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Developmentally regulated *O*-acetylated sialoglycans in the central nervous system revealed by a new monoclonal antibody 493D4 recognizing a wide range of *O*-acetylated glycoconjugates*

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We have previously detected an alkali-labile and developmentally regulated antigen in rat embryonic cerebral cortex, which may be 9-*O*-acetylsialylated GT3 ganglioside (Hirabayashi Y, Hirota M, Suzuki Y, Matsumoto M, Obata K, Ando S (1989) *Neurosci Lett* 106:193–98). In this study we established a mouse monoclonal antibody, 493D4, that recognizes 9-*O*-acetyl GT3 ganglioside, but not non-*O*-acetyl gangliosides. This antibody also reacted with 9-*O*-acetyl GD3 to a much lesser extent. By using this antibody, we found that *O*-acetyl GT3 as well as *O*-acetyl GD3 were expressed strongly in fetal murine cerebral cortex and decreased to an undetectable level after birth. With the assistance of TLC-immunostaining using 493D4 together with Q-Sepharose column chromatography, *O*-acetyl gangliosides of bovine brain were purified and the structural analysis showed the presence of *O*-acetyl GD3, *O*-acetyl LD1, *O*-acetyl GD2 and *O*-acetyl GD1b in the adult brain as extremely minor components. Interestingly, the antibody 493D4 could detect *O*-acetyl sialoglycoproteins in rat brain tissues. One of the major immunoreactive proteins was shown to be synaptophysin, an integral membrane protein specifically present in synaptic vesicles. This monoclonal antibody was therefore useful for sensitive detection of both *O*-acetylated gangliosides and glycoproteins with *O*-acetylated sialic acids.

***Keywords*: monoclonal antibody, *O*-acetylated sialic acid, ganglioside, synaptophysin, embryonic development**

***Abbreviations*: Ac, acetyl group; mAb, monoclonal antibody; HPTLC, high performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; FAB-MS, fast atom bombardment mass spectrometry; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Cer, ceramide; LD1, NeuAc(α2-8)NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)Glc(β1-1)Cer. Ganglioseries are abbreviated according to the system of Svennerholm [1]**

Introduction

Sialic acid is an acidic sugar constituent in most glycoconjugates including gangliosides and glycoproteins. In addition to acetylation or glycolylation of the amino group, sialic acid is also found to be modified by *O*-acetylation at the 4, 7, 8 and 9 positions. Some biological properties of these sugars seem to be associated with these modifications, *eg* they prevent hydrolysis by sialidases [2, 3], they serve as receptors of influenza C viruses and coronaviruses [4, 5], as antigens of bacterial capsules [6] and as a differentiation marker on chicken erythrocytes [7], and they modulate complement activation through the alternative pathway [8]. The expression of 9-*O*-acetylated gangliosides was associated with cell migration in rat brain development [9], and the expression of an *O*-acetylated ganglioside 9-*O*-Ac-GD3 in human melanoma cells served as a tumour-associated antigen [10]. The mAb 27A against *O*-Ac-GD3 induced mitogenic response in cultured peripheral blood mononuclear cells [11], whereas the mAb 8A2 against *O*-Ac-gangliosides caused retraction of goldfish retinal ganglion cell axons [12, 13]. Varki *et al.* [14] destroyed the 9-*O*-acetylated from of sialic acids in mouse embryos by expressing influenza C virus 9-*O*-acetyl esterase. An early expression of the esterase arrested the development at the two-cell stage. The selective expression of the esterase

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during late embryogenesis caused abnormalities only in the retina and adrenal gland. Based on these results, it is postulated that the modification of sialic acid residues with acetate may play roles in a wide range of biological processes.

Since gangliosides containing *O*-acetyl sialic acid are relatively minor components of total gangliosides and the *O*-ester linkage is unstable at neutral pH, the detection and purification of *O*-Ac-gangliosides remain difficult. Several monoclonal antibodies have been reported recognizing specifically gangliosides containing *O*-acetyl sialic acid residues [15–18]. The detection of gangliosides with *O*-acetyl sialic acids is significantly improved by TLC-immunostaining with these antibodies. However, these mAbs selectively recognize only a few gangliosides containing *O*-acetyl sialic acid. To discover all the *O*-Ac-gangliosides, the establishment of new mAbs with different specificity is required. Recently, an effort has also been made to produce a chimeric probe by fusion of the IgG Fc portion with the *O*-acetyl sialic acid binding domain of influenza C virus haemagglutinin [19].

Previously we have reported several mAb against C-series polysialogangliosides, one of which, mAb M6704, recognizes ganglioside GT3 [20]. In fetal rat cerebral cortex, GT3 ganglioside antigen could be exposed to mAb M6704 by base treatment [18]. Our data suggested that the antigen may be masked by *O*-acetylation of the sialic acid residues of GT3. The antigen was highly enriched at gestation day 14, and decreased afterwards. In this study, we established a new mAb (493D4) that can directly bind to *O*-acetylated sialic acid epitopes without the requirement of base pretreatment. TLC-immunostaining of gangliosides from murine embryonic cerebral cortex with mAb 493D4 showed that not only the expression of *O*-Ac-GT3 but also that of *O*-Ac-GD3 was regulated in a development consistent with the observation made by Chou *et al.* [17]. This antibody was found to recognize a wide range of *O*-Ac-gangliosides that did not react or reacted only weakly with several other antibodies known to recognize *O*-Ac-gangliosides. With the aid of this antibody, the structures of several *O*-Ac-gangliosides of adult bovine brain were identified.

Material and methods

Materials

HPTLC plates (silica gel 60) were purchased from E. Merck, Germany, plastic TLC plates (Polygram Sil G) from Macherey-Nagel, Germany, Iatrobeads from Iatoron Co., Japan, ganglioside standards from Iatoron Lab., Japan sialidase and mAb SVP38 from Sigma. The mAb P-path was kindly supplied by Dr M. Yamamoto, Faculty of Medicine, University of Tsukuba. 9-*O*-Ac-GD3 and 9-*O*-Ac-GT3 were isolated from bovine milk [21] and cod fish brain [22], respectively.

Derivation of mouse monoclonal antibody 493D4

Antibody 493D4 is a mouse IgM originally isolated in a hybridoma experiment to search for reagents useful for the analysis of the developing chicken visual system. A BALB/c mouse was immunized with a homogenate in saline of the optic nerves from 10-day chick embryos, and hybridoma supernatants were screened by fluorescence immunohistochemistry on frozen sections of fixed embryonic tissues. Hybridoma 493 D4 was established by limiting dilution. This antibody stains the optic nerve, optic tract and other structures of the brain and spinal cord of 10-day chick embryo. A histochemical study of the developing chicken nervous system using mAb 493D4 will be reported in future by one of the authors (S.C.F.).

Isolation of murine brain gangliosides

Cerebral cortices from mice of gestation day 14 to postnatal day 10 and adult mice were collected, lyophilized and stored at $-20\,^{\circ}C$ before use. The dry samples (20–100 mg) were homogenized with 10 ml chloroform (C): methanol (M) (2:1, by vol) twice, and C : M : water (W) (5 : 5 : 1, by vol) once. The combined extracts were subjected to Folch's partition. An aliquot of the upper phase containing gangliosides corresponding to 600 μg of mouse cerebral cortex (dry tissue weight) was applied to a plastic TLC plate for immunostaining as described below.

Isolation of bovine brain *O*-Ac-gangliosides

Total bovine brain gangliosides (12 g) obtained from Folch's partition upper phase were separated by Q-Sepharose chromatography into 16 fractions as described previously [23]. The powder (0.54 g) of disialoganglioside fractions (containing mAb 493D4 positive components) was dissolved in 2 ml of C : M (9 : 1, by vol) and applied to a Iatrobeads column (1.5×90 cm) which had been equilibrated with the same solvent. The column was eluted with 4 l of a linear gradient system from C : M : W (60 : 30 : 4.5, by vol) to C : M : W (60 : 30 : 8, by vol). Every 10 ml of the effluents were collected. Fractions containing mAb 493D4 positive components were pooled, evaporated to dryness, and further purified by HPLC, respectively, using a column of Senshu Pak AQUASIL SS-652N (3×15 cm: Senshu Scientific Company Ltd, Tokyo Japan). The gangliosides were eluted with 2 l of a linear gradient system from C : M : W (60 : 27 : 2, by vol) to C : M : W(60 : 32 : 5, by vol) with a flow rate of 5 ml min^{-1}. Final purification was achieved by preparative TLC using precoated silica gel HPTLC plates (Silica Gel 60 E. Merck) developed with C : M : 12 mM $MgCl_2$ (5 : 4 : 1, by vol, solvent A) or C : M : 12 mM $MgCl_2$: 15 M NH_4OH (50 : 40 : 73, by vol, solvent B). The compounds were located by iodine vapour and eluted with C : M : W (10 : 10 : 3, by vol) from the silica gel.

HPTLC

Analytical HPTLC was carried out on precoated HPTLC plates using the solvent A or solvent B. Gangliosides were visualized with resorcinol/HCl reagent [24].

Immunological analysis

TLC-immunostaining was performed by the method of Kusunoki *et al.* [25]. Gangliosides were applied onto a plastic plate (Poligram Sil G, Nagel, Germany) and developed with solvent A. The plate was subjected to immunostaining with mAb 493 D4 and HRP-conjugated anti-mouse IgG + IgM. After visualization, gangliosides were quantitated by dual-wavelength densitometric scanner, Shimadzu CS-9000 (Shimadzu, Kyoto, Japan). The enzyme-linked immunosorbent assay was performed by the method of Higashi *et al.* [26].

Alkali treatment of ganglioside antigens

Purified gangliosides (5 μg) were incubated in 10 ml of 15 M ammonium hydroxide and 50 ml of methanol overnight, respectively [27]. The ammonium hydroxide was removed by N_2 gas. Alkali-treated gangliosides were analysed by HPTLC.

Negative-ion fast atom bombardment mass spectrometry (FAB-MS)

FAB-MS of the purified gangliosides was performed using a Finnigan MAT TSQ 700 triple-stage quadrupole mass spectrometer (Finnigan MAT, San Jose, USA) equipped with a FAB-MS spectrometric ion source. Data were processed with a DEC Station 2100 computer. The FAB-MS spectra in the negative mode were recorded as described earlier [28].

Immunofluorescent histochemistry

Cerebellar cryosection (10 μm thickness) prepared from male Wistar rats (7–10 weeks old) were processed for indirect immunohistochemistry as described previously [29–31]. Following the blocking with PBS containing 10% normal goat serum (NGS) for 30 min at room temperature, the sections were incubated with mAb 493D4 or P-path (1 : 200 dilution in PBS containing 10% NGS) overnight at 4 °C. Fluorescein-5-isothiocyanate-conjugated goat affinity purified F(ab')2 fragment of IgG to mouse IgM (μ chain specific, 1 : 200 dilution, CappelTM, Organon Teknika Corp., Durham, NC) was used as a secondary antibody, and the bound antibodies were observed with a Zeiss fluorescence microscope equipped with an appropriate filter system. When the whole procedure was carried out in the absence of a primary antibody, no fluorescence was observed (data not shown). Sialidase treatment (*Vibrio cholerae*, 10 mU ml^{-1}; Sigma) completely abolished the immunoreactivities of both 493D4 and P-path mAbs in the resultant sections (data not shown).

Western blot analysis

Cerebellar microsomal membranes and cytosol fractions of Wistar rats (8–12 weeks old) were prepared as described previously [29] and kept at − 80 °C until used. In some experiments, the proteins were further fractionated by AEP-550 Biophoresis III system (Atto, Japan) which fractionally collects the proteins passing through SDS polyacrylamide gel. The proteins were denatured in the sample buffer containing 1% SDS and 1.25% 2-mercaptoethanol at 90 °C, and subjected to SDS-gel electrophoresis [32]. Separated proteins (20 μg per well) were electrophoretically transferred to nitrocellulose filters [33]. After blocking with 5% defatted milk in Tris-buffered saline (TBS), the filter blots were incubated with 493D4, P-path or anti-synaptophysin mAb SVP-38 (diluted 1 : 200) in TBS/DFM/TX (TBS containing 0.25% defatted milk and 0.1% Triton X-100) for 2 h at 30 °C. The blots were extensively washed in TBS/DFM/TX, incubated for 2 h with HRP-conjugated goat anti-mouse IgM + IgG (H + L) (diluted 1 : 1000; Jackson Laboratories, USA) in the same buffer, and developed with 4-chloro-1-naphthol with *N*,*N*-diethylphenylenediamine and 0.006% H_2O_2 according to the previous report [34].

Results

Developmental expression of *O*-Ac-GD3 and *O*-Ac-GT3 in murine cerebral cortex

We have reported that mAb M6704 recognizes a GT3 ganglioside antigen derived from base-treated gangliosides of rat prenatal cerebral cortex [18]. In this study, we demonstrated that mAb 493D4 directly recognizes the parent molecule, *O*-Ac-GT3, with no need of modification by base treatment. The mAb 493D4 does not cross-react with non-*O*-Ac-gangliosides including GT3. By TLC-immunostaining of gangliosides of murine cerebral cortex at gestation days 14–21 and postnatal days 1–10, mAb 493D4 detected several antigens (Figure 1A). One of them comigrated with 9-*O*-AcGT3 and another one, 9-*O*-Ac-GD3. Structure analysis indicates that these two gangliosides are identical to *O*-Ac-GT3 and *O*-Ac-GD3 as reported by Ren *et al.* [35]. The expression of *O*-Ac-GT3 and *O*-Ac-GD3 appears to be developmentally regulated in murine cerebral cortex (Figure 1B). *O*-Ac-GT3 was highly expressed at gestation days 14–16 and thereafter the amount of *O*-Ac-GT3 gradually decreased. *O*-Ac-GT3 levels were very low after birth and in the adult. The expression of *O*-Ac-GD3 reached a maximum at gestation days 16–17 and disappeared a few days after birth.

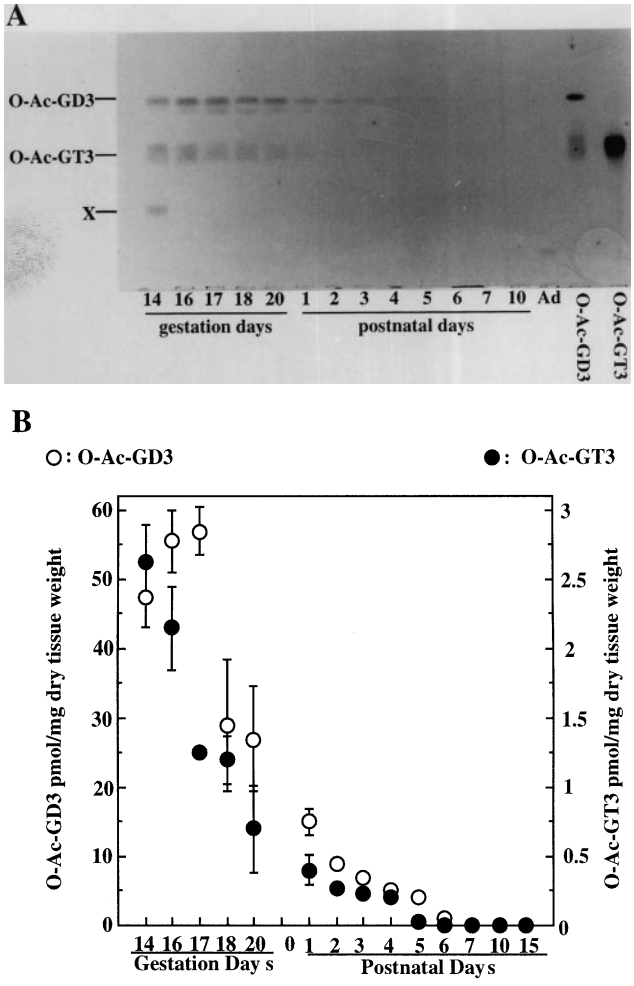

Figure 1. Changes of *O*-Ac-ganglioside level during development of mouse cerebral cortex. In (A) gangliosides of cerebral cortices from mice of gestation day 14 to postnatal day 10 as well as adult mice were extracted, isolated by Folch's partition and separated by TLC. The TLC plate was immunostained with mAb 493D4 as described in 'Materials and Methods'. Ad, adult; X, unknown ganglioside. The positions of 9-*O*-Ac-GD3 and 9-*O*-Ac-GT3 were indicated. In (B) the amounts of *O*-Ac-GD3 and *O*-Ac-GT3 were quantitated by densitometer. The data are mean ± SD from five experiments.

Isolation and characterization of mAb 493D4-reactive gangliosides of bovine brain

In the following study, we characterized the *O*-Ac-gangliosides of bovine brain using the two different mAbs, 493D4 and P-path. Total ganglioside extract from the bovine brain was separated by preparative Q-Sepharose chromatography (Figure 2A), which has been used to purify several minor gangliosides of bovine brain [36, 37]. After fractionation of gangliosides by Q-sepharose chromatography, the amount of each ganglioside applied on a TLC plate could be greatly increased and therefore the efficiency of TLC-immunostaining for detection of minor gangliosides was significantly enhanced. More than 20 components could be detected by TLC-immunostaining with mAb 493D4 in the separated ganglioside fractions of bovine brain (Figure 2B). The mAb P-path known to recognize *O*-Ac-ganglioside, reacted mainly with *O*-Ac-disialogangliosides (Figure 2C), while mAb 493D4 recognized not only *O*-Ac-disialogangliosides but also *O*-Ac-gangliosides in trisialo- and polysialoganglioside fractions (Figure 2B). In disialoganglioside fractions (fractions 4–8, Figure 2A and B), we detected four main antigens. One antigen comigrated with *O*-Ac-GD3 (in lane 4). The three other major positive components, designated as X1, X2 and X3, were further purified by HPLC equipped with a column of Senshu Pak AQUASIL SS-625N and finally preparative TLC. The purified antigens X1 and X2 had a similar mobility and migrated between GD1a and GM1, while X3 moved between GD1a and GD1b when HPTLC plate was developed with solvent A, a neutral developing solvent (Figure 3A). When the plate was developed with solvent B, an alkaline developing solvent, the mobilities of GM1 and GD1b were significantly reduced while the mobilities of X1 and X2 were not changed and therefore they migrated faster than GM1 (Figure 3B). In contrast, the mobility of X3 was significantly reduced to an extent comparable to GD1b. The mobility of *O*-Ac-GT3 was changed in a different manner from that of X1, X2 or X3 (Figure 3A and B), indicating that none of X1, X2, or X3 was *O*-Ac-GT3.

The yields of X1, X2, and X3 were approximately 1.4 mg, 0.94 mg and 1.2 mg from 12 g of total bovine brain ganglioside fraction.

Identification of alkali-labile antigens by base treatment

To confirm that these antigens contain alkali-labile *O*-acetyl group, purified antigens X1, X2 and X3 were treated with ammonium hydroxide to remove *O*-acetyl group from sialic acid residue [27] and then chromatographed on HPTLC plates with the solvent A (Fig. 4). The antigen X1 comigrated with LD1, X2 with GD2 (not shown) and X3 with GD1b. These results suggest that the antigens X1, X2 and X3 are probably *O*-acetylated derivatives of LD1, GD2 and GD1b, respectively.

Negative FAB-MS of three 493D4-positive gangliosides

The spectra of negative FAB-MS are shown in Figure 5.

X1 (Figure 5A); The molecular ions were indicated by the peak at *m/z* 1900(+Na) corresponding well to that of *O*-Ac-LD1 containing a Cer composed of long chain base d18 : 1 and fatty acid C18 : 0. The fragment ions at *m/z* 1545, *m/z* 1092, *m/z* 888, and *m/z* 726, corresponded to the sequential elimination of *O*-Ac-NeuAc, NeuAc + Gal, GlcNAc, and Gal from *O*-Ac-LD1. Although sialidase treatment abolished reactivity of X1 with 493D4, it did not produce an antigen stained by TLC-immunostaining with anti-asialo

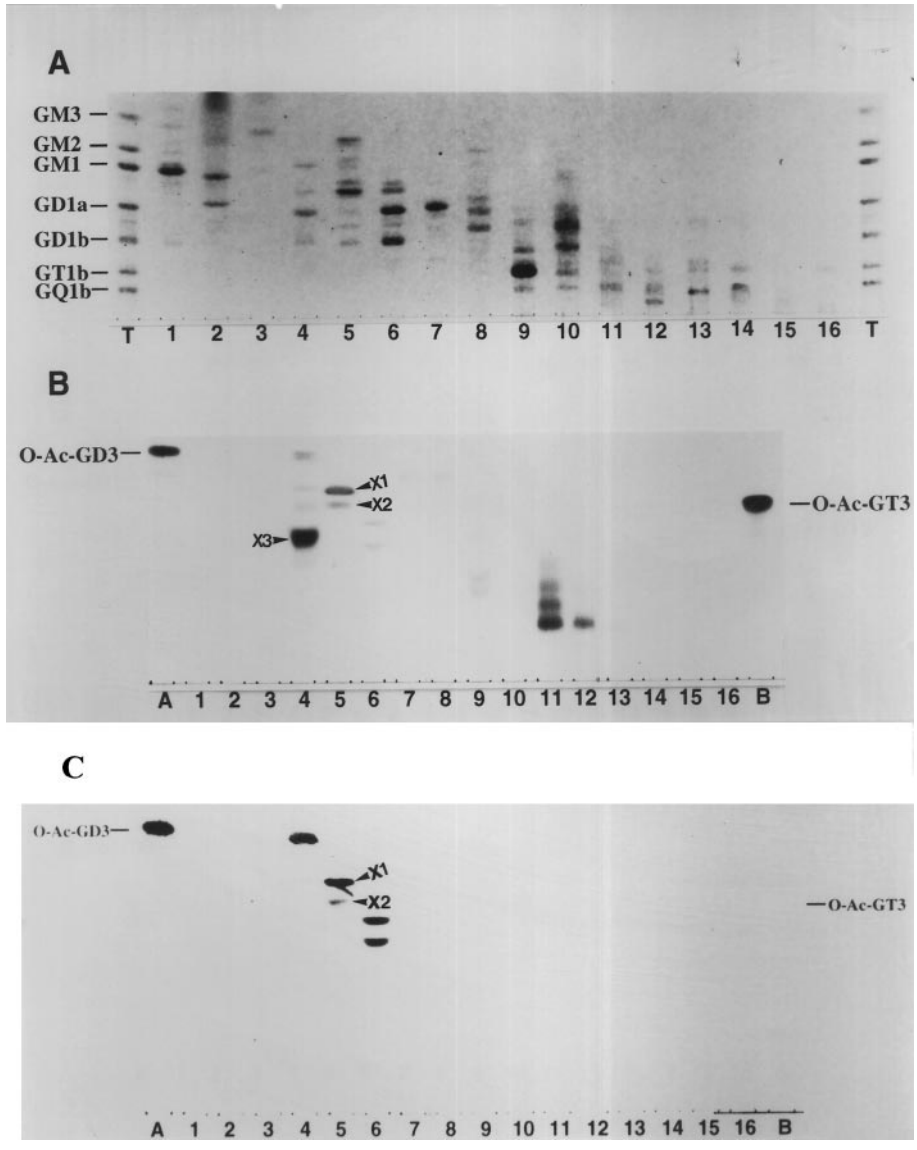

Figure 2. HPTLC and TLC-immunostaining of bovine gangliosides. Total ganglioside extract from the bovine brain was separated by Q-Sepharose chromatography into 16 fractions. In (A), the gangliosides from each fraction (5 μg) were applied onto an HPTLC plate and developed with solvent A. Gangliosides were visualized with resorcinol/HCl reagent. In (B) and (C), the gangliosides from each fraction (1 μg) were applied to a polygram Sil G plate and developed with solvent A. Gangliosides were visualized by immunostaining with mAb 493D4 (B), and mAb P-path (C), respectively, as described in 'Materials and methods'. The lane numbers (1–16) correspond to the fraction numbers of Q-sepharose chromatography. Fractions 1–3: monosialogangliosides; fractions 4–8, disialogangliosides; fractions 9–16, polysialogangliosides. Lane A, 9-*O*-Ac-GT3; Lane B, 9-*O*-Ac-GT3.

GM1 (data not shown), excluding the possibility that X1 is O-Ac-GDlc. A possible structure for X1 is shown in Figure 5A. Several unidentified peaks were attributed to contaminants.

X2 (Figure 5B): A molecular ion peak was at *m/z* 1744 corresponding to that of *O*-Ac-GD2 containing a Cer composed of long chain base d20:1 and fatty acid C18:0. The fragment ions at *m/z* 1411, 1120, 916, 754, and 592 corresponded to the sequential elimination of *O*-Ac-NeuAc, NeuAc, GalNAc, Gal and Glc from *O*-Ac-GD2. Thus, the structure of X2 may be that shown in Figure 5B.

X3 (Figure 5C): A major molecular ion was indicated by the peak at *m/z* 1929 corresponding to that of *O*-Ac-GD1b containing a Cer composed of long chain base d20:1 and fatty acid C18:0. The fragment ions at *m/z* 1573, 1282, 1119, 917, and 592 corresponded to the sequential elimination of *O*-Ac-NeuAc, NeuAc, Gal, GalNAc, Gal + Glc from *O*-Ac-GD1b. A possible structure for is shown in Figure 5C.

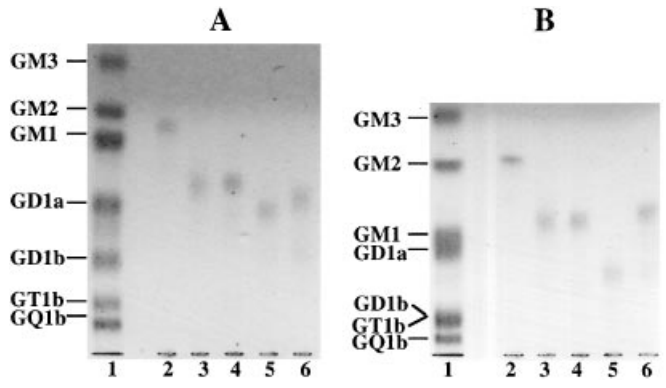

Figure 3. HPTLC of X1, X2 and X3. Each of purified X1 (lane 3), X2 (lane 4), X3 (lane 5) was applied onto HPTLC plate and developed with solvent A (A) or solvent B (B). Gangliosides were visualized by resorcinol/HCl reagent. Lane 1, ganglioside standards; Lane 2, 9-*O*-Ac-GD3; Lane 6, 9-*O*-Ac-GT3.

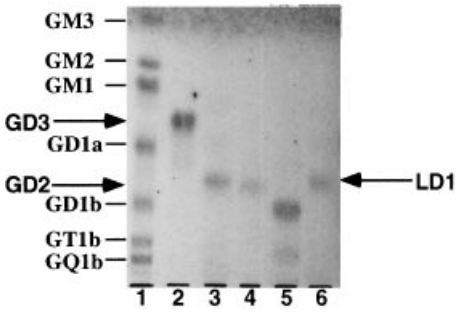

Figure 4. HPTLC of purified X1, X2 and X3 after alkaline treatment, X1, X2 and X3 were treated with ammonium hydroxide and analysed by HPTLC with developing solvent A as described in 'Materials and methods'. Lane 1, standard gangliosides; Lane 2–5, alkaline-treated *O*-Ac-GD3, X1, X2 and X3, respectively; Lane 6, standard LD1. Gangliosides were visualized by resorcinol/HCl reagent.

Reactivity of five *O*-Ac-gangliosides with mAb 493D4 and P-path

In Figure 2, we have observed the difference in the staining patterns of bovine brain gangliosides by mAbs 493D4 and P-path. Specificity and affinity of the two mAbs to *O*-Ac-GD3, *O*-Ac-LD1, *O*-Ac-GD2, *O*-Ac-GD1b and *O*-Ac-GT3 were further quantitatively compared by TLC immunostaining and ELISA. The mAb 493D4 recognized all those *O*-Ac-gangliosides But with a much higher affinity to *O*-Ac-GT3 than to the other four (Figure 6A). The mAb P-path mainly reacted with *O*-Ac-LD1 and *O*-Ac-GD3 with a much lower affinity to *O*-Ac-GD2 and no reactivity with *O*-Ac-GD1b and *O*-Ac-GT3 (Figure 6B). Other regular (non-acetylated) gangliosides have no reaction with the two mAbs (Figure 6A and B). Essentially the identical results were obtained by ELISA assay of the five *O*-Ac-gangliosides using the two mAbs (Figure 7A and B).

Recognition of glycoproteins by mAb 493D4

It is interesting to note that mAb 493D4 also recognizes its antigenic determinant on a few glycoproteins. In the rat cerebellum, the 38 and 52 kDa proteins of microsomal membrane fraction and 52 kDa protein of cytosol fraction were strongly stained on Western-blot of SDS-PAGE by mAb 493D4 (Figure 8A). When detergent-solubilized membran proteins of the rat cerebellum were fractionated electrophoretically by an AEP-550 Biophoresis III system (see Materials and methods), both mAb 493D4 and mAb SVP38 (against synaptophysin) stained the 38 kDa protein in the same fractions with a highly similar pattern (Figure 8B and C), suggesting that the 38 kDa protein may be synaptophysin. The 38 kDa and 52 kDa protein-associated determinant for mAb 493D4 was alkaline-labile and destroyed by sialidase treatment (data not shown).

No immunologically positive spots could be detected on Western-blot when mAb P-path was used instead of mAb 493D4 (data not shown).

Differential localization of 9-*O*-acetylated sialoglycan in the cerebellar cortex

To examine the cellular localization of glycoconjugate antigens carrying 9-*O*-acetylated sialic acid in brain, we performed an immunohistochemical analysis of the rat cerebellum using P-path and mAbs 493D4. In cryocut sections of the cerebellum stained with mAb P-path, the immunoreactivity was associated exclusively with the Purkinje cell dendrites at the molecular layer and their cell bodies (Figure. 9A). The labelling appeared as a punctuated pattern associated with the membranes of dendrites and cell bodies except for nuclei. Similar but more sparse staining patterns for P-path antigens were observed in the mouse cerebellum [16]. By contrast, the immunoreactivity of mAb 493D4 was detected on both parallel fibres in the molecular layer and cerebellar glomeruli in the granule cell layer (Figure 9B). Cerebellar Purkinje cells an interneurons in the molecular layer were not stained by this mAb. These staining profiles were quite similar to those of rabphilin-3A and rab 3A, synaptic vesicle associated proteins [38], and coincided with the fact that mAb 493 recognized synaptophysin on immunoblot as demonstrated above. Moreover, treatment of cerebellar tissues with a chloroform/methanol mixture did not cause significant changes in immunorectivity of mAb 493D4 with the tissues, indicating that the antibody reacted exclusively with synaptophysin but not *O*-Ac-gangliosides (data not shown). The present immunohistochemical analyses defined differential distributions of 9-*O*-acetylated sialoglycans reactive with mAb P-path or 493D4 in the central nervous system.

Discussion

We have developed a procedure using preparative Q-Sepharose chromatography to purify extremely minor

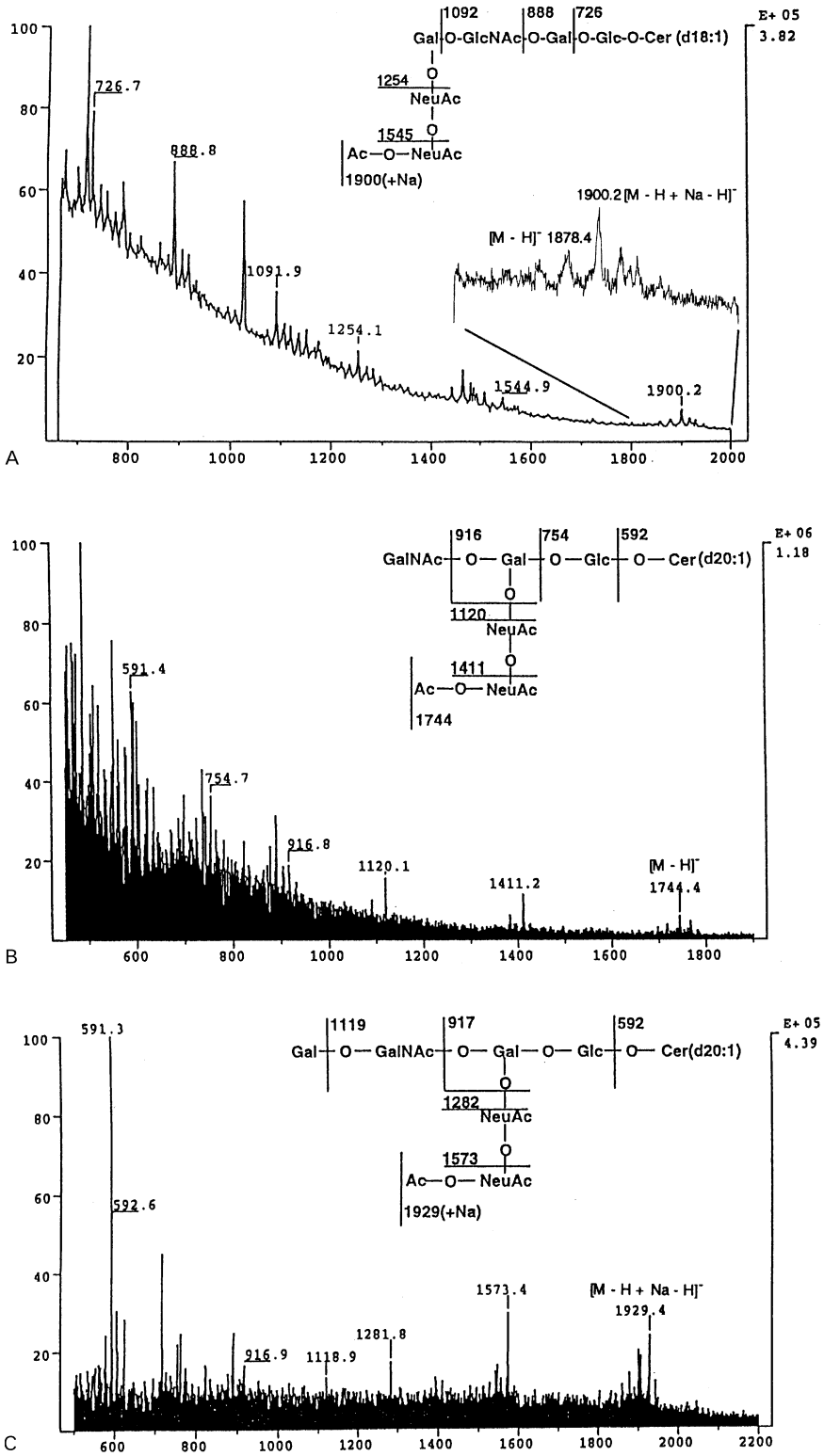

Figure 5. Negative FAB-MS spectra of X1, X2 and X3. Negative FAB-MS of X1 (A), X2 (B) and X3 (C) was carried out as described under 'Materials and methods'.

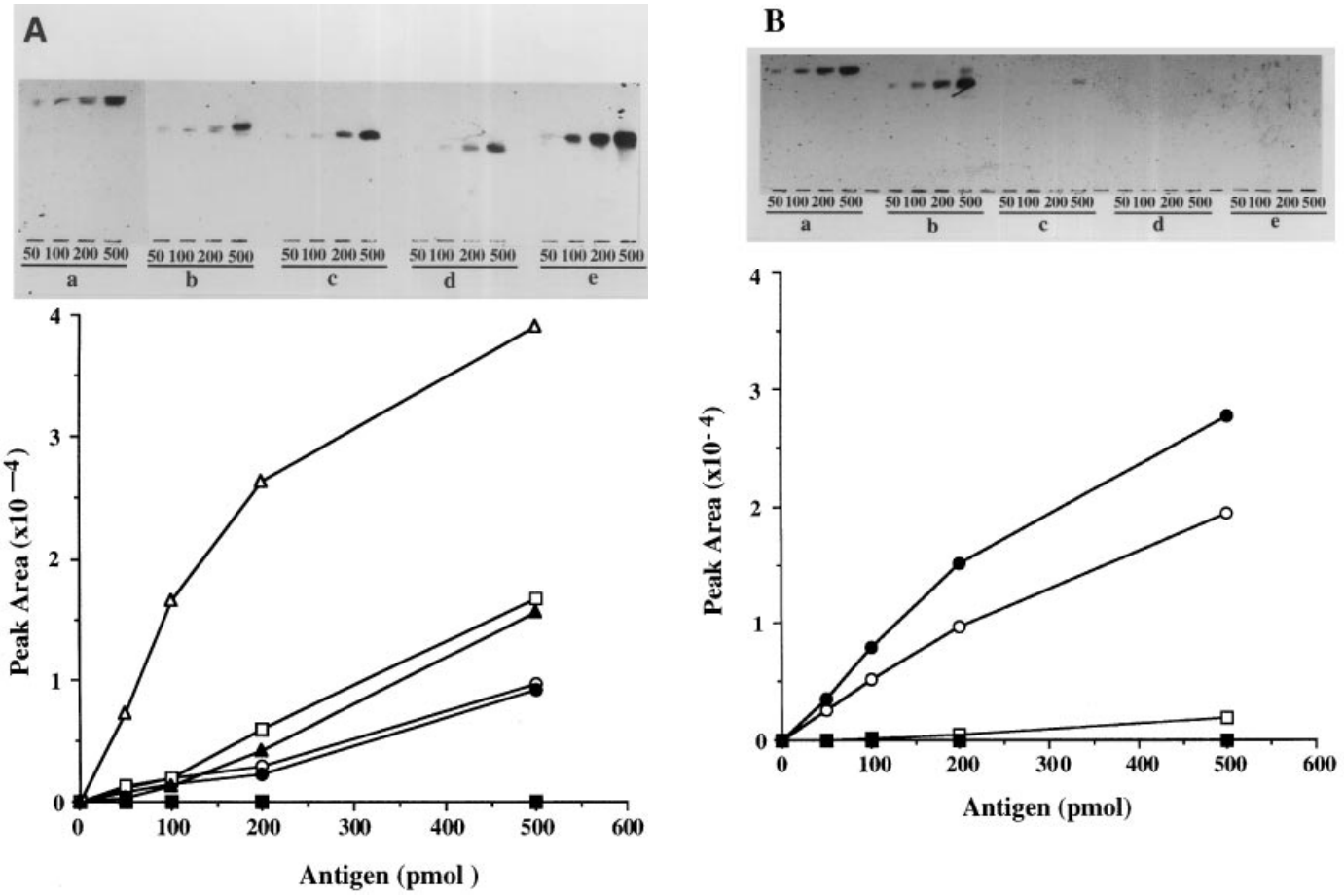

Figure 6. TLC-immunostaining of *O*-Ac-GD3, *O*-Ac-LD1, *O*-Ac-GD2, *O*-Ac-GD1b, and *O*-Ac-GT3 by mAbs 493D4 and P-path. Different amounts (50, 100, 200 and 500 pmol) of the *O*-Ac-gangliosides were separated on plastic TLC plates and immunostained with mAbs 493D4 (A) and P-path (B), respectively. The patterns of immunostained *O*-Ac-gangliosides are shown by photography and the quantitative determination with densitometer by graphs (a and ○, 9-*O*-Ac-GD3; b and ●, *O*-Ac-LD1; c and □, *O*-Ac-GD2; d and ▲, *O*-Ac-GD1b; e and △, 9-*O*-Ac-GT3). Other regular gangliosides (■) were also analysed by TLC immunostaining in the same way.

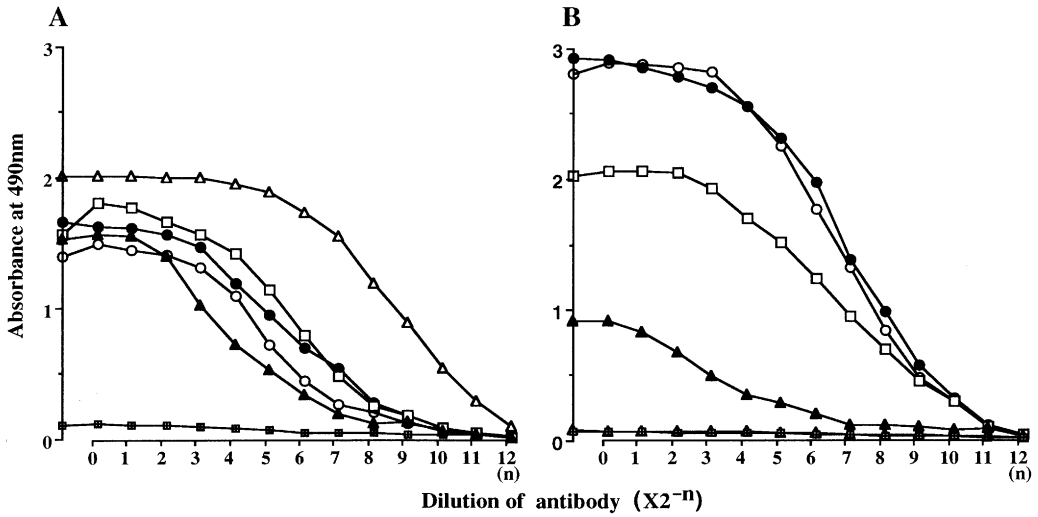

Figure 7. Reactivity of *O*-Ac-GD3, *O*-Ac-LD1, *O*-Ac-GD2, *O*-Ac-GD1b and *O*-Ac-GT3 with mAbs 493D4 and P- path in ELISA. Purified 9-*O*-Ac-GD3 (○), *O*-Ac-LD1 (●), *O*-Ac-GD2 (□), *O*-Ac-GD1b (▲), 9-*O*-Ac-GT3 (△) and other regular gangliosides (■) were coated on 96-well polystyrene plate (10 pmol per well). ELISA was performed with mAb 493 D4 (A) or P-path (B) serially diluted by the method described under experimental procedures.

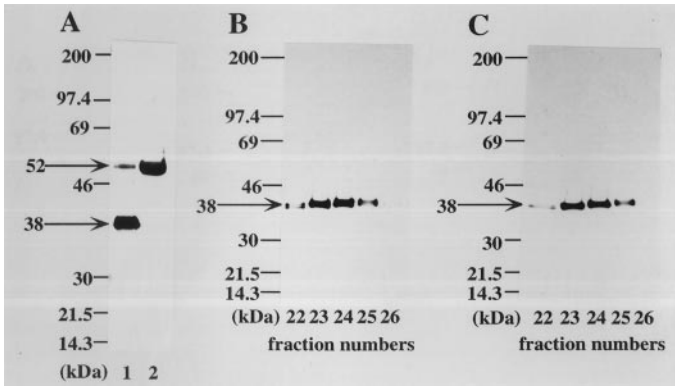

Figure 8. Western blotting of rat cerebellar proteins with mAb 493D4 and SVP38. Proteins of cerebellar microsomal membranes (1) and cytosol fractions (2) of Wistar rats (8–12 weeks old) (A) and fractions 22–26 of cerebellar proteins separated by AEP-550 Biophorosis III system (Atto, Japan) (B, C) were applied to SDS-gel electrophoresis and transferred to nitrocellulose membranes. In A and B, the proteins were detected with mAb 493D4 and in C, mAb SVP38.

gangliosides from complex mixtures of bovine brain gangliosides [23]. We purified several minor gangliosides, determined their chemical structures and examined expression profile in CNS. It is noteworthy that some minor gangliosides are expressed in a cell type specific and developmentally regulated manner [36–40]. For example, Chol-1α gangliosides are localized on cholinergic neurons [41–43]. We have observed that 14 day fetal rat cerebral cortex highly expresses an antigen that may be GT3 ganglioside containing *O*-acetyl sialic acid [18]. To directly detect the presence of this antigen in tissue a mAb 493D4 was established which has high affinity to 9-*O*-Ac-GT3. This mAb can also recognize a number of other gangliosides containing *O*-acetyl sialic acid, but not those major and regular gangliosides containing non-*O*-acetyl sialic acid. Among documented antibodies, mAb JONES [44] and mAb 27A are specific for 9-*O*-Ac-GD3 [45]. D1.1 [15] (data not shown) and P-path [16] mAbs recognize mainly 9-*O*-Ac-gangliosides in the disialoganglioside fractions of Q-Sepharose chromatography. In contrast, mAb 493D4 reacts with *O*-acetyl gangliosides present in fractions of Q-Sepharose chromatography from disialogangliosides to tetrasialogangliosides.

In the present study, four major *O*-Ac-gangliosides in disialoganglioside fractions of Q-sepharose chromatography were purified from adult bovine brains. Although we could not determine the exact position of *O*-acetyl group because of their low yields, we identified them as

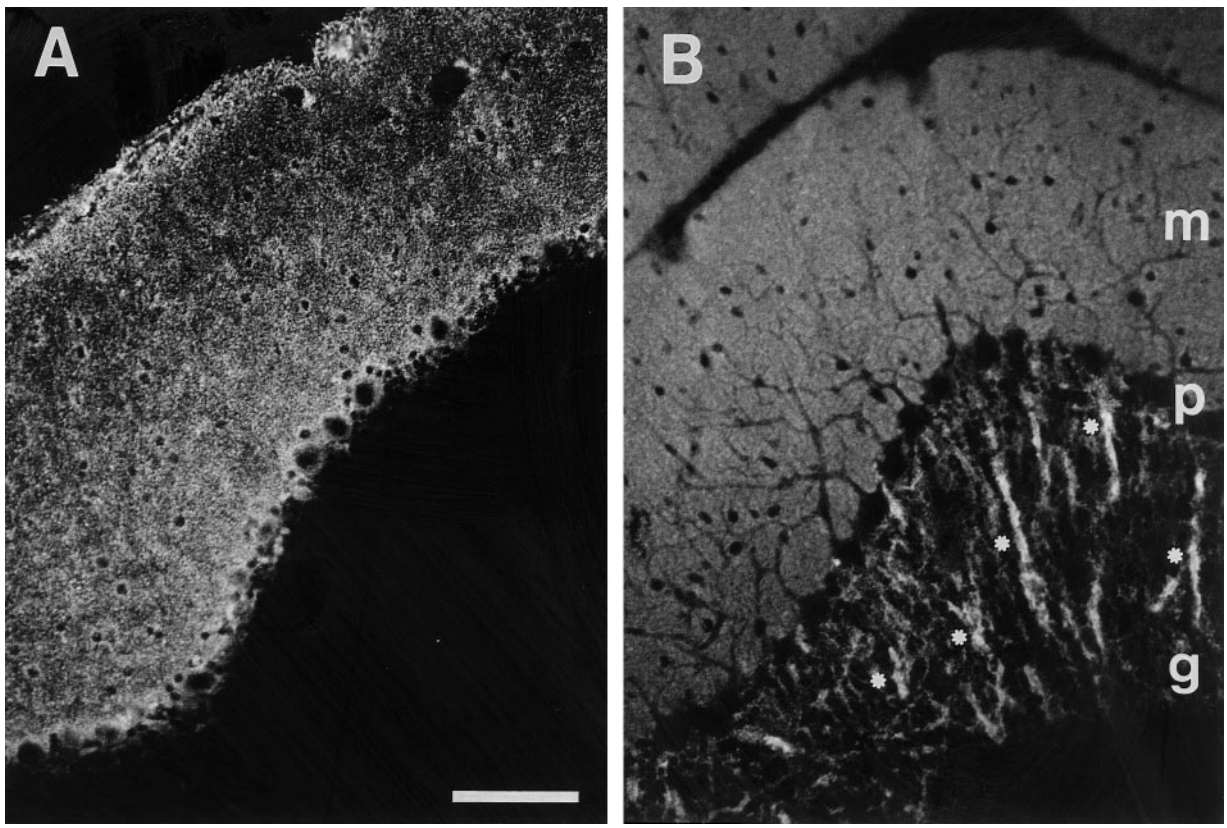

Figure 9. Immunofluorescence localization of 9-*O*-acetylated sialoglycans in rat cerebellum. Cryosections of the rat cerebellum were processed for indirect immunofluorescence analysis with mAb P-path (A) and 493D4 (B). Asterisks in (B) point to cerebellar glomeruli labelled with mAb 493D4 in the granule cell layer. Abbreviations: m, molecular layer, p, Purkinje cell layer; g, granule cell layer. Bar in (A) indicates 100 μm.

O-Ac-GD3, *O*-Ac-LD1, *O*-Ac-GD2 and *O*-Ac-GD1b with the assistance of TLC-immunostaining using mAb 493D4. Chou *et al.* [17] reported the presence of *O*-Ac-LD1 in mouse cerebellum as indicated by base treatment and immunostaining with antibodies against *O*-Ac-gangliosides. In this study, the *O*-Ac-LD1 was purified and the structure was analysed by FAB-MS. Our data exclude the possibility that this composition of ganglioside is a lactone form of LDI and indicate the presence of *O*-Ac-LD1 in adult bovine brain.

This is the first report of the detection and structural characterization of *O*-Ac-GD1b. In all four *O*-Ac-disialogangliosides, *O*-Ac-GD3, *O*-Ac-LD1, *O*-Ac-GD2 and *O*-Ac-GD1b, the *O*-acetylation of sialic acid occurred at the terminal α2-8-linked sialic acid.

In addition to *O*-Ac-disialogangliosides so far characterized, the presence of *O*-Ac-GT1b and *O*-Ac-GQ1b has been reported in the CNS tissues. These two polysialylated gangliosides were possibly formed through sialylation of *O*-Ac-GD1b by sialyltransferase(s). The *O*-acetyl ester of *O*-Ac-GT3 is also located on the terminal α2-8-linked sialic acid rather than the internal α2-8-linked trisialyl residue [18, 35]. Thus it is suggested that *O*-acetyl transferase(s) may *O*-acetylate the terminal α2-8-linked sialic acid of the tandem disialyl and trisialyl residue.

The expression of *O*-Ac-gangliosides has been documented as being regulated developmentally for review see [48]. The C-series *O*-Ac-gangliosides are extremely interesting. In rat and mouse, *O*-Ac-GDE and *O*-Ac-GT3 were highly expressed in fetal cerebral cortex, but decreased significantly after birth to a low level in the adult [16, 27, Figure 1]. In adult bovine brain, *O*-Ac-GD3 and *O*-Ac-GT3 could be detected only after enrichment by purification procedures. The mAb JONES, which was more specific to *O*-Ac-GD3, showed a striking dorsal-ventral gradient of expression across the developing retina [44, 49, 50]. The transient expression of *O*-Ac-GD3 was also observed in the development of other organs, for example, rat kidney [45, 51]. In the adult, C-series *O*-Ac-gangliosides are only expressed in the restricted tissues or cell types, for example, the podocytes of the glomeruli of the rat kidney [51] and bovine milk products [52]. However, the *O*-Ac-GD3 is expressed in a considerable amount in human melanomas [15, 53, 54]. *O*-Ac-GD3 may have a significance in the immunotherapy of human melanoma [55].

The Western blotting of cerebellar proteins and the immunohistochemical analysis with mAb 493D4 and mAb SVP38 showed quite similar profiles suggesting that the 38 kDa protein recognized by mAb 493D4 might be synaptophysin. The synaptophysin has been reported to be a 38 kDa protein with *N*-glycosylation [56]. However, the physiological function of synaptophysin is as yet unclear [57]. The reactivity of synaptophysin with mAb 493D4 and its sensitivity to alkaline treatment and sialidase hydrolysis indicates that synaptophysin may contain *O*-Ac-sialic acid. Treatment of glycoproteins with endoglycoceramidase did not destroy the reactivity of the glycoprotein with mAb 493D4 (data not shown), suggesting that the determinant on the glycoprotein is not an associated *O*-Ac-gangliosides but synaptophysin itself. These results show that this monoclonal antibody is quite useful for sensitive detection of both *O*-acetylated gangliosides and glycoproteins with *O*-acetylated sialic acids.

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