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α -(2 → 3)- and α -(2 → 6)-Sialyltransferase activities present in three variants of Ehrlich tumor cells: identification of the products derived from *N*-acetyllactosamine and β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn[☆]

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Abstract

We compared several sialyltransferase activities related to synthesis of O-linked and N-linked sialylglycoproteins in Ehrlich ascites tumor cells that grow normally in murine ascites, but are not adherent nor grow in tissue culture (na-EAT cells), with those in cells that were selected to grow in tissue culture and adhere to extracellular matrices (a-EAT cells). Crude Golgi preparations from both cell types contained predominantly β -D-Gal-(1 → 3)-D-GalNAc α -(2 → 3)-sialyltransferase activity. Sialylation of *N*-acetyllactosamine, lacto-*N*-tetraose, and benzyl α -D-GalNAc occurred at from 1 to 4% of that activity. Analysis, by ion-exchange HPLC at high pH, of sialylated *N*-acetyllactosamine showed that na-EAT cells sialylated β -D-Gal-(1 → 4)-D-GlcNAc mostly by α -(2 → 3)-sialyltransferase, whereas β -D-Gal-(1 → 4)-D-GlcNAc α -(2 → 6)-sialyltransferase activity was prominent in a-EAT cells. In addition, preparations from na-EAT cells formed significant quantities of an unknown tritiated product from CMP-[9-³H]sialic acid, suggesting at least one other difference in enzyme levels between the cell types. a-EAT cells reestablished in murine ascites for 11 passages retained the sialyltransferase levels characteristic of a-EAT cells. When viable cells were labeled with

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D-[³H]glucosamine, na-EAT cells formed larger amounts of sialic acid in O-linked glycoproteins than did a-EAT cells.

Keywords: α -(2→3)-Sialyltransferase; α -(2→6)-Sialyltransferase; Sialyltransferase; Sialylglycoproteins; Ehrlich tumor cells

1. Introduction

Changes in the quantity and type of sialic acid linkages in cell-surface glycoconjugates have been implicated in transformation of normal cells to cancerous ones as well as in cancer metastasis [1–5]. Cancer-related changes in oligosaccharide structures often include the production of highly branched and more extensively sialylated glycoproteins [6–10]. Several glycosyltransferases are related to onco-developmental regulation. This allows the stage-specific expression of onco-fetal oligosaccharide structure to occur [5,10,11].

A model for such transformation may be the conversion of Ehrlich ascites tumor (EAT)² cells between a rapidly growing nonadherent form and a more slowly growing adherent type [12–14]. When EAT cells grown in the murine ascites fluid, termed nonadherent EAT (na-EAT)³, were incubated in culture flasks coated with bovine corneal endothelial cell basement membrane, a very small fraction ($\sim 10^{-5}$) of the cells attached to the bottom of the flask and grew. Those cells were harvested and subcultured several times using flasks coated with the same basement membrane, establishing a variant termed adherent Ehrlich tumor (a-EAT) cells. a-EAT cells grew very slowly when first returned to mouse peritoneal cavities, but in subsequent passages grew as fast as normal na-EAT cells. The a-EAT cells also appeared different from na-EAT cells by lectin agglutination assay, until about 25 passages after being returned to mouse ascites [13]. Accordingly, cell-surface differences in carbohydrates of these cell lines were investigated using various lectins [12–14]. In these studies, the binding behavior of *Maackia amurensis* lectin (MAL) and elderberry bark lectin (SNA) were quite different for the two cell types. MAL, which interacts with sialyl- α -(2→3)- β -D-Gal-(1→4)-D-Glc/GlcNAc structures [14], bound to both cell surfaces, whereas SNA, which interacts with sialyl- α -(2→6)-D-Gal/GalNAc structures [15], bound to a-EAT cell surfaces but only very poorly to na-EAT ones.

There are a few reports of sialyltransferases involved in sialylation of cell surfaces of Ehrlich ascites tumor cells. However, these papers give no information regarding the sialyltransferases relative to the synthesis of specifically sialylated glycoproteins [16–18].

In recent work from our laboratory [19], the activities of sialyltransferases in crude Golgi membrane preparations in the two types of EAT cells were compared on the basis of their reactivity with specific acceptor oligosaccharides, as well as with their lectin reactivity and sensitivity to sialidases of the sialylated products.

² Abbreviations used: EAT, Ehrlich ascites tumor; na-EAT, EAT cells grown in murine ascites; a-EAT, EAT cells grown in culture; EAT c/m, a-EAT cells reestablished in murine ascites; MAL, *Maackia amurensis* lectin; SNA, elderberry (*Sambucus nigra* L.) bark lectin; CHO cells, Chinese hamster ovary cells.

³ In some previous publications, we used the terms wild-type (EAT-wt) and cultured (EAT-c) to designate na-EAT and a-EAT cells, respectively.

The present study extends that work by using anion-exchange HPLC techniques at high pH to identify and quantitate the products of the sialyltransferase activities in each cell type demonstrating that reversion of a-EAT to a nonadherent form occurs without apparent change in the sialyltransferase activity. Additional data on the distribution of *N*-acetylneuraminic acid and between *N*- and *O*-linked oligosaccharides was obtained by metabolic labeling with D-[³H]glucosamine.

2. Materials and methods

Materials.—Fetal calf serum, modified Eagle medium, RPMI 1640 medium, glutamine and gentamicin were purchased from Gibco BRL. CMP-[9-³H]Neu5Ac (25.1 Ci/mmol) was obtained from New England Nuclear Corp., while D-[6-³H]Glucosamine hydrochloride (25.4 Ci/mmol) and NaB³H₄ were from Amersham Chemical Co. Benzyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-α-D-galactopyranoside [β-D-Gal-(1 → 3)-α-D-GalNAc-(1 → O)-Bn], benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside [α-D-GalNAc-(1 → O)-Bn], and lacto-*N*-tetraose were obtained from Sigma. *N*-Acetyllactosamine (β-D-Gal-(1 → 4)-D-GlcNAc) was synthesized in our laboratory. Purified preparations of sialyltransferases from rat and porcine liver were from Boehringer, Mannheim. Bio-Gel P Bio-Gel P-10, and Dowex AG resins were obtained from Bio-Rad, and QAE-Sephadex 25 from Pharmacia Fine Chemicals. Chinese hamster ovary cells (CHO cells) were kindly provided by the Genetics Institute, Andover, MA, USA.

Cells.—EAT cells, serially passed in female CD-1 mice (Charles River, 20–30 g body weight) were used as na-EAT. a-EAT cells, the same culture as used previously [19], were grown in 75-cm² culture flasks using modified Eagle's medium with 10% fetal calf serum, 2 mM glutamine, and 0.1% gentamicin. The cells were harvested in a buffer containing 10 mM EDTA and centrifuged. EAT-c/m cells were a-EAT cells that were passed in female CD-1 mice for ca. 11 passages. Although initially slow-growing, these cells eventually grew as fast as the na-EAT cells, requiring weekly passage [13].

Preparation of crude enzyme [5,19].—The Golgi membrane fraction containing sialyltransferase activities were prepared essentially as previously described [19]. Briefly, na-EAT or EAT-c/m cells (2–4 mL packed cell volume) were freed of red blood cells as described [20], and a-EAT cells were washed with 0.15 M saline. Washed cells were homogenized in a Brinkmann Polytron Homogenizer with 1–3 vol of homogenizing buffer containing 15 mM Tris-HCl, pH 7.5, 15 mM NaCl, and 1 mM MgCl₂ in 25% glycerol for 15 s with cooling on ice. The homogenate was centrifuged at 1 000g for 10 min and the supernatant solution recentrifuged at 16 000g for 10 min. The resulting supernatant solution was centrifuged at 100 000g for 1 h, the pellet therefrom was dissolved in a small amount (generally 1.0 mL) of membrane lysis buffer containing 20 mM HEPES, pH 7.5, 4% Lubrol PX, 1% Triton CF-54 in 50% glycerol, and lysed overnight at 4°C. This solubilized Golgi membrane fraction was used as the enzyme preparation.

Sialyltransferase assay [19,20].—The assay mixture contained, in a total volume of 100 μL, 20 mM MES buffer pH 6.5, 10 mM MnCl₂, 25% glycerol, 1 nmol of CMP [9-³H]Neu5Ac (60,000 dpm), 10 μg of selected acceptor, and 10 μL of the enzyme preparation. β-D-Gal-(1 → 3)-α-D-GalNAc-(1 → O)-Bn, α-D-GalNAc-(1 → O)-Bn,

acetyllactosamine, and lacto-*N*-tetraose were used as acceptors. After incubation at 30°C or 37°C for various time periods from 5 min to 60 min, depending on the acceptor, the reaction was stopped with 1.0 mL of ice-cold 5 mM sodium phosphate buffer, pH 6.8. Each reaction mixture was applied to a column (0.5 × 4 cm) of Dowex 1 × 8 phosphate (200–400 mesh). The effluent was recycled through the column 4 times. The final effluent was added to 4 mL of a scintillation cocktail for determination of radioactivity by liquid scintillation counting.

Preparation of standard [³H]sialylated trisaccharides.—For the synthesis of α -D-[9-³H]Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc, 600 μ L of a mixture containing 300 μ g of *N*-acetyllactosamine, 0.6 mU of rat liver α -(2 → 6)-sialyltransferase, 17.5 mM cacodylate buffer pH 6, 225 mM NaCl, 0.04% Triton CF-54, and 80 nmol of CMP-[9-³H]Neu5Ac in 25% glycerol was incubated overnight at 37°C. Similarly, labeled α -Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc was prepared under the same conditions except that a CHO Golgi membrane fraction was used as the enzyme source. When β -D-Gal-(1 → 3)-D-GalNAc or β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn was the disaccharide acceptor, 30 μ g of acceptor was incubated overnight at 37°C with 0.6 mU of porcine liver α -(2 → 3)-sialyltransferase, 80 nmol of CMP-[9-³H]Neu5Ac, 50 mM MES buffer, pH 6.5, 5 mg of BSA, 0.005% Triton X-100, and 25% glycerol in 1.0 mL (total volume). Reactions were stopped by the addition of 2 mL of water and the mixtures were applied to a column (1 × 10 cm) of QAE Sephadex Q-25 (formate form). The column was washed with 100 mL of water, followed by elution of the sialylated oligosaccharides with 50 mM ammonium formate. The first radioactive fractions eluted were pooled and lyophilized, applied to a Bio-Gel P-4 column (1 × 20 cm), and eluted with 100 mM ammonium formate. The first radioactive peak was lyophilized and used as a standard sialyloligosaccharide preparation.

Preparation of α -Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc-OT and α -Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc-OT [21].—To 100 μ g of α -Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc or α -Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc (Oxford Glycosystems) was added 20 μ L of 0.05 N NaOH and 32 nmol of NaB³H₄. After 4 h, the reaction was stopped by the addition of 0.1 mL of acetic acid and dried in vacuo. The material was dissolved in 1 mL of water, applied to a Dowex 50 × 8 column (H⁺ form, 0.4 × 2 cm), and eluted with 5 vol of water. The effluent was lyophilized, followed by evaporating 3 times with MeOH. The resulting products were dissolved in water and used as standards for HPLC analysis.

Preparation and purification of sialylated-oligosaccharide and -oligosaccharitol using na-EAT and a-EAT enzymes.—A mixture of 10 μ g of β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn, β -D-Gal-(1 → 3)-D-GalNAc or 100 μ g of *N*-acetyllactosamine in 100 μ L of the solubilized Golgi preparation was incubated under the same enzyme assay conditions as above for 5 to 17 h, after which the reaction was stopped by the addition of 2 mL of water, and the same purification procedure used as for the preparation of the standard sialyl oligosaccharide. In later experiments, the procedure was modified by using a column (0.7 × 2 cm) of Dowex 1 × 8 (acetate, 100–200 mesh) instead of QAE Sephadex, and eluting the products with M acetic acid. The first fractions containing radioactivity (sialylated oligosaccharides) were lyophilized, taken up in a small volume of water, and analyzed directly without the additional P-4 column step. Free sialic acid eluted later from the Dowex

1 column. A portion of the sialylated products was reduced with NaBH_4 using the same procedure used for sialylated sugar alcohols.

Analysis of sialylated oligosaccharides by HPLC.— $[9\text{-}^3\text{H}]$ Sialyloligosaccharides and $[^3\text{H}]$ sialylsaccharitols were analyzed by HPLC on a column (4×250 mm) of CarboPac PA-100 (Dionex). Elution was effected isocratically with 100 mM NaOH + 50 mM NaOAc in water [22,23], at a flow rate of 0.5 mL/min. Fractions of 5 drops of eluate were collected directly into scintillation vials containing 5 mL of scintillation fluid for liquid scintillation counting.

Metabolic labeling of na-EAT and a-EAT cells [5,10].—Samples of cells (30×10^7) were washed twice with RPMI 1640 medium without glutamine and incubated at 37°C for 2 h in 1 mL of the medium + 70 μCi of D- $[^3\text{H}]$ glucosamine. Half of the samples were harvested, and to the remainder was added 10 mL of medium, followed by overnight incubation. Following incubation, cells were harvested with trypsin-EDTA, washed 3 times with buffered saline, and lysed with membrane lysis buffer overnight. The lysed cells were treated with 0.05 N NaOH containing M NaBH_4 for 15 h at 45°C [24]. After the reaction was stopped by the addition of acetic acid, the solution was applied to a column (1×20 cm) of Bio-Gel P-10 and eluted with 100 mM ammonium formate. The void and total volume fractions were collected and lyophilized. The lyophilized products were hydrolyzed with 0.1 N HCl at 80°C for 1 h [25]. After neutralization, the hydrolyzates were applied to a QAE-Sephadex Q-25 column (0.4×4 cm, formate form), the column was washed with 5 vol of water, and sialic acid was eluted with 50 mM ammonium formate and analyzed by liquid scintillation counting.

3. Results

Sialyltransferase activities.—The sialyltransferase activities toward $\beta\text{-D-Gal-(1} \rightarrow 3)\text{-}\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$, *N*-acetyllactosamine, lacto-*N*-tetraose, and $\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$ in na-EAT and a-EAT are shown in Table 1. na-EAT, a-EAT, and EAT-c/m cells contained high levels of sialyltransferase activity toward $\beta\text{-D-Gal-(1} \rightarrow 3)\text{-}\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$ as acceptor, but low levels of activity towards *N*-acetyllactosamine, lacto-*N*-tetraose, and $\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$. Quantitatively, the level of sialyltransferase activity toward $\beta\text{-D-Gal-(1} \rightarrow 3)\text{-}\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$ was as much as 5-fold greater in na-EAT cells, and slightly greater in EAT-c/m cells, than in a-EAT cells. This activity was

Table 1
Sialyltransferase activities in na- and a-EAT cells

Acceptor	Specific activity, nmol/mg protein/h		
	na-EAT	a-EAT	EAT-c/m
$\beta\text{-D-Gal-(1} \rightarrow 3)\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$	5.0	1.0	2.0
$\beta\text{-D-Gal-(1} \rightarrow 4)\text{-D-GlcNAc}$	<0.03	0.05	0.04
Lacto- <i>N</i> -tetraose	<0.03	0.05	n.d.
$\alpha\text{-D-GalNAc-(1} \rightarrow \text{O)-Bn}$	0.04	0.04	n.d.

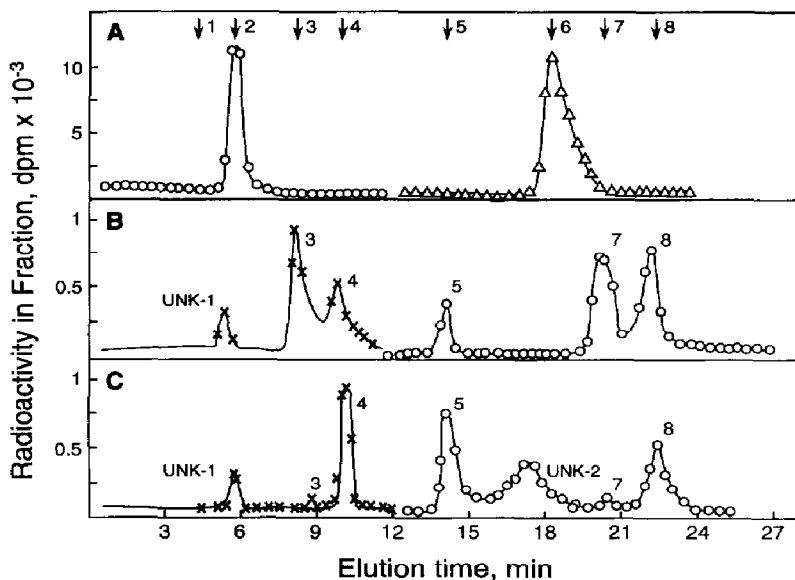


Fig. 1. HPLC analysis of sialylated oligosaccharides. Column: Dionex PA-100 (4 × 250 mm) with guard column; eluent, 0.1 M NaOH + 0.05 M NaOAc; flow rate, 0.5 mL/min. Fractions: 5 drops (20 s) were collected for radioactivity determination. Arrows show elution positions of standard sialylated sugars: 1, neutral oligosaccharitol, β -D-Gal-(1 → 3)-D-GalNAc-OT; 2, α -[9- 3 H]Neu5Ac-(2 → 3)- β -D-Gal-(1 → 3)-GalNAc-(1 → O)-Bn synthesized by porcine sialyltransferase; 3, α -Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc-OT made by reduction of α -Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc; 4, α -Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc-OT made by reduction of α -Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc; 5, [9- 3 H]Neu5Ac; 6, α -[9- 3 H]Neu5Ac-(2 → 3)- β -D-Gal-(1 → 3)-D-GalNAc synthesized by porcine sialyltransferase; 7, α -[9- 3 H]Neu5Ac-(2 → 6)- β -D-Gal-(1 → 4)-D-GlcNAc synthesized by rat liver β -D-Gal-(1 → 4)-D-GlcNAc α -(2 → 6)-sialyltransferase; 8, α -[9- 3 H]Neu5Ac-(2 → 3)- β -D-Gal-(1 → 4)-D-GlcNAc synthesized by Golgi sialyltransferase of CHO cells. Panel A, products synthesized by na- or a-EAT preparations (which were identical) using β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn (2, \circ) and β -D-Gal-(1 → 3)-D-GalNAc (6, Δ) as acceptors. Panels B and C, products of a- and na-EAT preparations respectively, using β -D-Gal-(1 → 4)-D-GlcNAc as acceptor. Reduced products (\times) eluted before 12 min; nonreduced products (\circ) eluted after 12 min. The nonreduced product from EAT-c/m cells was identical to the a-EAT cell preparation.

almost 100 times higher than with *N*-acetyllactosamine, lacto-*N*-tetraose, or α -D-GalNAc-(1 → O)-Bn as acceptors in na-EAT preparations, but was generally ~10–25 times higher in a-EAT cells and ca. 50-fold in EAT-c/m cells.

HPLC analysis of sialylated products.— β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn can be sialylated at position 3 of Gal and/or position 6 of GalNAc, whereas *N*-acetyllactosamine can accept sialic acid at position 3 or 6 of Gal (Table 1). In order to confirm the structure of sialylated β -D-Gal-(1 → 3)-D-GalNAc and β -D-Gal-(1 → 4)-D-GlcNAc, the products generated by sialyltransferase were purified and analyzed by HPLC.

Elution profiles of sialylated β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn and β -D-Gal-(1 → 3)-D-GalNAc synthesized by na- or a-EAT sialyltransferases were identical, as shown in panel A of Fig. 1. The products from sialylation of β -D-Gal-(1 → 3)- α -D-GalNAc-(1 → O)-Bn and β -D-Gal-(1 → 3)-D-GalNAc (panel A, peaks 2 and 6, respectively) by Golgi preparations from either na- or a-EAT cells had the same retention times (6.5 and 19 min) as the corresponding product from the porcine enzyme (arrows 2 and 6), which are

α -Neu5Ac-(2→3)- β -D-Gal-(1→3)- α -D-GalNAc-(1→O)-Bn and α -Neu5Ac-(2→3)- β -D-Gal-(1→3)-D-GalNAc, respectively. Thus, both na- and a-EAT cells contain high levels of β -D-Gal-(1→3)-D-GalNAc α -(2→3)-sialyltransferase activity.

When β -D-Gal-(1→4)-D-GlcNAc was sialylated by na- or a-EAT cell preparations, the reaction product was divided into two aliquots, one of which was reduced by NaBH₄. Both reduced and nonreduced products were analyzed by HPLC (Fig. 1, panels B and C). The elution profile of a-EAT sialylated products is shown in panel B. Three peaks are evident in the reduced products. The main products, peaks 3 and 4, had retention times of 8.5 and 10.5 min, the same as α -Neu5Ac-(2→6)- β -D-Gal-(1→4)-D-GlcNAc-OT (arrow 3) and α -Neu5Ac-(2→3)- β -D-Gal-(1→4)-D-GlcNAc-OT (arrow 4) made by reduction of α -Neu5Ac-(2→6)- β -D-Gal-(1→4)-D-GlcNAc and α -Neu5Ac-(2→3)- β -D-Gal-(1→4)-D-GlcNAc, respectively. The small peak at 5.5 min (panel B, UNK 1) resulted from an impurity either in the cell preparations or in the CMP-[³H]Neu5Ac, as it also occurred in controls without added acceptor. Similarly, the nonreduced product showed three peaks at retention times of 14.5, 20, and 21.5 min (panel B, peaks 5, 7, and 8), later than the reduced ones. Peak 7 was identified as α -Neu5Ac-(2→6)- β -D-Gal-(1→4)-D-GlcNAc on the basis of showing the same retention time as α -Neu5Ac-(2→6)- β -D-Gal-(1→4)-D-GlcNAc prepared by rat liver β -D-Gal-(1→4)-D-GlcNAc α -(2→6)-sialyltransferase. Peak 8 had the same retention time as the product synthesized by CHO sialyltransferase. As CHO cells express β -D-Gal-(1→4)-D-GlcNAc α -(2→3)-sialyltransferase, but lack β -D-Gal-(1→4)-D-GlcNAc α -(2→6)-sialyltransferase [26], the peak resulting from CHO enzyme is assumed to be α -Neu5Ac-(2→3)- β -D-Gal-(1→4)-D-GlcNAc. The peak eluting at 22.5 min. (Panel B, peak 8) is thus identified as α -Neu5Ac-(2→3)- β -D-Gal-(1→4)-D-GlcNAc. Peak 5 in panel B (14.5 min) is free Neu5Ac, as its retention time is identical to labeled Neu5Ac produced from acid hydrolysis of CMP-[³H]Neu5Ac. Samples prepared by elution from Dowex 1 (OAc⁻) instead of QAE-Sephadex contained the same two peaks of sialylated LacNAc but lacked free Neu5Ac, which eluted later from the Dowex 1 (OAc⁻) column.

The products from sialylation of LacNAc by na-EAT preparations (Fig. 1, panel C) were qualitatively the same as those from a-EAT preparations, but the proportion of α -Neu5Ac-(2→6)- β -D-Gal-(1→4)-D-GlcNAc and α -Neu5Ac-(2→3)- β -D-Gal-(1→4)-D-GlcNAc were different (Table 2). Considerably less α -Neu5Ac-(2→6)- β -D-Gal-

Table 2
Sialylation of LacNAc by Golgi preparations from EAT cells

Cell type	Sialyl-LacNAc synthesis, pmol/hr/mg ^a (%)		N-Linked activity, % of O-linked ^b
	α -(2→3)	α -(2→6)	
na-EAT	16.9 (88)	2.3 (12)	<1
a-EAT	23.5 (55)	19.3 (45)	9.7
EAT-c/m	29.9 (62)	18.4 (38)	5.3
na-EAT + a-EAT ^c	25.3 (75)	8.4 (25)	n.d.

^a Based on radioactivity in sialyl-LacNAc fractions after 16 h incubation at 37°C.

^b Measured as sialylation of β -D-Gal-(1→3)- α -D-GalNAc-(1→O)-Bn.

^c Assayed using equal volumes of each preparation having approximately equal O-linked sialylation activity.

(1 → 4)-D-GlcNAc was consistently observed in the products from na-EAT preparations. On the other hand, preparations from EAT-c/m cells were nearly indistinguishable from the a-EAT cells in this respect (Table 2, chromatographic data not shown). Also, when preparations from na- and a-EAT cells were mixed, an intermediate proportion of the two products was observed, indicating that the product ratios reflect the levels of the two sialyltransferase activities, and not the presence of interfering reactions, such as a specific neuraminidase, in the na-EAT cells. When products from na-EAT cell preparations were purified on QAE Sephadex, a substantial amount of an unidentified radioactive material at 17 min (panel C, unk. 2) was observed. This material eluted with Neu5Ac from Dowex1 (OAc⁻) columns. Unlike unknown 1 which was present in all samples, unknown 2 was predominant only in the products from na-EAT cell preparations. Its identification is currently underway.

The results show that na-EAT cells contained almost exclusively β -D-Gal-(1 → 3)- or (1 → 4)-GlcNAc α -(2 → 3)-sialyltransferase, with only very weak β -D-Gal-(1 → 4)-D-GlcNAc α -(2 → 6)-sialyltransferase activity.

Estimation of N-glycosidically and O-glycosidically bound sialic acid in na- and a-EAT cells.—In order to corroborate the *in vivo* activity of these sialyltransferases in the na- and a-EAT cells, the two cell lines were metabolically labeled with D-[³H]glucosamine, which is a precursor of GlcNAc, GalNAc, and Neu5Ac. Metabolically labeled cells were subjected to mild alkaline borohydride treatment to cleave O-linked glycoproteins [24] and the resulting oligosaccharides were separated on a column of Bio-Gel P-10. The void volume and total volume fractions were hydrolyzed with mild acid to cleave the sialic acid moieties, which were then separated from the neutral saccharides on QAE-Sephadex.

Table 3 shows the extent of labeling of sialic acid in na- and a-EAT cells after 2 and 16 h. Following 2 h of metabolic labeling, neither cell type contained detectable amounts of labeled sialic acid in O-linked oligosaccharides, but significant amounts of N-linked sialic acid were labeled. After 16 h incubation, however, O-linked oligosaccharides predominated in both cell types, ~10 times more radioactive sialic acid being incorporated into the O-linked fractions than into the N-linked ones. Thus, labeled sialic acid was incorporated into N-linked glycans more rapidly initially, but after a lag of at least 2 h, O-linked glycans were

Table 3
Incorporation of radioactivity from D-glucosamine into sialylated glycoconjugates in na-EAT and a-EAT cells

Cell type	Time, h	Sialic acid incorporated, pmol/mg protein		
		Total	N-Linked ^a	O-Linked ^b
a-EAT	2	0.02	0.02	0
	16	0.46	0.04	0.29
na-EAT	2	0.52	0.29	0
	16	9.3	0.84	9.0

^a Measured as radioactivity in acidic fraction released by mild acid hydrolysis from high-MW fraction following alkaline borohydride treatment of the cell lysate.

^b Measured as radioactivity in acidic fraction released by mild acid hydrolysis from low-MW fraction following alkaline borohydride treatment of the cell lysate.

much more rapidly sialylated, with an overall greater rate in na-EAT cells. This result is comparable with the enzyme activities of the two cell lines shown in Table 1.

4. Discussion

This study confirms the important finding that a-EAT cells express much higher levels of β -D-Gal-(1 \rightarrow 4)-D-GlcNAc α -(2 \rightarrow 6)-sialyltransferase than do na-EAT cells and further, establishes the nature of the products derived from use of β -D-Gal-(1 \rightarrow 4)-D-GlcNAc as acceptor.

One of the most characteristic features of na-EAT cells is their apparently high level of β -D-Gal-(1 \rightarrow 3)-D-GalNAc α -(2 \rightarrow 3)-sialyltransferase. This enzyme contributes to the transfer of sialic acid to O-glycosidically linked glycoprotein. High levels of this activity were also shown in human leukemia myeloblastoid cells [11]. This enzyme is presumably responsible for the large amount of sialic acid found in the degradation products (total P-10 column volume) after mild alkaline treatment of metabolically labeled cells (Table 3).

The trisaccharide product α -Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 3)-D-GalNAc generated by the action of β -D-Gal-(1 \rightarrow 3)-D-GalNAc α -(2 \rightarrow 3)-sialyltransferase can theoretically be further sialylated by subsequent action of a GalNAc α -(2 \rightarrow 6)-sialyltransferase [27,28]. However, we detected no disialylated products of α -Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 3)- α -D-GalNAc-(1 \rightarrow O)-Bn or α -Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 3)-D-GalNAc in HPLC (Fig. 1, panel A), since such products would have distinctly different elution times from monosialylated products.

Analysis of sialylated trisaccharide products by the CarboPac PA-100 column using the buffer system of Townsend et al. [23] clearly resolved α -Neu5Ac-(2 \rightarrow 6)- β -D-Gal-(1 \rightarrow 4)-D-GlcNAc, α -Neu5Ac-(2 \rightarrow 3)- β -D-Gal-(1 \rightarrow 4)-D-GlcNAc, and the corresponding sugar alcohols (Fig. 1, panel A). This procedure is a powerful tool for the analysis of products of sialyltransferase. It should be noted that when care was taken to avoid the presence of carbonate in the solvent (solvent prepared from freshly-distilled water using saturated NaOH, and protected during use with a CO₂ trap), resolution of the column and elution times with different batches of solvent remained virtually constant over a period of more than 2 years. On the other hand, if the CO₂ trap was omitted, resolution with a single batch of solvent was completely lost over a period of a few days.

In previous studies, it was shown that the lectin SNA bound to a-EAT cells and EAT-c/m cells passaged 15 times but did not bind to na-EAT cell surfaces or to EAT-c/m cells after 25 passages in mice, whereas a lectin reactive with α -(2 \rightarrow 3)-linked sialic acid, MAL, agglutinated all three types of EAT cells [12–14]. Our results with EAT-c/m cells after 11–13 passages are consistent with the lectin-binding data in showing that relatively higher levels of N-linked α -(2 \rightarrow 6)-sialyltransferase are still present, even though the cells have already largely lost their adherent properties.

The levels of sialic acid labeled via glucosamine incorporated by whole cells into N- and O-linked glycoconjugates (Table 3) were generally comparable to the levels of sialyltransferase activities of the two cell types (Table 1). The basis for the distinction between these two activities is the lability of O-glycosidic linkages with threonine or serine to mild alkaline borohydride treatment (β -elimination) [27,28]. Some reports [29,30] suggest that as much

as 20% of β -elimination products may arise from N-linked glycoproteins. However, since we observe no detectable labeled sialic acid released by this treatment from the 2-h samples, and relatively more N-linked sialic acid than expected based on the enzymic levels after 16 h, this source of error is not significant in our experiments.

Although the short-term metabolic labeling results generally corroborate the results of the sialyltransferase measurements, they do not correspond with the total sialic acid content of a-EAT and na-EAT cells reported previously [13]. It must be realized, however, that those measurements represent the accumulation of glycoproteins over a long period of growth under different culture conditions (1 week in synthetic medium vs. murine ascites fluid) and do not necessarily correspond to the short-term labeling conducted in these studies. Even in our studies, the pattern of labeling changed markedly between 2 and 16 h. It should also be noted that possible differences in pool sizes of sialic acid precursors could result in different specific radioactivity of the sialic acid in the two cell types. Therefore, comparison of total sialic acid incorporated in each cell type may not be directly comparable, but the relative amounts in O-linked and N-linked oligosaccharides should be unaffected.

Numerous reports indicate that sialic acid is involved in the adhesion properties of cell surface [31–34]. a-EAT cells adhere to the extracellular matrix components laminin and fibronectin, and to tissue culture plastic. On the other hand, na-EAT cells lack those adhesive properties. After sialidase treatment of a-EAT cells, the adhesion property remained unaffected, whereas the binding activity to MAL was lost [12]. Thus, it appears that the sialic acid present on EAT cell surfaces does not by itself determine cell adhesion properties.

Several kinds of tumor cells produce mucin associated with specific antigens and, probably, metastasis of tumor cells [34]. The abundance of β -D-Gal-(1 \rightarrow 3)-D-GalNAc α -(2 \rightarrow 3)-sialyltransferase activity in na-EAT cells may play a role in production of tumor-related mucin and associated metastasis of the tumor cells. On the other hand, the significance of the loss of β -D-Gal-(1 \rightarrow 4)-D-GalNAc α -(2 \rightarrow 6)-sialyltransferase activity in na-EAT cells is not clear, as this sialylation appears not to be directly responsible for cell adhesion. This enzyme is known to be released from liver cells into the extracellular space as an acute-phase reactant upon tissue damage or inflammation [35]. The inflammation associated with the growth of the ascites tumor may promote a protease-mediated release of this sialyltransferase [36] from the Golgi apparatus of the tumor cells. If so, however, such activity does not apparently lead to immediate loss of the enzyme activity, since the adherent cells reestablished in ascites culture for 11 passages retain the higher level of β -D-Gal-(1 \rightarrow 4)-D-GalNAc α 2 \rightarrow 6 sialyltransferase.

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