

Discovery of an α 2,9-PolyNeu5Ac Glycoprotein in C-1300 Murine Neuroblastoma (Clone NB41A3)*

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1 Table and 5 Figures

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SUMMARY

α 2,8-PolyNeu5Ac is expressed on NCAM during embryogenesis, and also re-expressed on certain tumors. PolyNeu5Ac is therefore an oncodevelopmental antigen, has important regulatory effects on the adhesive and migratory behavior of neural cells, and is thus crucial to synaptic plasticity. Until now, α 2,9-polyNeu5Ac, a linkage isomer of α 2,8-polyNeu5Ac, has long been thought to occur only in capsules of neuroinvasive *Neisseria meningitidis* group C bacteria. Here we report the unexpected discovery of α 2,9-polyNeu5Ac in a new cell adhesion-related glycoprotein on the membrane of C-1300 murine neuroblastoma cells (clone NB41A3). We also report the expression of α 2,9-polyNeu5Ac was affected by cell growth and retinoic acid-induced differentiation. Occurrence of the linkage isomer of α 2,8-polyNeu5Ac has left unrecognized by conventional methods using biological diagnostic probes for α 2,8-polyNeu5Ac. Thus, our discovery may change contemporary views of polysialobiology and pathology and open new avenues for the development of anti-neural tumor drugs.

INTRODUCTION

Polysialic acid (PSA) is a unique cell surface homopolymer that is expressed by clinically important serogroups B and C *Neisseria meningitidis* in α 2,8- and α 2,9-linked isomers respectively (1) (Fig. 1). These molecules encapsulate the organisms, which are responsible for dominant portion of all cases of meningococcal meningitis, and are critical to their pathogenesis. One of these linkage isomers, α 2,8-polyNeu5Ac, which also forms the capsule of neuroinvasive *E. coli* K1, is poorly immunogenic (2), probably due to the fact that it is a

self-antigen occurring as an oncodevelopmentally regulated mammalian antigen in the neural cell adhesion molecule (NCAM) and certain tumors. In contrast α 2,9-polyNeu5Ac is immunogenic enough to be used as a vaccine against meningitis caused by group C meningococci and is expressed by these organisms in both *O*-acetylated and *O*-deacetylated forms (2). Since the 1980s, the molecular biology, structural biology, physiological and pathophysiological functions of α 2,8-polyNeu5Ac on NCAM, have already been the subject of major reviews (3-7). Usually detection and identification of α 2,8-PSA in NCAM studies are made using monoclonal antibodies specific to α 2,8-polyNeu5Ac. Although such immunological reagents are useful for studies of developmentally regulated dynamic expression of α 2,8-polyNeu5Ac, they cannot be used to define DP, or to detect either changes in the distribution of glycoforms differing in DP, or the presence of PSA differing in sequences or inter-residue linkages. Prior to the identification of α 2,8-polyNeu5Ac-NCAM, we discovered α 2,8-polyNeu5Gc by chemical and biochemical methods which was the first example of animal PSA (8,9). Later we also unveiled divergent forms of PSA such as an α 2,8-poly(Neu5Ac, Neu5Gc) copolymer, α 2,8-polyKDN, and α 2,5-*Oglycolyl*-linked oligo/polyNeu5Gc (10,11). These PSA-glycoproteins were shown to play biological functions in fertilization and early embryogenesis, and until now, except for the last example found in sea urchin eggs, all inter-residue linkages in PSA chains found in vertebrate sources were α 2,8.

Insert Figure 1 near here.

In view of the fact that the presence of a large number of glycoforms for a single functional protein is important for the delicate and fine regulation of 'protein-based

functions' in higher animals, we thought that the NCAM of neuroblastoma cell lines would be an excellent model system to demonstrate how the DP of PSA chains and the structure of the core *N*-glycan, are modulating the adhesive and migratory behavior of neural cells. To define DP, and the change in distribution of glycoforms differing in DP, we developed an ultrasensitive fluorescent labeling method using a fluorogenic probe DMB (1,2-diamino-4,5-methylenedioxybenzene), which selectively reacts with any type of oligo/poly- α -ulosonic acids to introduce a strong fluorescent group at the reducing termini of these chains (DMB/HPLC-FD method) (12-17). This method permits us to achieve the same level of sensitivity as that attained by using antibodies.

Here we report the serendipitous discovery of α 2,9-linked polyNeu5Ac in a new cell adhesion-related glycoprotein on the membrane of C-1300 murine neuroblastoma cells (clone NB41A3). α 2,9-PolyNeu5Ac has long been thought, until now, to be a homopolymer only found as capsular polysaccharide of *N. meningitidis* group C bacteria.

EXPERIMENTAL PROCEDURES

Antibodies Used—(Sources for rat anti-mouse embryonic PSA-NCAM (CD56) mAb (12F8, IgM) and anti-rat NCAM mAb (5B8, IgG) were given previously (16). Serum pooled mouse polyclonal antisera against de-*O*-acetyl GCMP-tetanus toxoid conjugate Ab against α 2,9-polyNeu5Ac was prepared as described previously (18).

Cell Lines and Their Culture, Growth, and Harvest—(C-1300 murine neuroblastoma (clone NB41A3) cell line was obtained from American Type Culture Collection (ATCC), and actually purchased from Food Industry Research and Development Institute, Hsinchu,

Taiwan. Cells were cultured in Ham's F-10 medium, supplemented with 15% horse serum, 2.5% FBS and kept in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were transferred upon reaching confluence and those within 5 passages after purchasing from ATCC were used for analysis, and harvested by trypsination after washing with phosphate-buffered saline. The cells were collected by centrifugation at 3,000 rpm for 10 min. To examine growth-stage dependent change of polysialylation, NB41A3 cells were inoculated at the concentration of 5x10⁵/ml and were collected at different stages of growth (5-, 6-, and 7-day). The same amount of cells (250 mg wet weight) was subjected to PSA analysis to facilitate direct comparison. The cells harvested at different stages of growth and differentiation were examined for their morphological features under a phase-contrast microscope (Olympus, IX70, Olympus, Tokyo). All-trans-retinoic acid (RA; Sigma chemical Co., St. Louis, MO) was used at a final concentration of 10 μM in the culture medium.

Analysis of PSA and Total Neu5Ac in the Solubilized Glycoproteins—(PSA and total Neu5Ac in the glycoproteins solubilized with Triton X-100 were analyzed by DMB/HPLC-FD method (16). Peaks of oligo/polysialic acids were monitored with a fluorescence detector set at 372 nm for excitation and 456 nm for emission. Because each PSA can acquire only one DMB molecule, the total peak area for DMB-oligo/polysialic acid peaks (DP > 4) represents the total number of PSA chains and used as a value comparing the level of PSA in the cells.

ELISA was also applied to evaluate quantitatively the PSA expression level in the cells

using mouse polyclonal antisera against de-*O*-acetylated GCMP (α 2,9-polyNeu5Ac)-tetanus toxoid conjugate Ab and alkaline phosphatase-conjugated goat antimouse IgM, as the primary and secondary antibody, respectively. The absorbance at 405 nm (*p*-nitrophenol) of the reaction mixture represents the amount of PSA epitope. All procedures used in SDS-PAGE and Western blot were essentially similar to those previously described (16).

Isolation of α 2,9-PolyNeu5Ac-glycopeptides from NB41A3 Cells—(The membrane fraction of NB41A3 cells was first delipidated with a mixture of chloroform-methanol-10 mM Tris-HCl (pH 8.0), 4 : 8 : 3 (v/v). The lipidated residue was washed with cold 80% ethanol, suspended in 2 volumes of 0.1 M Tris-HCl (pH 8.0) containing 10 mM CaCl₂ and incubated with *Streptomyces griseus* non-specific proteinase (Sigma, 1 mg for 1 g of wet cells) for 3 days at 37°C under a toluene atmosphere. The incubation mixture was clarified by centrifugation and applied to a DEAE-Toyopearl 650M (Tosoh, Tokyo) column (1.2 × 8 cm). Compounds bound to the column were eluted stepwisely with 4 column volume each of 0.1, 0.2 and 0.4 M NaCl in 10 mM Tris-HCl buffer (pH 8.0). PSA was found only in the 0.4 M NaCl fraction.

Periodate Oxidation/Borohydride Reduction/Mild Acid Hydrolysis for C7/C9 Analysis—(Each sample containing 200 ng Neu5Ac of NB41A3 α 2,9-polyNeu5Ac-glycopeptides, authentic α 2,8-polyNeu5Ac and α 2,9-polyNeu5Ac was dissolved in 20 μ l of 0.1 M NaOH and left at 37°C for 4 h to remove possible *O*-acetyl group. To a 10 μ l aliquot of each sample solution (pH was adjusted to 5.5 with 1 M acetic acid), 10 μ l of water and 10 μ l of 0.15 M sodium metaperiodate were added and incubated at 4°C. After 24, 48 and 96 h, reaction was stopped by adding 5 μ l of 3% glycerol. Then, 10 μ l of freshly prepared 0.04 M sodium

borohydride in 0.01 M sodium hydroxide was added and the mixtures were kept at room temperature for 30 min. Excess borohydride was destroyed with 10 μ l of 0.2 M acetic acid. The products were hydrolyzed in 0.1 M trifluoroacetic acid at 80°C for 4 h, derivatized with DMB for 2.5 h at 55°C and analyzed for C7 and C9 nonulosonates by HPLC/FD as described elsewhere (17).

RESULTS AND DISCUSSION

The expression of PSA and NCAM in the solubilized membrane fractions was analyzed for the murine neuroblastoma, by DMB/HPLC-FD and by Western blot analysis using anti- α 2,8-polyNeu5Ac mAb (12F8) and anti-NCAM mAbs (5B8 and VIN-IS-53). For 5 cell lines (4 different neuroblastoma cell lines (IMR-32, SK-N-MC, SK-N-SH and Neuro-2A) and one pheochromocytoma cell line (PC-12) examined in our previous report, these two diagnostic tests gave consistent results (16): IMR-32 and PC-12, PSA-positive/NCAM-positive; neuro-2A, PSA-negative/NCAM-positive. However, conflicting results were obtained regarding PSA expression in NB41A3 cells that were found to be NCAM immunoassay negative with anti-NCAM mAb (Table I). NB41A3 cells were α 2,8-polyNeu5Ac-negative on Western blots using 12F8 (Fig. 2C) but PSA-positive on the DMB/HPLC-FD analysis (Fig. 2A and B). PSA residues in NB41A3 cells were also shown to be completely hydrolyzed to unsubstituted Neu5Ac monomer by treatment with *Arthrobacter ureafaciens* sialidase. These surprising findings provided a hint about the possible occurrence of a linkage isomer of α 2,8-polyNeu5Ac, i.e. α 2,9-polyNeu5Ac, in NB413A cells, which has long been thought, until now, to be a homopolymer only found as capsular

polysaccharide of *N. meningitidis* group C bacteria (1). This prompted us to investigate the PSA structure of the NB413A membrane-bound glycoprotein.

Insert Table 1 near here.

Usually detection and identification of α 2,8-polyNeu5Ac are made using monoclonal antibodies specific to this form of polymer. However, it should be emphasized here that if we had used only immunochemical criteria for examining PSA expression, the present finding of α 2,9-polyNeu5Ac in NB413A cells would have definitely been missed. This emphasizes the importance of having additional analytical methods to detect PSA. Therefore we recommend that the DMB/HPLC-FD method should be routinely used as a screening test for PSA chains in biological samples. Confirmation of the presence of novel α 2,9-polyNeu5Ac in the NB413A-derived glycoprotein was obtained by Western blot analysis using a mouse polyclonal antibody induced by a protein conjugate of the *O*-deacetylated group C meningococcal polysaccharide (18). The cell membrane extract of NB413A PSA-gp revealed a broad anti- α 2,9-polyNeu5Ac positive band on SDS-PAGE, centering at an apparent molecular size of approximately 150,000, because of the presence of numerous glycoforms differing in the DP of their PSA chain(s) (Fig. 2D).

Insert Figure 2 near here.

Structural evidence for the presence of α 2,9-polyNeu5Ac was also obtained from the results of the analysis of C7 and C9 ulosonates obtained after periodate oxidation of the sample, followed by borohydride reduction and mild acid hydrolysis. Oxidation of the α 2,9-polyNeu5Ac chain with periodate resulted in its depolymerization and the destruction of all Neu5Ac residues to form their C7 analogs. The time-course of reaction was examined for

three samples, NB41A3 PSA-glycopeptide, authentic α 2,9-polyNeu5Ac, and α 2,8-polyNeu5Ac, by monitoring C7 and C9 monomers using the DMB/HPLC-FD method (Fig. 3A-C). The reaction proceeded slowly (19), and the NB41A3 PSA-glycopeptide and authentic α 2,9-polyNeu5Ac samples gave the C7 analog of Neu5Ac as the only product (Fig. 3B and C) whereas α 2,8-polyNeu5Ac remained unoxidized and gave C9 Neu5Ac (Fig. 3A). These results prove that the interresidue linkage of polyNeu5Ac in NB41A3 PSA-gp is α 2,9.

Insert Figure 3 near here.

Supporting evidence for the presence of α 2,9-polyNeu5Ac was obtained, using the DMB/HPLC-FD method, by co-injecting NB41A3-derived PSA-gp with each of the authentic samples, which resulted in a subtle retention time difference between homologous α 2,8-oligo/polyNeu5Ac and α 2,9-oligo/polyNeu5Ac labeled with fluorescent quinoxalinone at their reducing termini. The results are reproduced in Fig. 3D and E, and, as expected, NB41A3 PSA exhibited a simple profile when cochromatographed with authentic standard α 2,9-polyNeu5Ac (Fig. 3D) whilst, when mixed with α 2,8-polyNeu5Ac, a series of doublet peaks was seen on the co-chromatogram (Fig. 3E).

Interestingly, while α 2,9-polyNeu5Ac has been known for well over a couple of decades as a constituent of the capsule of *N. meningitidis* group C (1), its occurrence in animal glycoproteins had yet to be identified. Indeed, to the best of our knowledge, there has been only one report concerning the finding of an α 2,9-diNeu5Ac residue, Neu5Ac α 2 \rightarrow 9Neu5Ac α 2 \rightarrow , in human ovarian teratocarcinoma (PA-) cells (20), but we failed to detect polySi PA-1 cells by the DMB/HPLC-FD method. α 2,8-PolyNeu5Ac is almost exclusively

associated with NCAM in animal, where it can modulate the homotypic adhesive properties of this member of the immunoglobulin superfamily. Based on its immunoreactivity, the newly identified α 2,9-PSA in NB41A3 neuroblastoma cells is expressed on a distinct glycoprotein species other than α 2,8-polyNeu5Ac-bearing NCAM, but its biological function has yet to be defined. However by analogy these two cell surface molecules are probably functionally equivalent, α 2,9-polyNeu5Ac playing a similar role to α 2,8-polyNeu5Ac as a cell adhesion regulatory molecule during cellular development and differentiation. This can be indicated by the observed change of α 2,9-polyNeu5Ac expression profiles with culture time in the presence or absence of RA as determined by the DMB/HPLC-FD method (Fig. 4), and this can be deduced from coordinated behavioral changes in both the expression level of PSA and cellular adhesive nature of NB41A3 cells (Fig. 5): The growth-stage dependent change in PSA expression was evaluated by two methods; one as the total peak areas in DMB/HPL-FD, and the other as the PSA level estimated by ELISA. The results in the absence and presence of RA respectively, are shown in Fig. 5A and B. The amount of total protein-bound Neu5Ac (ng/mg of wet cells) was also included. In the absence of RA the PSA level increased with cell culture time and reached the maximum values at confluence (day 7) (Fig. 5A). By contrast, in the presence of RA the level of PSA was high on the day 5 and gradually decreased during cell growth until it reached the lowest value at day 7 (Fig. 5B). We were not able to determine the PSA expression level at stages before the day 5 post-inoculation time, simply because the cells grew so slowly that not enough of them could be harvested.

Insert Figures 4 and 5 near here.

Cellular adhesive properties were examined in cultured NB41A3 cells. When NB41A3 cells were incubated in the absence of RA, they formed aggregates at the initial stages of proliferation (day 5 post-inoculation incubation time; Fig. 5C) and underwent dissociation of cell aggregates at later stages (day 7; Fig. 5C). These changes were associated with a parallel increase in the level of PSA per unit weight of wet cells (Fig. 5A). In sharp contrast to these observations, in the presence of 10 μ M RA, NB41A3 cells did not form aggregates at the initial stages of incubation (day 5; Fig. 5D) and a majority of the cells differentiated to a neuronal phenotype. They extended long neuritic processes during incubation, and finally on day 7 of culture a large portion of the cells formed large aggregates (Fig. 5D). Similar morphological changes were correlated with changes in the expression level of α 2,8-polyNeu5Ac chains in human neuroblastoma IMR-32 cells and pheochromocytoma PC-12 cells during growth in the absence and presence of RA (16). Since cell aggregation can be correlated with change in the expression level of PSA, the newly identified α 2,9-polyNeu5Ac-expressing glycoprotein may be considered as a molecule closely related to NCAM. α 2,8-PolyNeu5Ac chain was suggested to be associated with the voltage-sensitive Na-channels in adult rat brain merely by immunohistochemical and Western blot data (21). However, to our knowledge, there is no report showing that expression of PSA on Na-channels affects cell adhesion. Identification and structural determination of the new cell adhesion molecule is important and is underway. In addition we are pursuing the identification of a specific polysialyltransferase(s) (PST) that catalyze formation of α 2,9-polyNeu5Ac in NB41A3 cells.

Over the past decade, there has been a rapid advance in the knowledge of biochemistry,

molecular biology, and genetics of the PST genes and enzyme proteins. The discovery of a novel α 2,9-PSA-glycoprotein on NB41A3 cells will open up a new avenue of research to characterize the biosynthesis of α 2,9-polyNeu5Ac by identifying its associated enzyme(s), PST, and determining how this unique glycosylation influences the fate and function of this putative new cell adhesion glycoprotein on neuroblastoma cells.

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Footnotes

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Figure Legends

FIG. 1. **Depicts the two linkage isomers of polyNeu5Ac** i.e., (A) α 2,9-polyNeu5Ac which was only known to occur in the *Neisseria meningitidis* group C bacterial capsular polysaccharide until it was discovered in this study to be expressed by a C-1300 murine neuroblastoma clone (NB41A3), and (B) α 2,8-polyNeu5Ac which is expressed by *N. meningitidis* group B bacteria, the embryonic form of NCAM of chicken, *Xenopus laevis*, mammals, including neuroblastoma cells such as human neuroblastoma cell lines IMR-32 and CHP-134.

FIG. 2. **Detection of the PSA glycotope in NB41A3 cells as examined by the DMB/HPLC-FD method.** (A) A membrane fraction obtained from 100 mg of NB41A3 cells was directly subjected to analysis. Though the presence of PSA chains was not detected when examined using an α 2,8-polyNeu5Ac-specific monoclonal antibody (12F8), the DMB/HPLC-FD method revealed a series of quinoxalinone-labelled oligo/polyNeu5Ac formed during derivatization with DMB. (B) The initial evidence for the presence of a membrane-associated PSA glycotope other than α 2,8-polyNeu5Ac is indicated by the profile in (A) and was confirmed by the analysis of a partially purified sample of PSA-glycopeptide eluted with 0.4 M NaCl-10 mM Tris-HCl from a DEAE-Toyopearl 650M column. (C and D) An NB41A3 cell membrane fraction was analyzed by polyacrylamide gel electrophoresis (SDS-PAGE) and electro-blotted onto PVDF membranes. Immunochemical evidence for the occurrence of α 2,9-polyNeu5Ac epitope in NB41A3 cell membrane-bound PSA-gp was revealed by Western blot analysis. *Lane 1*, marker; *lane 2*, NB41A3 cell-

derived PSA-gp; *lane 3*, Day 6 embryonic chicken brain NCAM; *lane 4*, Day 14 embryonic chicken brain NCAM; *lane 5*, Day 20 embryonic chicken NCAM; *lane 6*, IMR-32 neuroblastoma NCAM. (c) Samples in lanes 3 through 6 all contained $\alpha 2,8$ -polyNeu5Ac and immunostained with a specific monoclonal antibody, 12F8 but NB41A3 cell-derived PSA-gp (*lane 2*) failed to stain with the anti- $\alpha 2,8$ -polyNeu5Ac mAb. (D) Western blots were developed employing the mouse polyclonal antisera against a de-*O*-Ac GCMP-tetanus toxoid conjugate vaccine [GCMP = Group C meningococcal polysaccharide ($\alpha 2,9$ -polyNeu5Ac)] with an alkaline-phosphatase-conjugated goat anti-mouse IgG + IgM (H + L) (Pierce), and anti-rat IgM (μ chain specific) (Southern Biotechnology Associates, Inc., Birmingham, AL). Color was developed using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt (Invitrogen). The polyclonal anti-GCMP was highly specific for $\alpha 2,9$ -polyNeu5Ac and unreactive with $\alpha 2,8$ -polyNeu5Ac, and only NB41A3 PSA-gp (*lane 2*) stained.

FIG. 3. Time course study of periodate oxidation/reduction/mild acid hydrolysis of a pair of authentic samples ($\alpha 2,8$ -polyNeu5Ac and $\alpha 2,9$ -polyNeu5Ac) and NB41A3 cell membrane-bound PSA-gp by monitoring the products using the DMB/HPLC-FD method. Chromatographic data were acquired at the times of periodate oxidation indicated. (A) $\alpha 2,8$ -polyNeu5Ac, the inner Neu5Ac residues are unattacked by periodate and only Neu5Ac was detected on hydrolysis following the periodate oxidation/reduction procedure; (B) $\alpha 2,9$ -polyNeu5Ac, as expected, underwent periodate oxidation to result in the destruction of the $\alpha 2,9$ -polyNeu5Ac chain to form C7-ulosonic acid upon subsequent reduction/hydrolysis. Thus,

the C9-ulosonate (Neu5Ac) gradually disappeared as the reaction slowly proceeded with simultaneous appearance and gradual increase of C7-ulosonic acid, and on prolonged periodate oxidation finally gave rise to complete conversion of C9- to C7-ulosonate; (C) To characterize the inter-residue linkages of polyNeu5Ac in NB41A3 cell-derived PSA-gp, periodate oxidation was also explored, and verification of the α 2,9-polyNeu5Ac structure was obtained by the fact that the C7-ulosonate appeared at the expense of C9-ulosonate as observed for the α 2,9-polyNeu5Ac sample (B). (D and E) Cochromatography of polyNeu5Ac in NB41A3 cell-derived PSA-gp as examined by the DMB/HPLC-FD method with reference compounds; (D) When cochromatographed with α 2,9-polyNeu5Ac, all the peaks coeluted. (E) In contrast, chromatographic profile did not coelute when cochromatographed with α 2,8-polyNeu5Ac.

FIG. 4. Changes with culture time of α 2,9-polyNeu5Ac expressed on the membrane-bound protein fraction from 250 mg of wet cells of NB41A3 as determined by the DMB/HPLC-FD method. (A) Cells were cultured in the absence of RA and harvested at (a) 5-, (b) 6- and (c) 7-day. (B) Cells were cultured in the presence of RA and similarly harvested at (a) 5-, (b) 6- and (c) 7-day.

FIG. 5. Comparison of PSA levels in NB41A3 PC-12 cells at different culture times (A) in the absence and (B) in the presence of RA at post-inoculation incubation time 5-days, 6-days and 7-days. The levels of PSA were evaluated by two different methods; (i) PSA analysis by DMB/HPLC-FD (PSA, in total area of PSA peaks) and (ii) ELISA using anti-

PSA (ELISA, in A₄₀₅ ×10). Total glycoprotein-bound Neu5Ac was also determined and expressed in ng/mg of wet cells. (**C** and **D**) Morphological changes of the cells cultured (**C**) in the absence and (**D**) in the presence of RA were examined under a phase-contrast microscope at different stages of at post-inoculation incubation time 5-days, 6-days and 7-days.

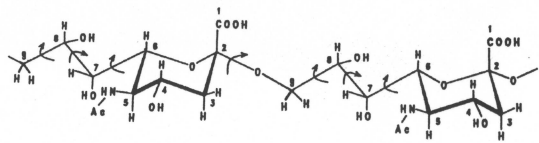
TABLE I

Diagnosis for polySia and NCAM in neuroblastoma cell lines (NB41A3 and IMR-32) and pheochromocytoma cell line (PC-12) by DMB/HPLC-FD and by Western blot analysis using anti- α 2,8-polyNeu5Ac mAb (12F8) and anti-NCAM mAb (5B8)

	polySia by DMB/HPLC -FD method	<u>Western blot</u>	
		Anti- α 2,8-polyNeu5Ac mAb, 12F8	Anti- NCAM mAb, 5B8
Mouse neuro- blastoma, NB41A3	+	-	-
Human neuro- blastoma, IMR-32	+	+	+
Rat pheochromo- cytoma, PC-12	+	+	+

Fig.1

A



B

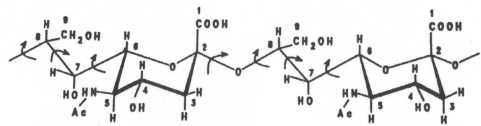


Fig.2

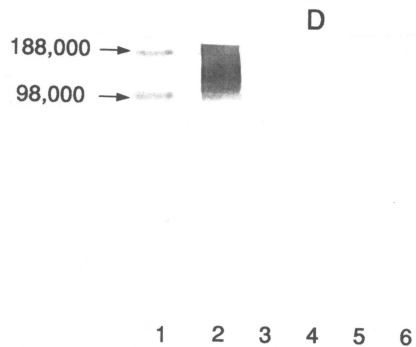
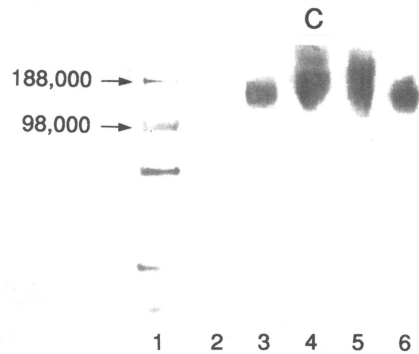
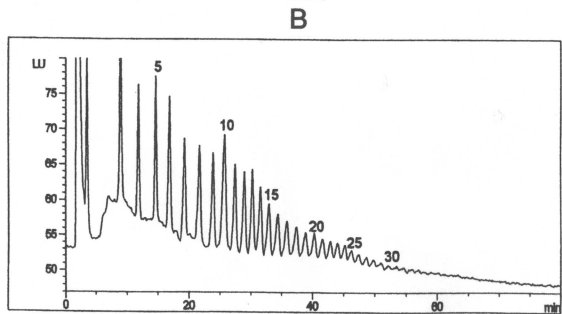
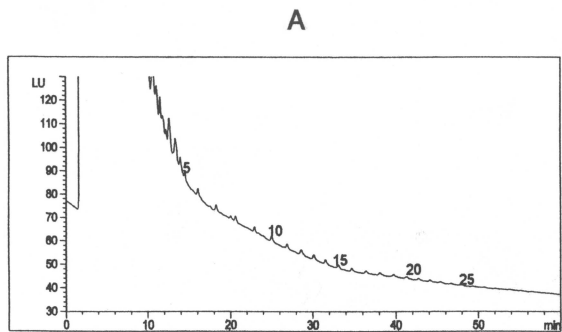


Fig.3

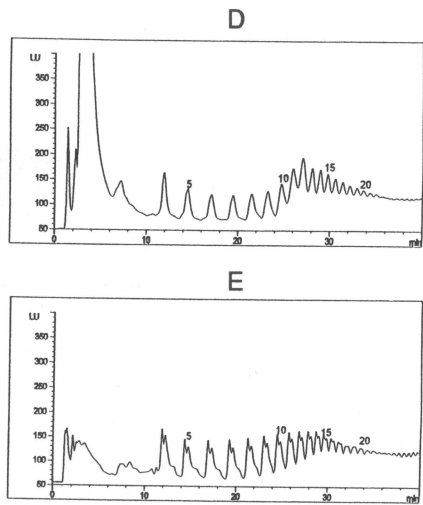
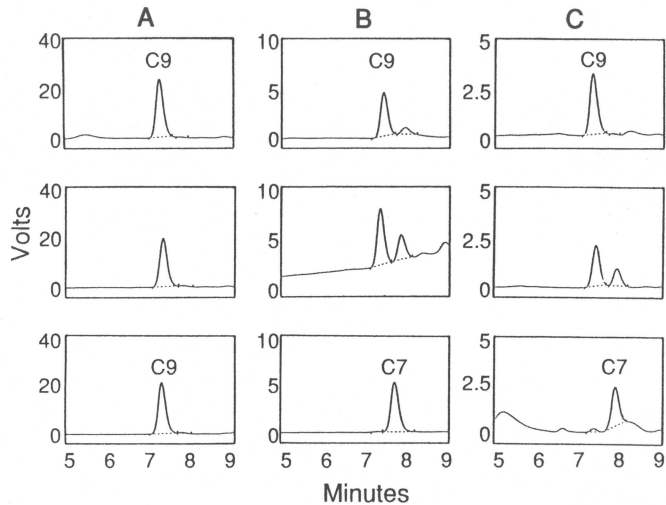
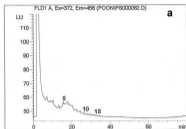


Fig.4

A



B

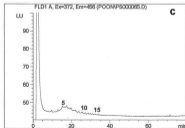
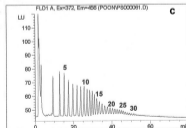
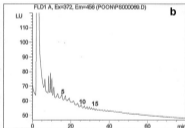
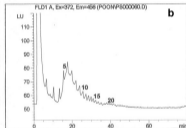
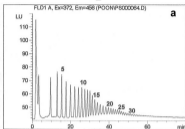
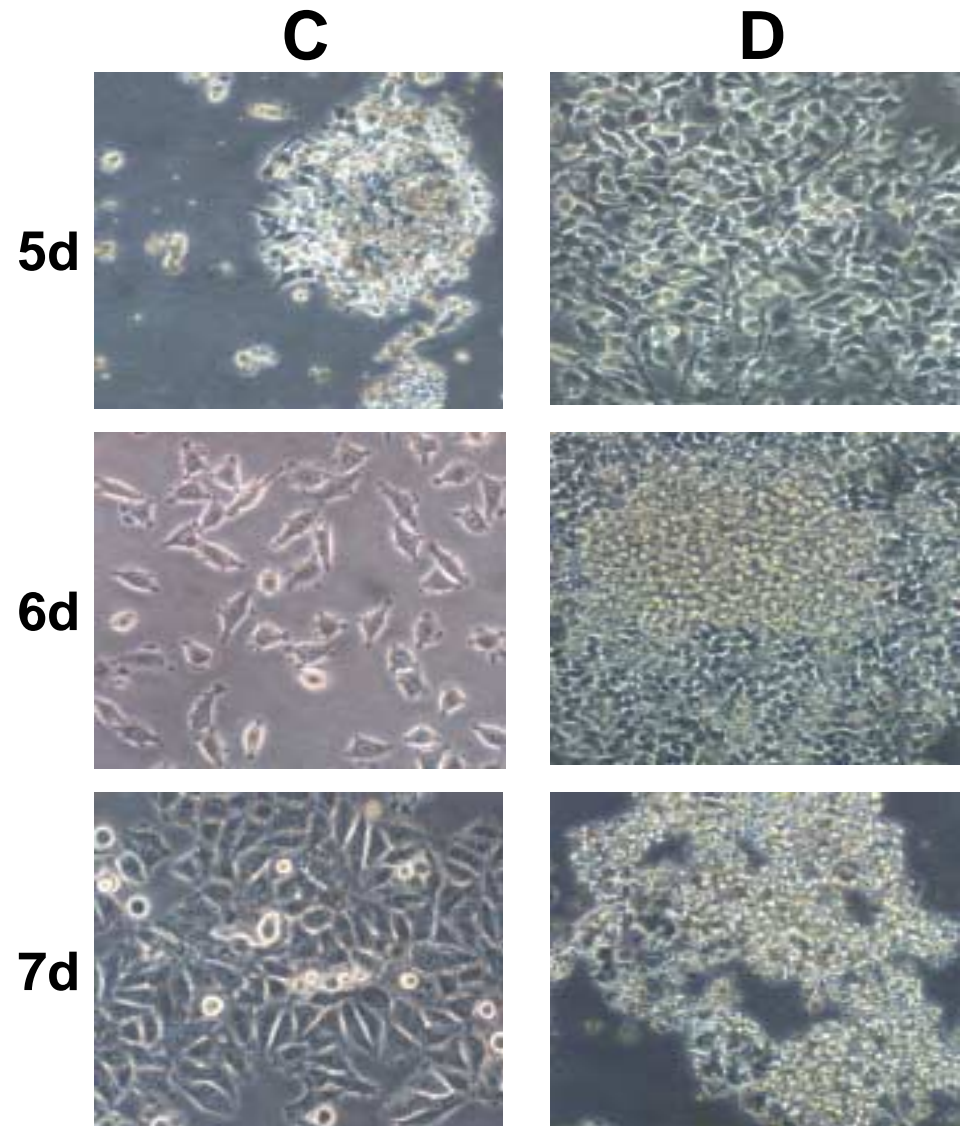
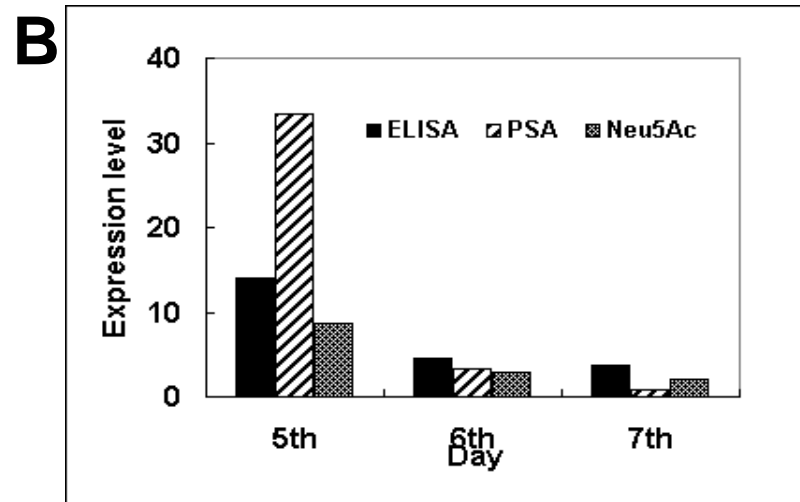
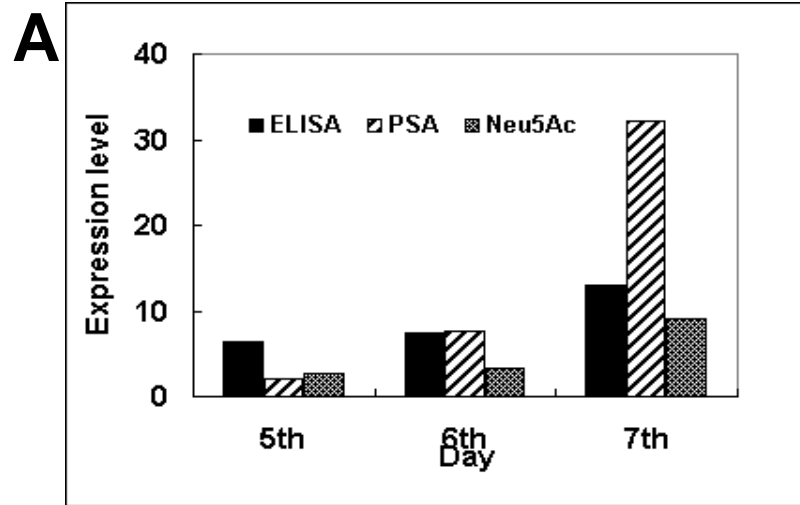


Fig.5



**Discovery of an α 2,9-PolyNeu5Ac glycoprotein in C-1300 murine neuroblastoma
(Clone NB41A3)**

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