endothelium in these tissues remained positive (Fig. 2*I*). In all cases, the CHE-Fc serves as a specific negative control for the CHE-FcD binding. To further check the specificity of CHE-FcD, we reasoned that prior treatment of tissue section with CHE-Fc should eliminate the 9-O-acetyl groups required for binding. This prediction was borne out well (data not shown), providing further confirmation of the specificity of detection by CHE-FcD.

Direct Detection of 9-O-Acetylated Sialic Acids on Glycoproteins and Glycolipids by CHE-FcD. Rat serum glycoproteins and human erythrocyte membrane proteins were used as known positive and negative controls, respectively. Blots from SDS/PAGE gels (Fig. 3) indicated that while human erythrocyte proteins were negative, only certain proteins from rat serum were specifically stained by the CHE-FcD (the CHE-Fc control gave a weak positive response with some bands, presumably because of a high density of O-acetylated sialic acids on these glycoproteins). Staining was abolished by prior de-O-acetylation, with alkali or CHE-Fc esterase treatment. We next studied membrane and soluble glycoproteins as well as gangliosides from rat brain, liver, and submaxillary glands. Blots of proteins showed selective expression of 9-O-acetylated sialic acids on specific polypeptides (Fig. 4). In some cases, minor bands barely detectable by Coomassie blue staining showed strong reactions with the CHE-FcD. Again, the unmodified CHE-Fc acted as a negative control (data not shown). Gangliosides from these tissues also showed expression of 9-O-acetyl residues on a subset of molecules, and in all cases the staining was abrogated by alkali pretreatment (Fig. 5). The positive control in this case was the 9-O-acetyl-G_{D3} molecule of human melanoma cells.

DISCUSSION

Muchmore and Varki (9) reported that DFP inactivation of influenza C virions stabilizes the hemagglutinin activity, permitting use of the virus particles to detect 9-O-acetylated

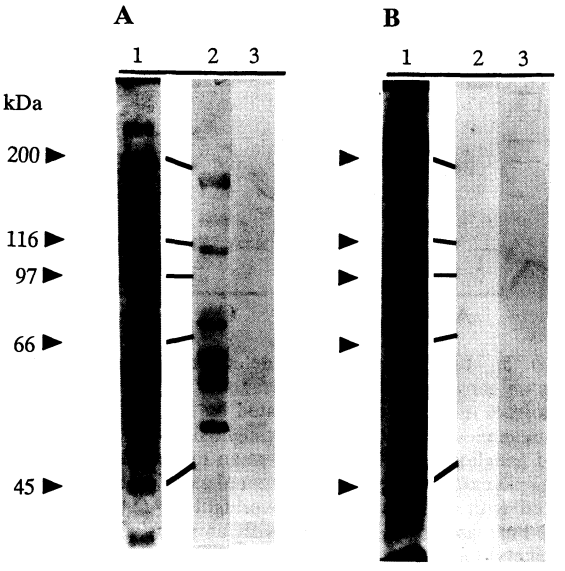

FIG. 3. Western blot analysis of 9-O-acetylated sialic acids on sialoglycoproteins. SDS/PAGE separated rat plasma proteins (*A*) and human erythrocyte ghost proteins (*B*) were stained as follows. Lanes: 1, Coomassie blue; 2, CHE-FcD; 3, CHE-Fc.

sialic acids on cells analyzed by flow cytometry. Since then, other groups have used influenza C virions for the direct demonstration of 9-O-acetylated sialic acids (4, 11). As discussed earlier, this approach has many limitations, as does the use of monoclonal antibodies described as specific for 9-O-acetyl groups. We have therefore constructed a soluble and versatile form of the InfCHE that retains both the hemagglutinin and esterase activity and also has the binding properties of IgG Fc (see Table 1). The esterase activity is very similar to that of the native protein, and pH conditions can be adjusted to remove either 9-O-acetyl groups alone or both 9- and 7-O-acetyl groups completely. DFP inactivation stabilizes and "unmasks" the hemagglutinin activity, giving a probe that specifically detects 9-O-acetylated sialic acids at ambient temperatures. Since CHE-FcD and CHE-Fc differ only by a single diisopropyl group, each can be used as control for the other. Thus, when the CHE-Fc is used to remove 9-O-acetyl esters, the CHE-FcD can be used as an inactive control. Conversely, when CHE-FcD is used to probe for 9-O-acetylated sialic acids, the CHE-Fc can be used as a control for nonspecific binding. The two forms can even be used sequentially; i.e., treatment with CHE-Fc can be used to remove the 9-O-acetyl esters prior to probing with CHE-FcD, leaving only nonspecific staining, if any. It should be noted that the CHE-Fc does have a masked hemagglutinin activity, which can give a weak positive response at ambient

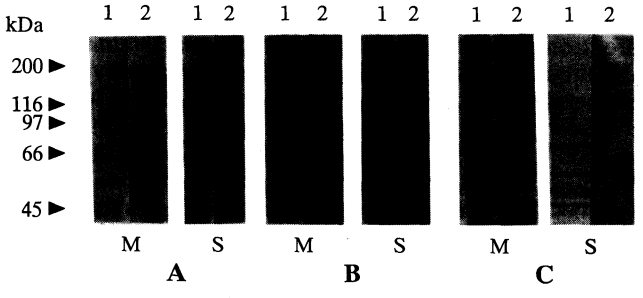

FIG. 4. Western blot analysis of 9-O-acetylated sialic acids on glycoproteins from rat tissues. SDS/PAGE separated membrane (lanes M) or soluble (lanes S) glycoproteins from various tissues were stained with Coomassie blue (lanes 1) or CHE-FcD (lanes 2). (*A*) Submaxillary glands. (*B*) Liver. (*C*) Brain.

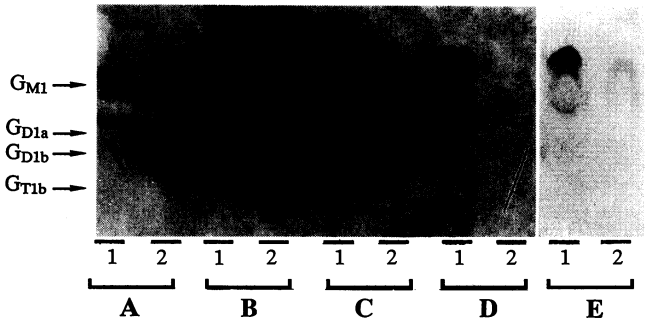

FIG. 5. Lipid TLC overlay detection of 9-O-acetylated sialic acids on gangliosides. Lipids extracts or partially purified gangliosides from tissues were fractionated by TLC and probed. (*A*) Rat brain gangliosides. (*B*) Rat liver gangliosides. (*C*) Rat submaxillary gland gangliosides. (*D* and *E*) Human melanoma cell line (Melur) total lipid extract. Lanes: 1, nontreated gangliosides; 2, gangliosides treated with ammonia vapors overnight. *A–D* were probed with CHE-FcD and *E* was probed with antibody D 1.1, specific for 9-O-acetyl-G_{D3}.

temperatures if the density of O-acetylated sialic acids is very high (e.g., with some glycoproteins blotted on nitrocellulose).

O-acetylated sialic acids were once thought to be rare species-specific modifications found only in certain mucins and gangliosides. We show here that 9-O-acetylation is found in a surprising variety of tissues in the rat. In the brain, this modification is widespread, showing significant regional variations. Whether these patterns are due to differential distribution of specific gangliosides or to different levels of expression of *O*-acetyltransferase(s) remains to be studied. O-acetylation is carried by multiple gangliosides, and probing of protein blots shows O-acetylation of specific glycoproteins as well. As predicted earlier (5), the liver shows extensive O-acetylation mainly in the glycoprotein fraction. Staining appeared to be localized to the plasma membrane, with an increase in the sinusoidal face and a centrilobular gradient. Since the pattern of 9-O-acetylation of glycoproteins in the liver membrane fraction is quite distinct from that in plasma, this is not an artifact caused by increasing vectorial secretion of plasma proteins. A striking and unexpected finding is that the endothelium of blood vessels stains positively (to various extents) in all tissues studied. Here too the possibility of adsorption of plasma proteins must be considered. However, in protein blots from tissues where only the endothelium is positive (e.g., submaxillary gland), the positive bands are distinct from those seen in the plasma. The positive staining seen in the colonic mucosa fits prior reports of 9-O-acetylated sialic acids in this tissue from many species (23, 24). The increase in staining at the apical regions of cells directly exposed to the lumen fits our prior hypothesis that these modifications may play a role in protecting the mucosa against bacterial sialidases (23). In the kidney, the glomeruli are strongly positive by immunohistochemical staining, while the tubular parenchymal cells are largely negative. While glomerular podocytes have previously been shown to contain 9-O-acetyl-G_{D3} by immunohistochemical localization with antibody 27A (25), the precise cell types being stained by CHE-FcD need to be assessed at higher resolution. Since the submaxillary gland mucins of many species have been reported to have high concentrations of 9-O-acetylated sialic acids (1), it was surprising to find no significant staining in the parenchyma of the rat submaxillary gland. Lipid overlays and protein blots confirmed that this is not due to inaccessibility of molecules on the tissue section. It is possible that the sialic acids of rat submaxillary mucins are 4-O- rather than 9-O-acetylated (26).

Interesting patterns of expression were also seen in several other organs, which require further investigation. For the retina and adrenal gland, previous work in transgenic mice (12) indicated that the specific removal of 9-O-acetyl groups caused abnormalities in development. Thus, with the results presented here and previous studies of neural gangliosides (1, 2), O-acetylation is emerging not only as a developmentally regulated molecule but as a major posttranslational modification, with widespread but selective expression in many tissues. The availability of the dual-function probe described here enhances the possibility of uncovering the specific biological roles of these modifications.

Finally, although the InfCHE has distant homology to the hemagglutinins of influenza A and B viruses, the latter are known to exist as tightly associated trimers in their functional native state (27). Our work indicates that a dimeric state is sufficient for expression of both the hemagglutinin and esterase activities of the InfCHE and that tryptic cleavage is not required for either activity.

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