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Presence of Cytidine 5′-Monophospho-*N*-acetylneuraminic Acid:Galβ1-3GalNAc-R α(2-3)-Sialyltransferase in Normal Human Leukocytes and Increased Activity of This Enzyme in Granulocytes from Chronic Myelogenous Leukemia Patients[1]

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ABSTRACT

We have examined granulocytes from patients with chronic myelogenous leukemia (CML) and from normal subjects to determine whether activity of a specific sialyltransferase might account for the aberrant sialylation of *O*-linked membrane oligosaccharides in CML cells. Total membrane preparations of morphologically mature CML and normal granulocytes were tested for sialyltransferase activity using the substrates galactosyl-β1-3-*N*-acetyl-D-galactosamine-α-*O*-nitrophenyl and *N*-acetyl-D-galactosamine-α-phenyl. *N*-Acetyl-D-galactosamine-α-phenyl was not an acceptor with either CML or normal cells. With galactosyl-β1-3-*N*-acetyl-D-galactosamine-α-*O*-nitrophenyl, sialyltransferase activity was 2.8 times higher in CML cells compared to normal cells. Product identification by high performance liquid chromatography showed that enzyme from both normal and CML granulocytes linked sialic acid to galactosyl-β1-3-*N*-acetyl-D-galactosamine-R by the α(2-3) and not the α(2-6) linkage. The enzyme CMP-*N*-acetylneuraminic acid: galactosyl-β1-3-*N*-acetyl-D-galactosamine-R α(2-3)-sialyltransferase has not previously been described in human granulocytes. The marked increase in activity of this enzyme in CML and the resulting increase in sialylation may contribute to the pathophysiological behavior of CML granulocytes.

INTRODUCTION

Chronic myelogenous leukemia is characterized by inappropriate early release of immature granulocyte precursors from the bone marrow and by a major delay of exit of mature granulocytes from the blood into the tissues (1). CML[3] granulocytes show a marked decrease in binding to PNA when compared to normal granulocytes; they are less adhesive to nylon wool (2) than normal cells; their cell membranes show decreased labeling after treatment with galactose oxidase-NaB^3H_4 and increased labeling after sodium periodate-NaB^3H_4 treatment (3). All these effects can be partially or completely reversed by treatment with *Vibrio cholera* neuraminidase. These studies suggest that the cell surface oligosaccharides are more fully sialylated in CML cells and this may account in part for the pathophysiological behavior of the circulating cells. The aberrant sialylation involves the major granulocyte glycoprotein with a molecular weight of 150,000 which has been shown to be important for granulocyte adhesion (4, 5). CML granulocytes show decreased binding of the synthetic chemotactic peptide FMLP (6).

Sialylation of glycoproteins is effected by sialyltransferases specific to the structure of the substrate (Table 1; Ref. 7). Two enzymes are known to transfer sialic acid from CMP-NANA to Galβ1-3GalNAc-R: (*a*) CMP-NANA:Galβ1-3-GalNAc-R α(2-3)-sialyltransferase (sialic acid to galactose) (EC 2.4.99.4), where R can be polypeptide or a nitrophenyl group, and (*b*) CMP-NANA:R_1β1-3GalNAc-R_2 α(2-6)-sialyltransferase I (sialic acid to *N*-acetylgalactosamine (EC 2.4.99.3), where R_1 can be H-, Gal-, or sialylα2-3Gal-and R_2 must be a polypeptide. The α3-sialyltransferase has been reported in rat, porcine and fetal calf liver, human placenta, and porcine and ovine submaxillary glands (8–12). The α6-sialyltransferase I has been found in the submaxillary glands of several species (9, 11, 13–16). The structures formed by these enzymes, sialylα-2-3-Galβ1-3GalNAc- and Galβ1-3(sialylα2-6)GalNAc-, respectively, have been detected on many mucins and on glycoproteins such as antifreeze glycoprotein, RBC glycophorins, etc. (7). Additionally, a third sialyltransferase has been described which acts on *O*-glycosyl oligosaccharides. This enzyme, CMP-NANA:sialylα2-3Galβ1-3GalNAc-R α(2-6)-sialyltransferase II, also transfers a residue of sialic acid to *N*-acetylgalactosamine (EC 2.4.99.7); R can be a polypeptide or a nitrophenyl group (10, 14). This enzyme has been found in fetal calf liver, but is probably quite widely distributed (10, 14). It can act neither on Galβ1-3GalNAc-R nor on GalNAc-R.

In a previous study (17) we compared the sialyltransferase activities of membranes prepared from CML granulocytes that appeared mature by microscopic criteria and from normal granulocytes of identical morphology. The CML preparations showed significantly higher sialyltransferase activities toward asialofetuin, asialo fucose-free blood group A-negative porcine submaxillary mucin and antifreeze glycoprotein, all of which are known to contain appreciable amounts of *O*-linked Galβ1-3GalNAc oligosaccharides. Asialo-ovine submaxillary mucin, which contains mainly *O*-linked GalNAc residues and only a small amount of Galβ1-3GalNAc, was a poor sialyltransferase substrate with either normal or CML cells. There was no significant difference in sialyltransferase activity between CML and normal cells when asialo-α_1 acid glycoprotein was used as an acceptor; this substrate contains only *N*-linked oligosaccharides with terminal Gal residues. These observations suggested that the increased sialyltransferase activity in CML cells was acting on *O*-linked Galβ1-3GalNAc groups.

The present studies were carried out to prove this hypothesis and to establish the nature of the sialic acid linkage to Galβ1-

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[3] The abbreviations used are: CML, chronic myelogenous leukemia; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; GalNAcOH, *N*-acetylgalactosaminitol; NANA, *N*-acetylneuraminic acid; PNA, peanut agglutinin; HPLC, high performance liquid chromatography; Galβ1-3GalNAc, galactosyl-β1-3-*N*-acetyl-D-galactosamine; NeuAc, *N*-acetylneuraminic acid; FMLP, formyl-methionine-leucine-phenylalanine.

Table 1 *Specificities of sialyltransferases acting on O-glycans*

Linkage	EC no.	Substrate	R	Refs.
α2–3 to Gal	2.4.99.4	Galβ1–3GalNAc-R	Polypeptide, nitrophenyl	8–12
α2–6 I to GalNAc	2.4.99.3	R_1β1–3GalNAc-R_2	R_1 can be H-, Gal-, or sialylα2-3Gal-; R_2 must be a polypeptide	9, 11, 13–16
α2–6 II to GalNAc	2.4.99.7	Sialylα2–3Galβ1-3-GalNAc-R	Polypeptide, nitrophenyl	10, 14

3GalNAc. For the identification of linkage, we have used antifreeze glycoprotein since it is capable of acting as a substrate for both the α(2–3) and the α(2–6) I sialyltransferases. The product of the α(2–3)-sialyltransferase reaction (sialylα2–3Galβ1–3GalNAc) is a substrate for the α(2–6) II sialyltransferase. We demonstrate that human granulocytes have a sialyltransferase that specifically transfers sialic acid in a α(2–3) linkage to the terminal Gal residue of *O*-linked Galβ1–3GalNAc. Furthermore we show that this enzyme activity (using the specific synthetic substrate Galβ1–3GalNAc-α-*ortho*-nitrophenyl) is significantly increased in CML granulocytes compared to normal granulocytes.

MATERIALS AND METHODS

Granulocytes. More than 95% morphologically mature granulocytes from both CML and normal samples were obtained from EDTA-anticoagulated peripheral venous blood after dextran sedimentation, ammonium chloride lysis, and layering leukocytes onto a double gradient of Hypaque and dextran, as previously described (2, 17). Cells were frozen and thawed three times in 0.2 M NaCl, washed in saline, treated with DNase (5 units/10^8 cells), washed again in saline, solubilized in 1% Triton X-100 at 4°C, and debris was removed by centrifugation (17, 18). The supernatant, a solubilized total cell membrane preparation, was used for assays. The protein contents of supernatants from normal and CML granulocytes were, respectively, 115 and 110 μg for 10^8 cells.

Materials. Antifreeze glycoprotein was prepared from Atlantic cod serum and supplied by Dr. Chow Hew, Department of Clinical Biochemistry, University of Toronto, by methods previously described (19). Galβ1–3GalNAc-α-*o*-nitrophenyl was supplied by Dr. K. L. Matta, Roswell Park Memorial Institute, Buffalo, NY, and has been characterized by high resolution proton nuclear magnetic resonance spectroscopy at 360 MHz. GalNAc-α-phenyl was purchased from Koch-Light Laboratories. Sialylα2–6GalNAcOH, [^{3}H]sialylα2–3Galβ1–3GalNAcOH, Galβ1–3([^{14}C]sialylα2–6)GalNAcOH, and [^{14}C]-sialylα2–3Galβ([^{14}C]sialylα2–6) GalNAcOH were kindly provided by Dr. Dirk H. van den Eijnden, Vrije Universiteit, Amsterdam, The Netherlands.

Sialyltransferase Assays. Enzyme incubations contained in a final volume of 50 μl: 20 μl cell lysate (40-60 μg protein in 1.0% Triton X-100 to give a final Triton X-100 concentration of 0.4%); 0.05 M Tris-HCl, pH 7.2; 0.5 mM CMP-[4-^{14}C]NANA (0.05 μCi, 25 mCi/mmol; New England Nuclear); and substrate 2 mM Galβ1–3GalNAc-α-*o*-nitrophenyl. The K_m for CMP-[4-^{14}C]NANA is 0.18 mM, and for Galβ1–3GalNAc-α-*o*-nitrophenyl it is 0.3 mM. The same number of morphologically mature CML and normal granulocytes were used for sialyltransferase assays. Reaction mixtures were incubated at 37°C for 1 h and the reaction was terminated by freezing. Radioactive low molecular weight product was separated (17, 18) at 4°C on columns (16.5 x 0.5 cm) of AG 2-X8, Cl^-form, 200-400 mesh (Bio-Rad Laboratories). Columns were washed with 3 M NaCl, followed by 15 mM Tris-HCl, pH 7.0, at 4°C. The first 5 ml of eluate contained all the labeled product. Products were counted by liquid scintillation using ACS (Amersham) as scintillation fluid. Endogenous acceptor controls showed less than 5% incorporation relative to exogenous acceptor assays; this incorporation was subtracted in calculation of enzyme activities. CML and normal granulocytes were handled in an identical manner and were tested simultaneously.

Preparation of Radioactive Oligosaccharide Alditols from Product. Sialyltransferase incubations were performed in a total volume of 0.5 ml containing 0.2 ml microsomes (rat liver, 0.7 mg protein, or pig submaxillary glands, 2.5 mg protein) or granulocyte lysates (0.8 mg protein in 1% Triton X-100 to give a final Triton X-100 concentration of 0.4%), 0.05 M Tris-HCl, pH 7.2, 0.5 mM CMP-[4-^{14}C]NANA (25 mCi/mmol), and antifreeze glycoprotein (5.3 mM galactose termini). Reaction mixtures were incubated at 37°C for 3 h, cooled to 0°C, and sialylated glycoprotein products were isolated by gel filtration on Sephadex G-25 columns (30 x 1cm; Pharmacia) equilibrated in water. Radioactive oligosaccharides were released by β elimination by incubation in 6 ml 0.05 N KOH-1 M $NaBH_4$ at 45°C for 16 h. The pH was adjusted to 5 by addition of 4 M acetic acid, and cations were removed by passage through columns (1.6 x 10 cm) of AG 50W/X2, H^+ form, 100-200 mesh (Bio-Rad Laboratories), at 0°C. The columns were washed with 80 ml 0.01 M acetic acid and the combined eluates and washings were freeze-dried. Borate was removed as methyl borate by repeated flash evaporation from methanol. The reduced oligosaccharides were further purified on a column of Bio-Gel P-4 (−400 mesh, 1.6 x 86 cm; Bio-Rad Laboratories) equilibrated in 0.05 M ammonium acetate at pH 5.4. The sialyl-oligosaccharide alditols were then analyzed by HPLC.

High Performance Liquid Chromatography. HPLC of sialyl-oligosaccharide alditols was carried out using a Waters system equipped with a Model 6000A solvent delivery system, Model 510 pumps, Model 680 automated gradient controller, Model 730 data module, and a Model U6K injector. Reduced oligosaccharides were fractionated at a flow rate of 1 ml/min under 1600-1700 psi of pressure on a 5-μm Alltech amino column (250 x 4.6 mm) protected with a CSK guard column packed with octadecylsilicic acid (Whatman). The chromatogram was developed isocratically for 20 min with 80% acetonitrile-20% 15 mM aqueous potassium phosphate at pH 5.2. A linear gradient was then applied which decreased the acetonitrile concentration to 32% at 100 min. Separations were monitored both by UV absorption at 195 nm (Waters Lamda-Max Model 480 LC spectrophotometer), and by subjecting fractions (volume, 1 ml) collected with a fraction collector to liquid scintillation counting in ACS. Radioactive peaks were usually confined to a volume of about 2 ml.

RESULTS

Product Identification. In order to establish the structures formed by the sialyltransferase activities in both CML and normal granulocytes, relatively large-scale incubations were carried out with cell extracts in the presence of CMP-[^{14}C]-NANA and antifreeze glycoprotein, as described in "Materials and Methods." Radioactive oligosaccharide alditols were released from the products by β elimination (alkaline $NaBH_4$). The oligosaccharides were fractionated on Bio-Gel P-4 columns followed by HPLC (Table 2). Under the conditions of HPLC used, the oligosaccharide products formed by the action of both the α3- and α6-sialyltransferase on Galβ1–3GalNAc can be readily separated and identified by comparison to standard compounds (9, 20).

Control experiments were first carried out by using as enzyme sources microsome preparations from rat liver and pig submaxillary gland. Rat liver microsomes produced only one radioactive oligosaccharide product, [^{14}C]sialylα2–3Galβ1–3GalNAcOH, (elution time, 47 min; Table 2), indicating the presence of α3-sialyltransferase and the absence of α6-sialyltransferase I. Rat liver has been reported to contain a sialyltransferase acting on antifreeze glycoprotein (21), but the prod-

Table 2 *Elution times of sialyl-oligosaccharides on HPLC*

Large-scale sialyltransferase incubations were performed as described in "Materials and Methods," using as enzyme source either rat liver microsomes, pig submaxillary gland microsomes, or granulocyte lysates. Sialylated glycoprotein products were isolated by gel filtration on Sephadex G25 columns. Oligosaccharides were released by β elimination. The reduced oligosaccharides were further purified on a column of Bio-Gel P4 and subjected to analysis by HPLC.

	Injected radioactivity (cpm)	elution time (min)	Eluted radioactivity (cpm)
Standards			
N-Acetylneuraminic acid		34	
Sialylα2–6GalNAcOH		41	
[^{3}H]Sialylα2–3Galβ1–3GalNAcOH	2,000	47	1,362
Mixture of [^{14}C]sialylα2–3Galβ1–3 GalNAcOH and Galβ1–3 ([^{14}C]sialylα2–6) GalNAcOH in a 15:85 ratio	2,000	47	202
		54	1,169
[^{14}C]Sialylα2–3Galβ1–3-([^{14}C]sialylα2–6)GalNAcOH	2,000	67	1,234
Enzyme products obtained with[a]			
Rat liver microsomes[b]	8,000	47	5,027
		54	0
		67	0
Pig submaxillary gland microsomes[c]	10,000	47	4,700
		54	1,435
		67	0
Normal granulocytes[d]	2,800	47	1,737
		54	0
		67	0
CML granulocytes[e]	4,500	47	3,339
		54	0
		67	0

[a] Endogenous activity showed less than 5% incorporation relative to exogenous activity in following samples.
[b] Enzyme activity was 15.1 nmol/mg/h.
[c] Enzyme activity was 7.4 nmol/mg/h (total).
[d] Enzyme activity was 3.2 nmol/mg/h.
[e] Enzyme activity was 5.2 nmol/mg/h.

uct was not definitely identified. However, pig and fetal calf liver have been shown to contain α3-sialyltransferase but not α6-sialyltransferase I (8, 10, 14).

Incubations with pig submaxillary gland microsomes produced two radioactive oligosaccharide products, [^{14}C]sialylα2–3Galβ1–3GalNAcOH (elution time, 47 min) and Galβ1–3([^{14}C]sialylα2–6)GalNAcOH (elution time, 54 min), in a 7:3 ratio (Table 2), confirming the presence of both α3-sialyltransferase (11, 12) and α6-sialyltransferase I (11, 13, 14) in this tissue. Although α6-sialyltransferase II is probably present in both rat liver and pig submaxillary gland (10, 14), this enzyme will not directly use antifreeze glycoprotein and other Galβ1–3GalNAc-R substrates.

Both normal and CML granulocytes produced only [^{14}C]-sialylα2–3Gal$\beta\alpha$1–3-GalNAcOH (elution time, 47 min). Galβ1–3([^{14}C]sialylα2–6)GalNAcOH, [^{14}C]sialylα2–3Galβ1–3([^{14}C]-sialylα2–6)GalNAcOH, and [^{14}C]sialylα2–6GalNAcOH could not be detected (Table 2). Both preparations therefore contain α3-sialyltransferase but lack α6-sialyltransferase I. Although the substrate for α6-sialyltransferase II, [^{14}C]sialylα2–3Galβ1–3GalNAc-protein, was produced by the action of α3-sialyltransferase, there was no evidence of α6-sialyltransferase II activity. The α3-sialyltransferase activities as measured by oligosaccharide production were 3.2 and 5.2 nmol/mg protein/h for normal and CML granulocytes, respectively (1.6-fold increase in CML cells), in excellent agreement with previous data on antifreeze glycoprotein (17).

Sialyltransferase Assays. Granulocyte membrane preparations from normal subjects and CML patients were assayed for sialyltransferase activity by using the synthetic low molecular weight substrate Galβ1–3GalNAc-α-*o*-nitrophenyl as acceptor

Table 3 *Sialyltransferase activity with substrate Galβ1–3GalNAc-α-*o*-nitrophenyl*[a]

Source of cells	No. of samples[b]	Sialyltransferase activity (nmol/mg/h)
CML untreated	6	4.2 ± 1.6[c]
CML on chemotherapy	9	3.4 ± 0.8
Normal donors	6	1.5 ± 0.7[d]

[a] Galβ1–3GalNAc-α-*o*-nitrophenyl concentration used was 2 mM.
[b] Samples were tested from 6 patients with untreated CML; from 3 patients with CML on chemotherapy with 3 samples from each at different times; and from 12 normal donors pooled in pairs to obtain sufficient cells for assay.
[c] Mean ± SD.
[d] $P < 0.05$ for normal compared to CML untreated or on chemotherapy.

(Table 3). Normal granulocytes exhibited activity with this substrate at 1.5 ± 0.7 nmol/mg/h, whereas CML granulocytes demonstrated activity of 4.2 ± 1.6 nmol/mg/h. Patients with CML receiving chemotherapy had a somewhat lower activity of 3.4 ± 0.8 nmol/mg/h. Activity in CML cells was significantly higher than in normal cells ($P < 0.05$). No sialyltransferase activity could be detected in normal or CML cell lysates when GalNAc-α-phenyl was used.

Incubations containing a mixture of membrane preparations from CML and normal cells showed additive sialyltransferase activity, indicating the absence of either a stimulator in CML cells or an inhibitor in normal cells (normal granulocytes, 909 cpm/0.079 mg protein/h, CML granulocytes, 1109 cpm/0.070 mg protein/h, and normal plus CML granulocytes, 2136 cpm/0.149 mg protein/h under standard assay conditions).

DISCUSSION

We have previously shown that sialyltransferase activity in CML granulocytes is approximately 1.5-fold higher than in normal granulocytes, using as substrates asialofetuin, asialo fucose-free blood group A-negative porcine submaxillary mucin, and antifreeze glycoprotein, all of which are known to contain appreciable amounts of *O*-linked Galβ1–3GalNAc oligosaccharides. Substrate rich in *O*-linked GalNAc groups (asialo ovine submaxillary mucin) was a poor acceptor for enzyme from CML and normal granulocytes. *N*-Linked oligosaccharides (asialo-α_1-acid glycoprotein) showed no difference in activity between CML and normal cells (17).

In this study, antifreeze glycoprotein (which contains only *O*-linked glycans of the Galβ1–3GalNAc type) was used as a substrate to further characterize this sialyltransferase activity. We were able to demonstrate that both CML and normal granulocytes contain a transferase which sialylates *O*-linked Galβ1–3GalNAc groups via an α(2–3) linkage to the terminal galactose residue. These results demonstrate for the first time, that human leukocytes contain the enzyme CMP-*N*-acetylneuraminic acid:Galβ1–3GalNAc-α(2–3)sialyltransferase. Furthermore, we have confirmed an increase in the α(2–3)sialyltransferase activity in CML granulocytes compared to normal granulocytes, when antifreeze glycoprotein is used as a substrate.

We were unable however to detect products containing the α(2–6) linkage (to GalNAc). This therefore suggests that neither normal nor CML granulocytes contain α(2–6)sialyltransferase I. Similarly, we were unable to detect the products of the α(2–6)sialyltransferase II. While this enzyme cannot utilize antifreeze glycoprotein directly as a substrate, it will nevertheless use the product of the α(2–3) transferase activity. However, we cannot rule out the possibility that insufficient substrate was generated by the action of the α(2–3) transferase to detect the α(2–6) transferase II activity. Alternatively, α(2–6) I and α(2–

6) II activities could be masked by the presence of specific sialidases in the leukocyte membrane preparations.

Using the synthetic substrate Galβ1–3GalNAc-α-orthonitrophenyl, we have shown that granulocytes from patients with chronic myelogenous leukemia, when compared to morphologically identical normal granulocytes, have a 2.8-fold increase in α(2–3) sialyltransferase activity.

The cause of enzyme increase in CML cells is not known. It is possible that CML and normal granulocytes differ in the rate of transferase synthesis, or in the availability of substrates or cofactors *in vivo*, or that the two cell types have different isoenzymic forms of α3-sialyltransferase. Increased activity of the enzyme probably causes the hypersialylation of CML granulocytes and decreased binding of CML cells to PNA, to nylon wool, and to FMLP, since these properties revert to normal after neuraminidase treatment (2, 3). It is of interest that sialyltransferase activity in CML patients treated with chemotherapy is lower than in untreated patients (Table 3), but still elevated 2-fold compared to normal granulocytes. Values for binding of granulocytes from treated CML patients to PNA, nylon wool, and FMLP are intermediate between values for untreated CML cells and normal granulocytes (22).

Further study of patients will be undertaken to show whether the onset of blastic crisis correlates with an increase in α(2–3) sialyltransferase activity. Aberrant sialylation may account for the abnormal release of CML precursor cells from the bone marrow, and for the prolonged circulation time and functional abnormalities of CML granulocytes (1, 23) indicating a pathophysiological role for α3-sialyltransferase in CML.

Our transferase data correlate well with a recent structural study by Fukuda *et al.* (24), showing the presence in both normal and CML granulocytes of *O*-glycans containing NeuAcα2–3Galβ1–3GalNAc termini. These authors did not detect NeuAcα2–6GalNAc in these *O*-glycans.

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