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Sialyltransferases of Developing Rat Brain

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The activity of four different sialyltransferases acting on N- or O-linked chains of glycoproteins was studied in brains of 19 days-old embryos, 1 day-old newborns and adult rats. By using asialofetuin, fetuin and *N*-acetyllactosamine as acceptors, it has been possible to measure independently the following enzyme activities: CMP-NeuAc:Galβ1-3GalNAc α(2-3)-sialyltransferase (EC 2.4.99.4), CMP-NeuAc:Galβ1-4GlcNAc α(2-3)-sialyltransferase (EC 2.4.99.6), CMP-NeuAc:Galβ1-4GlcNAc α(2-6)-sialyltransferase (EC 2.4.99.1) and CMP-NeuAc:NeuAcα2-3Galβ1-3GalNAc α(2-6)-sialyltransferase (EC 2.4.99.7). The specific activity of the first three enzymes which act on asialylated acceptors showed a 2.6-fold decrease in a parallel manner after ontogenic development, while the activity of NeuAcα2-3Galβ1-3GalNAc α(2-6)-sialyltransferase was four times lower in adult than in embryonic brain, showing a stronger dependence on ontogenic development. Despite the higher level of sialyltransferases able to act on glycoproteins, in fetal brain these glycoproteins do not contain a higher amount of sialic acid.

Glycoproteins and glycolipids are important components of the outer plasma membrane and participate in the complex phenomena which regulate the social behavior of the cells [1]. Cell-cell interactions involve, beside the well known protein-carbohydrate binding, also carbohydrate-carbohydrate interactions: recent data indicate the existence of an homophilic binding between Le^x carbohydrate structures of embryonal carcinoma cells [2] and its importance in compaction of mouse embryo at the morula stage [3].

Owing to its strong anionic charge, *N*-acetylneuraminic acid plays a special role among the different sugars which form the oligosaccharide chains of glycoconjugates. Embryonic and undifferentiated tissues often show a different sialylation pattern compared to their adult, differentiated counterparts [4]. Moreover, the increased sialylation of tumor cells may explain, at least in part, their altered adhesion properties [5, 6].

Since the main regulation of protein glycosylation occurs at the level of glycosyltransferase expression, the comparison of such enzyme activities in tissues at different stages of development is of interest because it allows the identification of enzymes involved in the

Abbreviations: HPLC, high performance liquid chromatography; N-CAM, neural cell adhesion molecule.

biosynthesis of stage-specific oligosaccharide structures. Rat brain expresses several different sialyltransferases acting on N- or O-linked chains of glycoproteins [7]; in this work the changes in activity of four of these enzymes during the ontogenic development has been investigated.

Materials and Methods

Materials

Fetuin, *N*-acetyllactosamine, CMP-NeuAc and sialyllactose were from Sigma (St. Louis, MO, USA), CMP-[^{14}C]NeuAc (262 Ci/mol) was from Amersham (Amersham, UK), Bio-Gel P-10 was from Bio-Rad (Richmond, CA, USA). Asialofetuin was prepared by mild acid hydrolysis (0.1 N H_2SO_4, 80°C, 1 h) of the native glycoprotein, followed by exhaustive dialysis against water and lyophilization. Monosialo- and disialo-O-linked oligosaccharides from fetuin were prepared as described in [8].

Rat Brain Homogenates

Brains were obtained from 19-days old rat fetuses, one day old newborn and adult (over 40 days) rats. Animals were killed by decapitation, their brains immediately placed in ice and homogenized in 5 vol of ice cold water using a Potter homogenizer. Homogenates were filtered through a metal strainer, their protein concentration was determined [9] using bovine serum albumin as a standard and brought to 12 mg/ml. These homogenates were used as enzyme sources.

Sialyltransferase Assay using Fetuin and Asialofetuin

Routine assays were performed in the presence of 80 mM sodium cacodylate buffer pH 6, 10 mM $MnCl_2$, 0.5% Triton X-100, 70 µM CMP-[^{14}C]NeuAc (37 dpm/pmol), 400 µg of fetuin or asialofetuin and 25 µl of brain homogenate in a final volume of 55 µl. The assays with fetuin were incubated at 28°C for 3 h while those with asialofetuin at 26°C for the same time. At the end of the incubation period, reactions were stopped by addition of 1 ml of 1% phosphotungstic acid in 0.5 M HCl and centrifuged at 1500 x *g* for 10 min. Protein pellets were washed twice with the same phosphotungstic acid solution and once with methanol. Thereafter they were solubilized with Soluene (Packard) and counted for radioactivity. The incorporation on endogenous substrates as determined in the absence of exogenous glycoproteins was negligible. All experiments were run in duplicate. To check the presence of heat-stable inhibitors in adult brain, a portion of adult brain homogenate was boiled 2 min and centrifuged at 1500 x *g* for 30 min. Sialyltransferase assays were then performed as described above, but in the presence of 12.5 µl of the supernatant and 12.5 µl of enzyme source.

Determination of the Sialic Acid Content of the Homogenates

The sialic acid content of the homogenates was determined according to the Warren method [10] as described [11].

Release of [^{14}C]NeuAc-labeled Oligosaccharides from Fetuin and Asialofetuin

The sialyltransferase reaction mixture was as above but the specific activity of CMP-[^{14}C]NeuAc was 74 dpm/pmol. After washing with phosphotungstic acid solution and methanol, protein pellets were dissolved in 1 ml of 0.05 M NaOH containing 1 M $NaBH_4$ and incubated for 48 h at 45°C. At the end of the incubation time, samples were acidified by addition of concentrated acetic acid and lyophilized. They were suspended with methanol containing 1% acetic acid, evaporated twice, dissolved in water and applied to a Bio-Gel P-10 (200-400 mesh) column (80 x 1 cm) equilibrated and eluted with 0.1 M pyridine-acetic acid buffer pH 5. The radioactivity in aliquots of the fractions was determined.

Sialyltransferase Assay using Galβ1-4GlcNAc (N-Acetyllactosamine)

The assay mixture contained 80 mM sodium cacodylate buffer pH 6, 10 mM $MnCl_2$, 0.5% Triton X-100, 250 µg of *N*-acetyllactosamine, 70 µM CMP-[^{14}C]NeuAc (37 dpm/pmol) and 25 µl of tissue homogenates in a final volume of 50 µl. After 3 h at 26°C the reactions were stopped by addition of 550 µl of water, samples were boiled 2 min and then filtered through a 0.2 µ membrane. Boiling completely releases *N*-acetylneuraminic acid from unreacted sugar nucleotide donor and no CMP-NeuAc could be recovered thereafter. NeuAcα2-3Galβ1-4GlcNAc was separated from the corresponding NeuAcα(2-6)- isomer and from *N*-acetylneuraminic acid by HPLC.

HPLC Analysis

HPLC analysis was performed with a Waters apparatus equipped with a Merck Lichrosorb-NH_2 column (12.5 x 0.4 cm). Various proportions of acetonitrile and 15 mM KH_2PO_4 in water were used as mobile phase at a rate of 1 ml/min [12]. The analysis of the O-linked chains released from fetuin and asialofetuin was performed under 80/20% isocratic conditions for 20 min followed by a linear gradient which decreased the acetonitrile concentration to 40% in 50 min. Fractions (2 ml) were collected and counted for radioactivity. Separation of *N*-acetylneuraminic acid from α(2-3)- and α(2-6)-sialyllactosamine was obtained using 83/17% isocratic conditions for 25 min; after which time the proportion was changed to 78/22% and kept isocratically for 10 min. Fractions (1 ml) were collected and counted for radioactivity. Unlabeled standards were detected spectrophotometrically at 200 nm.

Results

Sialyltransferase Activities towards Fetuin and Asialofetuin, and Sialic Acid Content of Fetal, Newborn and Adult Rat Brain

Fetuin carries three N-linked chains and three O-linked chains. Most of the N-linked chains are trisialylated with sialic acid in both α(2-3)- and α(2-6)- linkage on the subterminal galactose. About two thirds of the O-linked chains show the following structure: NeuAcα2-3Galβ1-3GalNAc, while the main structure of the remainder is NeuAcα2-3Galβ1-3(NeuAc-α2-6)GalNAc [13]. Asialofetuin may therefore serve as an acceptor for the different sialyltransferases present in rat brain, which act either on N- or O-linked chains of

Table 1. Sialyltransferase activity towards fetuin and asialofetuin, and sialic acid content of fetal, newborn and adult rat brains.
Sialyltransferase activities and sialic acid content of the homogenates were determined as described in the Materials and Methods section.

Brain	Incorporation (pmol/h mg protein)		Sialic acid content (μg/mg protein)
	fetuin	asialofetuin	
fetal	555 ± 16[a]	394 ± 12[a]	8.7 ± 0.55[a]
newborn	445 ± 14[a]	371 ± 11[a]	9.5 ± 0.65[a]
adult	140 ± 13[b]	149 ± 15[b]	11.0 ± 0.60[b]

[a] Mean value of two brain preparations ± SD.
[b] Mean value of five animals ± SD.

glycoproteins. In addition, rat brain can also sialylate native fetuin [14]. Responsible for this property is a sialyltransferase which adds sialic acid in α(2-6)- linkage to the innermost *N*-acetylgalactosamine residue of the two monosialylated O-linked chains of native fetuin [15, 16].

The rat brain sialyltransferase activities towards both fetuin and asialofetuin were found, in preliminary experiments, to be optimal at pH 6 and linear over 6 h of incubation time with up to 1 mg of the glycoprotein acceptor in the assay. Optimal temperatures were 26° C and 28° C for the activity towards asialofetuin and fetuin, respectively.

Comparison of the sialyltransferase activities towards fetuin and asialofetuin displayed by fetal, newborn and adult rat brain showed that adult brain possesses a significantly lower activity towards both substrates (Table 1). The fetal/adult sialyltransferase ratio when related to the amount of protein in the assay was found to be 2.6 and 4 when asialofetuin and fetuin, respectively, were used as acceptors. The protein content of fetal wet brain tissue is lower than that of the adult (6% vs. 9% in the samples used in this study). However the difference between fetal and adult remains highly significant even when the activity was correlated to the wet tissue weight. This indicates that ontogenic development has, in rat brain, a general effect on the expression of the sialyltransferases acting on glycoproteins and that the sialyltransferase which acts on sialylated acceptors is more greatly affected by the development. Despite the significantly higher activity of the sialyltransferases acting on asialoglycoproteins, the sialic acid content of fetal brain tissues is slightly lower than that of the adult (Table 1).

The higher sialyltransferase activity towards fetuin and asialofetuin measured in fetal brain may be due, in principle, to an enhanced expression of the same enzymes active in the adult or to the activity of different enzymes whose expression might be restricted to fetal life. To clarify this point, the structure of the sugar chains sialylated by fetal and adult sialyltransferases on fetuin and asialofetuin was investigated.

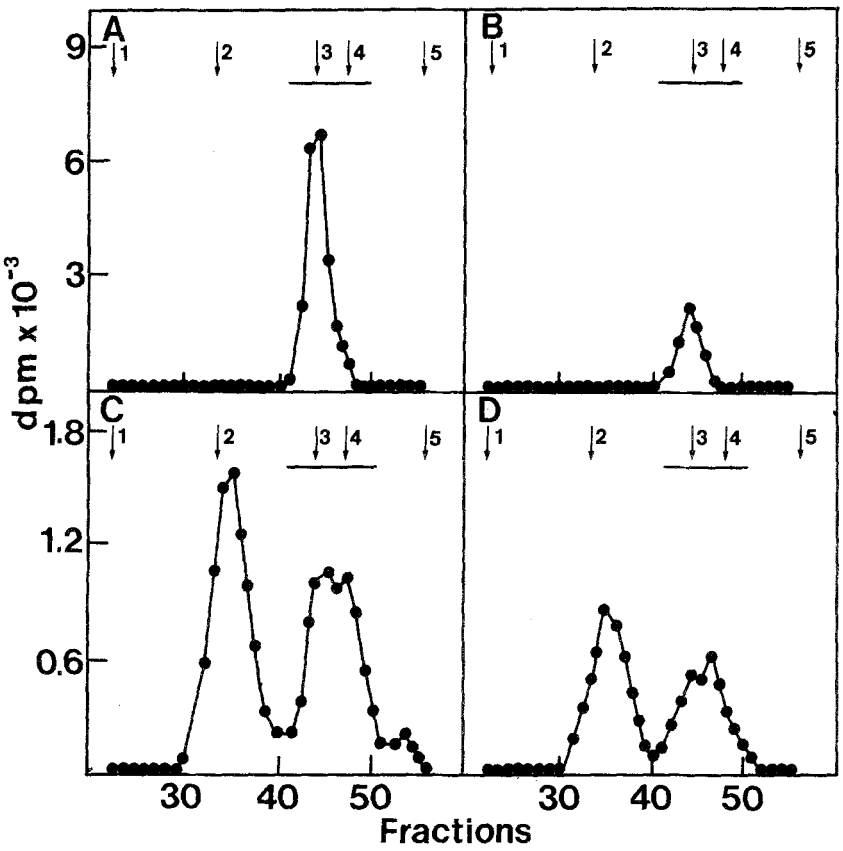

Figure1. Bio-Gel P-10 filtration of [^{14}C]NeuAc-labeled fetuin and asialofetuin oligosaccharides. Labeled oligosaccharides were released by mild alkaline borohydride treatment from fetuin (A and B) or asialofetuin (C and D) labeled using fetal (A and C) or adult (B and D) sialyltransferases. Arrows indicate the elution position of: 1, Blue dextran; 2, fetuin N-linked glycopeptide; 3, disialylated O-linked tetrasaccharide from fetuin; 4, sialyllactose; 5, mannose.

Characterization of [^{14}C]NeuAc-labeled Sugar Chains of Fetuin

When [^{14}C]NeuAc-fetuin, labeled using fetal or adult sialyltransferases was subjected to mild alkaline borohydride treatment followed by Bio-Gel P-10 chromatography, all the radioactivity was recovered in a single peak with an elution volume consistent with that of an authentic disialylated O-linked tetrasaccharide (Figs. 1A and B), thus indicating that only O-linked chains may act as acceptors for fetal and adult rat brain sialyltransferases. When analyzed by HPLC these oligosaccharides displayed the same retention time of the disialylated tetrasaccharide marker obtained after mild alkaline borohydride treatment of fetuin (Figs. 2A and B). It may be concluded that sialylation of native fetuin catalyzed by fetal

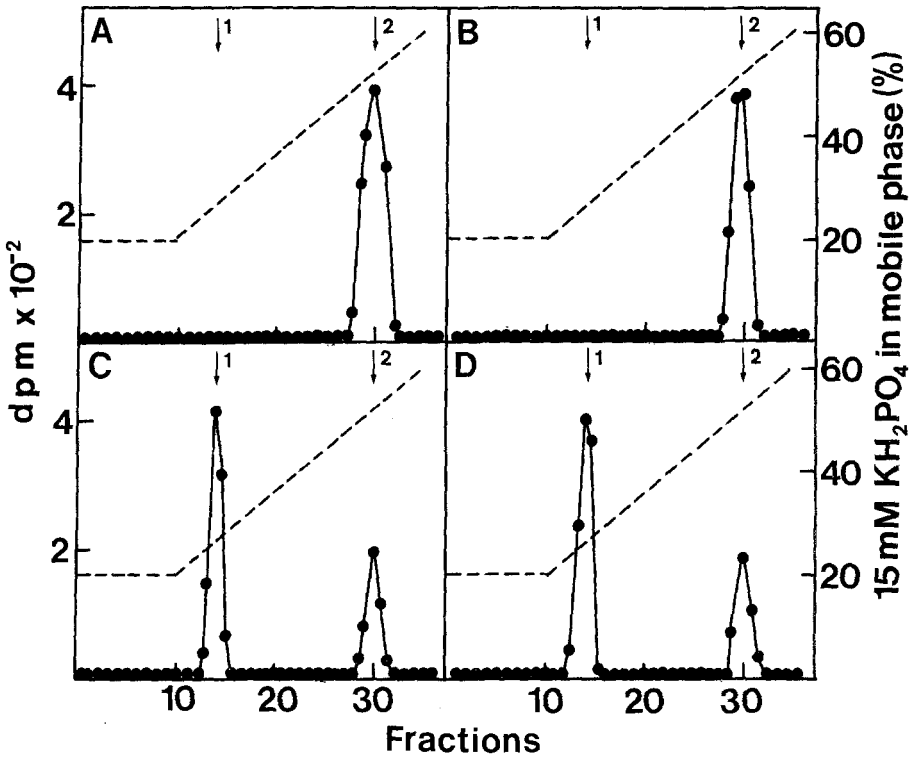

Figure 2. HPLC analysis of O-linked oligosaccharides from [^{14}C]NeuAc labeled fetuin and asialofetuin. Fractions under the bar of Fig. 1 were pooled, lyophilized, and a 4000 dpm aliquot of each sample was analyzed by HPLC as described in the Materials and Methods section. O-Linked oligosaccharides were obtained from [^{14}C]NeuAc-fetuin (A and B) or asialofetuin (C and D) labeled either using fetal (A and C) or adult (B and D) sialyltransferases. Arrows indicate the elution positions of the following oligosaccharide markers: 1, NeuAcα2-3Galβ1-3GalNAcol; 2, NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAcol.

and adult rat brain gives rise to the production of a single compound with the structure: NeuAcα2-3Galβ1-3(NeuAcα2-6)GalNAc, and that the incorporation in fetuin may be taken as a measure of the activity of a single enzyme: CMP-NeuAc:NeuAcα2-3Galβ1-3GalNAc α(2-6)-sialyltransferase, the specific activity of which is four times higher in fetal brain.

Characterization of [^{14}C]NeuAc-labeled Sugar Chains from Asialofetuin

Figs. 1C and D show the Bio-Gel P-10 chromatography profile after mild alkaline borohydride treatment of [^{14}C]NeuAc-asialofetuin labeled using either fetal or adult sialyltransferases. The absence of labeled material in the void volume fractions and the presence of a

Table 2. Sialyltransferase activity towards *N*-acetyllactosamine expressed by fetal and adult rat brains.
Incorporation of [^{14}C]NeuAc on Galβ1-4GlcNAc catalyzed by fetal and adult brain homogenates was determined by HPLC as described in the Materials and Methods section.

	Sialyltransferase activity (pmol/h mg protein)	
Brain	α(2-3)-transferase	α(2-6)-transferase
fetal	667 ± 70[a]	58 ± 6[a]
newborn	523 ± 27[a]	40 ± 5[a]
adult	323 ± 42[b]	22 ± 3[b]

[a] Mean value of two brain preparations ± SD.
[b] Mean value of five animals ±SD.

radioactive peak in the position of the N-linked glycopeptide may be explained by the fact that mild alkaline treatment may cause, in some glycoproteins, the release of N-linked chains as well as the release of O-linked chains [17, 18], while the small peak eluting in the totally included volume of the column in Fig. 1C probably reflects the presence of a small amount of degradation products. The percentage distribution of radioactivity in the peaks corresponding to N- and O-linked chains is not significantly different in the preparations from fetal or adult brain (53/47% and 56/44%). This indicates that brain maturation results in a parallel decreased expression of sialyltransferases acting on both kinds of chain. HPLC analysis of the oligosaccharides recovered in the position of O-linked chains revealed that about 60% of these oligosaccharides from both preparations has a retention time consistent with the following structure: NeuAcα2-3Galβ1-3GalNAcol, while the retention time of the remaining 40% is consistent with the structure of the disialylated tetrasaccharide from fetuin.

Sialyltransferase Activities towards Galβ1-4GlcNAc

Sialylation of the N-linked chains is mainly due to the action of two enzymes: CMP-NeuAc:Galβ1-4GlcNAc α(2-3)- and α(2-6)-sialyltransferase. In order to assess which of the two enzymes is predominant and whether the ontogenic development affects their expression at a different level, their activity was separately measured by using the disaccharide Galβ1-4GlcNAc (*N*-acetyllactosamine) as an acceptor. The use of this disaccharide allows a comparison of these two sialyltransferase activities in fetal and adult tissues because : i) *N*-acetyllactosamine is structurally identical to the terminal non-reducing ends of asialofetuin N-linked chains; ii) it is an efficient acceptor of both enzymes [19]; iii) the two isomer formed, NeuAcα2-3Galβ1-4GlcNAc and NeuAcα2-6Galβ1-4GlcNAc, can be easily separated by HPLC. On the contrary, the α(2-3/6)-sialyltransferase ratio measured with *N*-acetyllactosamine as an acceptor does not necessarily reflect the proportion of α(2-3)-/α(2-6)-linked sialic acid residues attached to the N-linked chains of asialofetuin by rat brain sialyltransferases. Indeed, both these enzymes display a preference for some branches of the polyantennary structures and their activity is strongly influenced by the presence of the peptide backbone [19-21].

Under the conditions used for the assay on *N*-acetyllactosamine, the sialic acid incorporation catalyzed by fetal sialyltransferases is 2.1 times higher than that catalyzed by adult enzymes (Table 2). The main sialyltransferase acting on this disaccharide is, by far, the α(2-3)- enzyme, which account for 92% of the total activity in fetal and newborn brain and 94% in the adult, thus indicating that the α(2-3)-/α(2-6)-sialyltransferase ratio remains practically unchanged during development.

Sialyltransferase Inhibitors in Adult Rat Brain

The purification of a heat-stable sialyltransferase inhibitor in adult rat brain has been recently reported [22]. In order to establish whether this inhibitor is responsible for the lower sialyltransferase activity in adult rat brain, the sialyltransferase assay on fetuin and asialofetuin was performed in the presence of supernatants of boiled adult rat brain homogenate. Under these conditions the percentage inhibition measured never exceeded 15% of control experiments (data not shown). This indicate that the differences in sialyltransferase activity of fetal and adult brain cannot be explained on the basis of the activity of this inhibitor.

Discussion

It is now well established that the structure of the oligosaccharide portion of glycoproteins results from the action of a large number of glycosyltransferases [23] and that the expression of some of these enzymes is developmentally regulated [24]. Data from our group indicate that in human and rat colon the α(2-6)-sialyltransferase acting on *N*-acetyllactosamine sequences and a β(1-4)-*N*-acetylgalactosaminyltransferase acting on (2-3)-sialylated acceptors are onco-developmentally regulated [11, 25, 26]. In brain, the embryonic form of neural cell adhesion molecule (N-CAM) which carries long chains of polysialic acids is substituted, a few days after birth, by the adult form of N-CAM, which lacks the long polysialic acid chains [27, 28]. The disappearance of these structures is due to the shutdown of the specific poly-α(2-8)-sialosyl-sialyltransferase and allows the establishment of stronger interactions between the cells of the nervous system [29-31].

Data reported in this paper indicate that four other main sialyltransferases of rat brain are also developmentally regulated. If one considers that the activity of the enzymes acting on N- and O-linked chains of asialofetuin undergoes a parallel reduction during postnatal development and that also the ratio between the two enzymes acting on *N*-acetyllactosamine (very likely the same enzymes that sialylate the N-linked chains of fetuin) is not affected by brain maturation, it may be postulated that the expression of the three enzymes acting on asialylated glycoprotein acceptors is subjected to a common regulatory mechanism.

Despite the fact that fetal brain possesses a higher level of four important sialyltransferases acting on glycoproteins, the degree of sialylation of the bulk of its glycoproteins is not higher than that of the adult. A possible explanation for this data is that during the fetal and postnatal period, the rapid growth of the brain requires a more intense protein synthesis. A higher sialyltransferase activity might be required to sialylate efficiently the larger number of glycoprotein molecules synthesized.

The only sialyltransferase present in fetal or adult rat brain able to sialylate native fetuin is CMP-NeuAc:NeuAcα2-3Galβ1-3GalNAc α(2-6)-sialyltransferase. This enzyme, which has been found active also in fetal calf liver [32], shows, among the four rat brain sialyltransferases tested, the strongest dependence on ontogenic development.

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