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Biosynthesis of sialylated and fucosylated selectin ligands of HL-60 cells in vitro

Midchain α3-fucose units inhibit terminal α6-sialylation but not α3-sialylation of polylactosamines

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Abstract Polylactosamines Neu5Acα2-3′Lexβ1-3′Lexβ1-3′Lex and Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lex [Lex, Galβ1-4(Fucα1-3)GlcNAc; LN, Galβ1-4GlcNAc] decorate selectin counterreceptors in human HL-60 cells. Here, we show that HL-60 cell lysates catalyze distal α3-sialylation of LNβ1-3′LNβ1-3′LN and LNβ1-3′Lexβ1-3′Lex efficiently, outlining two potential sets of biosynthetic pathways leading to the selectin ligands. In one set, α3-sialylation precedes internal fucosylation of the polylactosamine backbone, whereas in the other one, internal fucosylation is initiated before α3-sialylation. In contrast to α3-sialylation, LNβ1-3′Lexβ1-3′Lex was α6-sialylated much less efficiently than LNβ1-3′LNβ1-3′LN by HL-60 cell lysates. Hence, internal fucosylation may regulate the extent of α6-sialylation of polylactosamines in these cells.

Key words: HL-60 cell; In vitro biosynthesis; Selectin ligand

1. Introduction

Several sialylated and multifucosylated polylactosamines are counterreceptors for the selectin family of cell adhesion molecules in the vascular system. (i) The sequence Neu5Acα2-3′Lexβ1-3′Lex [Lex, Galβ1-4(Fucα1-3)GlcNAc] of *N*-glycans in neutrophils and in the monocytic cell line U937 shows high affinity for E-selectin [1]. (ii) Multifucosylated gangliosides, like Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lexβ1-3′LNβ1-3′Lβ1-ceramide [LN, *N*-acetyllactosamine; L, lactose] of human promyelocytic leukemia cells of line HL-60 also possess high affinity for E-selectin [2]. (iii) A related sequence, Neu5Acα2-3′Lexβ1-3′Lexβ1-3′Lex, is the major sialylated and multiply fucosylated polylactosamine epitope decorating the P-selectin counterreceptor PSGL-1 of HL-60 cells [3], and is assumed to be important in the recognition between PSGL-1 and P-selectin. (iv) A synthetic polylactosamine carrying two Neu5Acα2-3′Lexβ1-3′Lex epitopes is a highly potent inhibitor of L-selectin-mediated adhesion of lymphocytes to lymph node endothelium [4]. (v) Studies of ‘knock-out’ mice have shown that besides Fuc-TVII [5], also the expression of Fuc-TIV is required for biosynthesis of fully functional leukocyte selectin ligands in vivo [6].

Currently, the relative timing of sialylation and fucosylation events in the biosynthesis of the sialylated and fucosylated polylactosamines is only partially understood. The fucosylation of the distal LN unit probably occurs after the sialylation [7], because no sialyltransferase capable of α3-sialylation of the terminal Lex unit has been reported. By contrast, α3-sialylated linear i-chains of polylactosamines can be α3-fucosylated at distal sialylated LNs by Fuc-TVII and at internal positions by Fuc-TIV [8–13]; these two enzymes are the major fucosyltransferases of leukocytes [14,15]. However, Fuc-TIV also transfers to all LN units of non-sialylated i-chains of polylactosamines [10,12], raising the possibility of *internal* fucosylation prior to the sialylation step, either during the elongation of the backbone or right afterwards.

Here, we report that both the non-fucosylated polylactosamine LNβ1-3′LNβ1-3′LN and the internally pre-fucosylated LNβ1-3′Lexβ1-3′Lex were efficiently α3-sialylated by HL-60 cell lysates. This implies that internal backbone fucosylation, intrinsically, may either precede or follow distal α3-sialylation during the biosynthesis of selectin ligands in HL-60 cells. In contrast to α3-sialylation, LNβ1-3′Lexβ1-3′Lex was α6-sialylated less efficiently than LNβ1-3′LNβ1-3′LN. This, in turn, suggests that the expression of α6-sialylated polylactosamines may be regulated in part by internal fucosylation of the chains.

2. Materials and methods

2.1. The cell lysates

Human HL-60 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI 1640 medium (Life Technologies Ltd., Paisley, UK), supplemented with 10% fetal calf serum, 10 mM HEPES, 2 mM L-glutamine and 100 μg/ml gentamicin at 37°C in 5% CO_2. They were subcultured twice a week at a ratio of 1:3. The cells were lysed in the presence of a mixture of protease inhibitors (16 μg/ml benzamidine HCl, 10 μg/ml phenanthroline, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM PMSF; Pharmingen,

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Abbreviations: Fuc, L-fucose; Fuc-T, α1,3-fucosyltransferase; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HPAEC, high-pH anion exchange chromatography; L, Galβ1-4Glc; Lex, Galβ1-4(Fucα1-3)-GlcNAc; LN, Galβ1-4GlcNAc; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; ManNAc, *N*-acetyl-D-mannosamine; NDV, Newcastle disease virus; Neu5Ac, *N*-acetylneuraminic acid; PAD, pulsed amperometric detection; PSGL-1, P-selectin glycoprotein ligand-1

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San Diego, CA, USA) in 1% Triton X-100 on ice (lysate 1) or sonicated in ice cold water, and then lysed in 1% Triton X-100 on ice (lysate 2). Protein concentrations were determined with the BCA Protein Assay Reagent Kit 23225 (Pierce, Rockford, IL, USA).

2.2. *Oligosaccharides*

The acceptor saccharides LNβ1-3′LNβ1-3′LN [16] and LNβ1-3′Lexβ1-3′Lex were described previously [17]. The marker Neu5-Acα2-3′LNβ1-3′LNβ1-3′LN was synthesized as described in [17]. Its MALDI-TOF mass spectrum revealed a major signal at *m/z* 1403.6 that was assigned to the expected pseudomolecular ion [M-H]$^-$ (calc. monoisotopic *m/z* 1403.5). Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lex marker was also described in [17]. Its MALDI-TOF mass spectrum revealed a major peak at *m/z* 1695.6 that was assigned to the [M-H]$^-$ ion of the expected nonasaccharide (calc. monoisotopic *m/z* 1695.6); another signal at *m/z* 1531.5 was assigned to [M-H]$^-$ of an analog that had lost one fucose unit by β-elimination at the reducing end (calc. monoisotopic *m/z* 1531.5). Neu5Acα2-6′LNβ1-3′LNβ1-3′LN marker was synthesized from LNβ1-3′LNβ1-3′LN using α6-sialyltransferase of rat liver (Boehringer-Mannheim, Germany). The MALDI-TOF mass spectrum showed a major signal at *m/z* 1403.3 (calc. monoisotopic *m/z* for [M-H]$^-$ of Neu5AcGal$_3$GlcNAc$_3$ 1403.5). The ^{1}H-NMR spectrum revealed signals of the sialylated Gal H1, Neu5Ac H3-eq and Neu5Ac H3-ax at 4.455, 2.670, and 1.722 ppm, respectively; they closely resembled the signals of Neu5Acα2-6′LNβ1-3′LNβ-OR reported by Kashem et al. [18]. The Neu5Acα2-6′LNβ1-3′Lexβ1-3′Lex marker was synthesized from LNβ1-3′Lexβ1-3′Lex by the α6-sialyltransferase of rat liver. Its ^{1}H-NMR spectrum revealed signals of the sialylated Gal H1, Neu5Ac H3-eq and Neu5Ac H3-ax at 4.455, 2.668 and 1.722 ppm, respectively. For Neu5Acα2-6′LNβ1-3′Lexβ-OR, very similar data have been reported [18]. The MALDI-TOF mass spectrum revealed a major signal at *m/z* 1695.2 that was assigned to the [M-H]$^-$ ion of the expected nonasaccharide (calc. average *m/z* 1696.6); another signal at *m/z* 1531.5 was assigned to the [M-H]$^-$ ion of the β-elimination product that had lost one fucose unit at the reducing end (calc. average *m/z* 1532.4).

2.3. *Sialyltransferase reactions catalyzed by HL-60 cell lysates and general plan of the product analysis*

The unlabelled polylactosamine acceptors (2–20 nmol), the donor CMP-[^{14}C]Neu5Ac [5 nmol, 500 000 dpm; a mixture of CMP-Neu5-Ac, disodium salt (Sigma, St. Louis, MO, USA) and CMP-[^{14}C]Neu5Ac, ammonium salt, Amersham International (Buckinghamshire, UK)] and the HL-60 cell lysate (5 μl, 44–50 μg protein) were incubated in a total volume of 10 μl in 50 mM Na-cacodylate pH 6.5 for 60 min at 37°C. The incubation conditions resembled those described for different acceptors by other researchers [19,20], and the extent of [^{14}C]Neu5Ac transfer was limited to less than 0.3% consumption of the donor. The reactions were terminated by cooling on ice and by adding ice cold ethanol (10 μl). Finally, 100 μl water was added and the samples were frozen and stored at −25°C until the time of the analysis.

The product oligosaccharides were isolated by gel filtration HPLC, ensuing anion exchange HPLC, and were quantitated by liquid scintillation counting. The formation of ^{14}C-labelled α-monosialoproducts was established by cleavage with sialidase from *Arthrobacter ureafaciens*, and by MALDI-TOF mass spectrometry. For subsequent differentiation between linkage isomers, the products were treated with Newcastle disease virus (NDV) sialidase, which cleaves α3-bonded, but not α6-linked Neu5Ac units; the liberated [^{14}C]Neu5Ac and the resistant [^{14}C]oligosaccharide were isolated chromatographically from the enzymatic digests. The NDV-sialidase-resistant fraction obtained from [^{14}C]sialylated LNβ1-3′LNβ1-3′LN was identified in a co-chromatography experiment with synthetic Neu5Acα2-6′LNβ1-3′LNβ1-3′LN.

2.4. *Chromatographic methods*

Gel filtration was performed in a column of Superdex Peptide HR 10/30 (Pharmacia, Uppsala, Sweden) at a flow of 1 ml/min with 50 mM NH$_4$HCO$_3$. The runs of marker oligosaccharide samples ranging between 10 pmol and 10 nmol were monitored by UV absorption at 205 or 214 nm. *N*-Acetylglucosamine eluted at 17.1 min, *N*-acetylneuraminic acid at 16.0 min. Neu5Acα2-3′LNβ1-3′LNβ1-3′LN and Neu5Acα2-6′LNβ1-3′LNβ1-3′LN markers peaked at 12.6 ± 0.1 min and the peaks of both sialoglycans were confined between 11.9 ± 0.1 min and 13.3 ± 0.1 min, regardless of the sample size. The fucosylated sialoglycan markers Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lex and Neu5-Acα2-6′LNβ1-3′Lexβ1-3′Lex peaked at 12.4 ± 0.1 min and the peaks were confined consistently between 11.7 ± 0.1 min and 13.1 ± 0.1 min. Anion exchange chromatography on a Mono Q (5/5) column (Pharmacia) was performed essentially as in [21]. Sharp, symmetrical peaks of Neu5Acα2-3′ and Neu5Acα2-6′ derivatives of LNβ1-3′LNβ1-3′LN, as well as Neu5Acα2-3′ and Neu5Acα2-6′ derivatives of LNβ1-3′Lexβ1-3′Lex (10 pmol to 10 nmol) eluted between 8.6 and 9.7 min from the column; Neu5Ac marker eluted at 11.1 min. High-pH anion exchange chromatography (HPAEC) was performed on a (4 × 250 mm) Dionex CarboPac PA-1 column (Dionex, Sunnyvale, CA, USA), using a linear gradient of 25–200 mM NaOAc in 100 mM NaOH over 150 min as previously described [17]. The marker saccharides were detected by monitoring the whole run with a pulsed amperometric detector (PAD); radioactive sialyltransferase products were collected by recovering the appropriate peaks manually; these were neutralized with 0.4 M acetic acid, and were quantitated by scintillation counting.

2.5. *Enzymatic degradations*

Degradations with *A. ureafaciens* sialidase (EC 3.2.1.18, Boehringer-Mannheim) were performed as previously described [22]. Hydrolyses with NDV sialidase (EC 3.2.1.18, Oxford Glycosysytems, Abingdon, UK) were carried out by dissolving dry monosialylated saccharides (2–5 pmol) in 19 μl 50 mM sodium acetate pH 5.5 and adding 1 mU of the enzyme in 1 μl of 10 mM phosphate buffer pH 7.0. The reaction mixtures were incubated overnight at 37°C [23–25]. The oligosaccharide products were separated from the liberated [^{14}C]Neu5Ac by Superdex chromatography. The NDV sialidase cleaves only α3-bonded sialic acids from monosialoglygans [23,25], but in oligo- and polysialic acid groups it also cleaves α8-linkages [24].

2.6. *Analytical methods*

Aliquots of the fractions containing radiolabelled oligosaccharides and liberated [^{14}C]Neu5Ac were scintillation counted; in experiment 1 the aqueous samples were mixed with two volumes of Optiphase Hisafe (Wallac, Turku, Finland) and in experiment 2, the samples were deposited on small strips of filter paper and counted as described in [26]. For MALDI-TOF mass spectrometry with a BIFLEX instrument (Bruker-Franzen Analytik, Bremen, Germany), neutral oligosaccharides (5–15 pmol) were mixed in a 2,5-dihydroxybenzoic acid matrix using the positive ion delayed extraction mode [17]. Sialylated oligosaccharides were investigated in a 2,4,6-trihydroxyacetophenone/acetonitrile/aqueous ammonium citrate matrix as described in [27], utilizing the linear negative ion mode with delayed extraction; marker samples of 5–10 pmol were used in the experiments, but the samples of the actual products amounted only to 0.8 pmol. NMR spectroscopy was performed in D$_2$O at 23°C as described in [17].

3. Results

3.1. *Sialyltransferase activities present in HL-60 cell lysates*

Two polylactosamines, the fucose-free hexasaccharide LNβ1-3′LNβ1-3′LN and the doubly fucosylated octasaccharide LNβ1-3′Lexβ1-3′Lex, were incubated with CMP-[^{14}C]Neu5Ac and HL-60 cell lysates. The product oligosaccharides were isolated by gel filtration and anion exchange chromatography and were quantitated by liquid scintillation counting. The data of Table 1 show that significant transfer rates of sialic acid were observed with both acceptors.

3.2. *Characterization of the [^{14}C]sialylated products by MALDI-TOF mass spectrometry and sialidase treatments*

The MALDI-TOF mass spectrum of the [^{14}C]sialylation product of LNβ1-3′LNβ1-3′LN revealed in the molecular ion region a principal signal of *m/z* 1403.3 (Fig. 1A) that was assigned to the molecular ion [M-H]$^-$ of the monosialoglycan Neu5Acα2-3/6′LNβ1-3′LNβ1-3′LN (calc. monoisotopic *m/z* 1403.5). In the same way, the [^{14}C]sialylation product of LNβ1-3′Lexβ1-3′Lex revealed a major signal of *m/z*

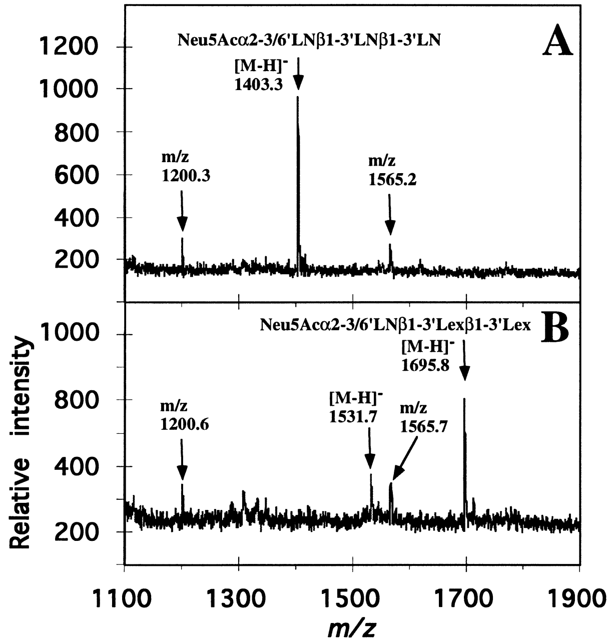

Fig. 1. The molecular ion regions of MALDI-TOF mass spectra of 0.8 pmol samples of [^{14}C]sialopolylactosamines generated by HL-60 cell lysates. A: Purified monosialylated oligosaccharide products from LNβ1-3′LNβ1-3′LN. B: Purified monosialylated oligosaccharide products from LNβ1-3′Lexβ1-3′Lex.

1695.7 (Fig. 1B) that was assigned to the molecular ion [M-H]$^-$ of Neu5Acα2-3/6′LNβ1-3′Lexβ1-3′Lex (calc. monoisotopic *m/z* 1695.6). A smaller peak at *m/z* 1531.7 was tentatively assigned to a sialylated β-elimination product devoid of the fucosyl unit at the reducing end (calc. monoisotopic *m/z* 1531.5). These features of Fig. 1B closely resemble those obtained from the marker compounds Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lex and Neu5Acα2-6′LNβ1-3′Lexβ1-3′Lex. Both product spectra of Fig. 1 also revealed small unidentified signals around *m/z* 1200 and 1565.

A treatment with the sialidase from *A. ureafaciens* released more than 95% of [^{14}C]Neu5Ac from both products (Table 1), establishing the presence of α-linked sialic acid. The [^{14}C]monosialylated product oligosaccharides were treated with NDV sialidase to liberate the α3-bonded [^{14}C]sialic acid. The data of Table 1 show that comparable amounts of α3-sialylated products were formed from the fucose-free LNβ1-3′LNβ1-3′LN and the internally pre-fucosylated LNβ1-3′Lexβ1-3′Lex by the sialyltransferase activities of HL-60 cell lysates. Hence, the presence of internal α3-bonded fucosyl groups in the acceptor polylactosamine did not inhibit α3-sialylation to any major extent. This was true at 0.20 mM and 2.0 mM acceptor concentrations.

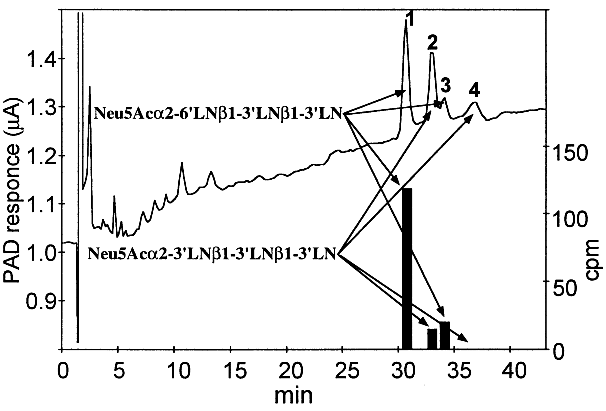

Fig. 2. HPAEC chromatography of a mixture consisting of (i) the NDV sialidase-resistant, ^{14}C-labelled sialyltransferase products from LNβ1-3′LNβ1-3′LN and (ii) the unlabelled markers Neu5Acα2-3′LNβ1-3′LNβ1-3′LN and Neu5Acα2-6′LNβ1-3′LNβ1-3′LN. The trace shows the detector response and the columns represent the radioactivity of individual fractions collected manually. Peak 1 contains Neu5Acα2-6′LNβ1-3′LNβ1-3′LN and peak 3 its ManNAc epimer, whereas peaks 2 and 4 correspond to the Neu5Acα2-3′LNβ1-3′LNβ1-3′LN and the ManNAc epimer, respectively.

3.3. Chromatographic identification of Neu5Acα2-6′LNβ1-3′LNβ1-3′LN in the NDV sialidase-resistant fraction obtained from LNβ1-3′LNβ1-3′LN

The NDV sialidase-resistant fraction of sialyltransferase products is often considered to consist of α6-sialylated saccharides. To confirm this notion, we mixed non-radiolabelled marker saccharides Neu5Acα2-3′LNβ1-3′LNβ1-3′LN and Neu5Acα2-6′LNβ1-3′LNβ1-3′LN with the α-[^{14}C]sialylated, NDV sialidase-resistant products of LNβ1-3′LNβ1-3′LN (239 cpm) generated by HL-60 cells, and subjected the mixture to HPAE chromatography. As shown in Fig. 2, most of the radioactivity, 139 cpm, was recovered in peaks 1 and 3, representing Neu5Acα2-6′LNβ1-3′LNβ1-3′LN and its reducing end ManNAc epimer, Neu5Acα2-6′LNβ1-3′LNβ1-3Galβ1-4ManNAc, respectively. (The ManNAc epimer was probably generated by base-catalyzed epimerization during the HPAE chromatography [28].) By contrast, peaks 2 and 4, represent-

Table 1
Rates of sialyltransfer reactions catalyzed by HL-60 cell lysates (pmol/mg/h)

Lysate number	Acceptor	Total α-transfer		α3-Transfer	α6-Transfer
		[^{14}C]Neu5Ac transferred to acceptors[a]	α-linked [^{14}C]Neu5Ac in isolated products[b]	α3-linked [^{14}C]Neu5Ac in isolated products[c]	α6-linked [^{14}C]Neu5Ac in isolated products[d]
1	LN-LN-LN, 2.0 mM	320 ± 4	317 ± 2	228 ± 3	91.2 ± 0.9
1	LN-Lex-Lex, 2.0 mM	255 ± 13	248 ± 0.4	214 ± 6	39.9 ± 4.1
2	LN-LN-LN, 2.0 mM	177 ± 8	172 ± 3	101 ± 2	73.9 ± 6.5
2	LN-Lex-Lex, 2.0 mM	115 ± 7	113 ± 1	87.9 ± 0.6	26.6 ± 6.8
2	LN-LN-LN, 0.20 mM	22.8 ± 0.4	22.4 ± 0.1	12.6 ± 0.1	10.0 ± 0.2
2	LN-Lex-Lex, 0.20 mM	15.6 ± 0.1	15.1 ± 0.03	10.8 ± 0.2	4.6 ± 0.1

[a] All values represent data from two parallel experiments and their means.
[b] [^{14}C]Neu5Ac released from isolated products by *A. ureafaciens* sialidase.
[c] [^{14}C]Neu5Ac released from isolated products by NDV sialidase.
[d] α-linked [^{14}C]Neu5Ac resistant to NDV sialidase.

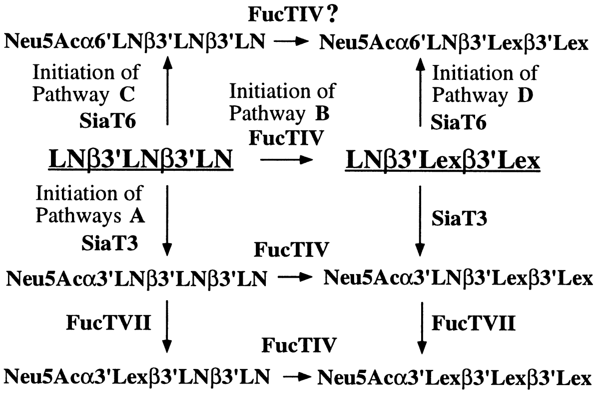

Fig. 3. Pathways of in vitro biosynthesis of α3-sialylated and α6-sialylated polylactosamines in human HL-60 cell lysates. SiaT3 and SiaT6 refer to the α3- and α6-sialyltransferase reactions described in the present experiments, respectively. FucTIV and FucTVII refer to transferase reactions of free oligosaccharides catalyzed by recombinant Fuc-TIV and Fuc-TVII, respectively, as described by Niemelä et al. [12]. It is not yet known whether the recombinant Fuc-TIV enzyme will catalyze the conversion of Neu5Acα2-6′LNβ1-3′LNβ1-3′LN to Neu5Acα2-6′LNβ1-3′Lexβ1-3′Lex, but it is known that the sialylated, distal LN unit does not react [11].

ing residual Neu5Acα2-3′LNβ1-3′LNβ1-3′LN and its putative reducing end ManNAc epimer, respectively, together yielded only 15 cpm of radioactivity. The data imply that almost 90% of the NDV sialidase-resistant α-$[^{14}C]$sialoglycans formed from LNβ1-3′LNβ1-3′LN by HL-60 cell lysates were chromatographically identical with Neu5Acα2-6′LNβ1-3′LNβ1-3′LN.

3.4. Relative rates of synthesis of α6-sialylated products from LNβ1-3′LNβ1-3′LN and LNβ1-3′Lexβ1-3′Lex

The rates of Neu5Acα2-6′LNβ1-3′LNβ1-3′LN synthesis in six independent experiments are shown in Table 1. The activities of the HL-60 cell α6-sialyltransferase(s) found in the LNβ1-3′LNβ1-3′LN experiments were significant but distinctly lower than those of α3-sialyltransferases. This was true at 0.2 mM and 2 mM acceptor concentrations. These data are similar to those reported in HL-60 cell lysates by [19] using asialo-α_1-acid glycoprotein as the acceptor.

The synthesis rates of the putative fucosylated sialoglycan Neu5Acα2-6′LNβ1-3′Lexβ1-3′Lex in the HL-60 cell lysate experiments were consistently less than half of the rates of Neu5Acα2-6′LNβ1-3′LNβ1-3′LN synthesis. Hence, the presence of internal α3-bonded fucosyl groups in the acceptor polylactosamines inhibited α6-sialylation markedly. It is not yet clear whether both midchain fucose units were inhibitory.

4. Discussion

4.1. The α3-sialyltransferases of HL-60 cells recognize mainly the distal LN unit of the polylactosamines

In the present experiments, the HL-60 lysates α3-sialylated both the fucosylated polylactosamine LNβ1-3′Lexβ1-3′Lex and the fucose-free analog LNβ1-3′LNβ1-3′LN at similar rates, revealing only a 1.1–1.2-fold preference for the latter (Table 1). This suggests that the α3-sialyltransferase(s) of HL-60 cells, representing mainly ST3Gal IV [29–32], recognized mainly the distal LN units, the other parts of the two acceptors being structurally quite different [33]. In contrast, rat liver ST3Gal III may recognize a longer epitope than the distal LN, because it shows 1.5–1.9-fold reaction rates for LNβ1-3′LNβ1-OR over LNβ1-3′Lexβ1-OR [18].

4.2. Two sets of synthetic pathways leading to multiply fucosylated α3-sialopolylactosamines are possible in HL-60 cells

The present data complete two distinct sets of in vitro biosynthetic pathways leading from polylactosamine backbones to the selectin ligands Neu5Acα2-3′LNβ1-3′Lexβ1-3′Lex and Neu5Acα2-3′Lexβ1-3′Lexβ1-3′Lex in HL-60 cells. Successful α3-sialylation of LN-β1-3′LNβ1-3′LN, together with the previously reported data on fucosylation of Neu5Acα2-3′LN-β1-3′LNβ1-3′LN by the concerted action of Fuc-TVII and Fuc-TIV [12], rounds off the set of pathways A of Fig. 3. On the other hand, Fuc-TIV reactions [12] lead from the finished polylactosamine backbone to the internally fucosylated LNβ1-3′Lexβ1-3′Lex, and the present data on the successful α3-sialylation of this intermediate represent the middle step of pathway B of Fig. 3; this step is concluded by the Fuc-TVII reaction described previously [12]. In theory, internal fucosylations catalyzed by Fuc-TIV can take place in HL-60 cells already during the elongation of the polylactosamine backbone; analogous reactions have been described in the generation of LNβ1-3′Lex [18] and LNβ1-3′Lexβ1-3′Lex determinants in vitro [17]. However, a Fuc-TIV reaction at the distal LN of a growing polylactosamine backbone [12] would inhibit further chain extension and α3-sialylation in vitro [34] and in vivo [10].

4.3. HL-60 cell lysates catalyze α6-sialylation of long i-type polylactosamine backbones but analogous gangliosides are not expressed by these cells

The present experiments showed that HL-60 cell lysates readily catalyzed the reaction LNβ1-3′LNβ1-3′LN → Neu5Ac-α2-6′LNβ1-3′LNβ1-3′LN, initiating pathway C of Fig. 3. The detection of α6-sialyltransferase activity is in line with the work of others who used asialoglycoproteins as acceptors [19,20], and also with the reported presence of Neu5Acα2-6′LNβ1-3′Lβ1-ceramide among the gangliosides of these cells [35]. However, the distinct α6-sialyltransferase activity of HL-60 cell lysates towards long chain polylactosamine acceptors is interesting in view of the reported absence of long chain α6-sialopolylactosamines among the gangliosides of these cells [35]. In contrast to the α6-sialylated gangliosides, a whole variety of α3-sialylated gangliosides of long polylactosamine chains are present among the gangliosides of HL-60 cells [35], suggesting that functional α3-sialyltransferase, but not functional α6-sialyltransferase, may co-localize with polylactosamine extension enzymes or reside at quite distal locations in the membranes of Golgi or *trans*-Golgi network.

4.4. Internally fucosylated polylactosamines show diminished acceptor activity for α6-sialyltransferase(s) of HL-60 cells

In the present α6-sialyltransferase reactions catalyzed by HL-60 cell lysates, LNβ1-3′Lexβ1-3′Lex showed less than half of the acceptor activity of the fucose-free analog LNβ1-3′LNβ1-3′LN. Hence, the enzyme(s) of HL-60 cells resembles the α6-sialyltransferase from rat liver, known to react more slowly with the internally fucosylated LNβ1-3′Lexβ1-OR than with the fucose-free LNβ1-3′LNβ1-OR [18]. Indeed, reduced α6-sialylation of internally fucosylated polylactosamines (pathway D in Fig. 3) may be common because all α6-sialyltransferases reacting with LN appear to belong to the ST6Gal I family [29]. Fucosylation at the terminal LN unit is known to prevent α6-sialylation completely [36].

In view of the impaired ability of ST6Gal I-deficient mice to mount immune responses [37], it will be interesting to learn whether the polylactosamine sequences Neu5Acα2-6′LNβ1-3′LNβ1-3′LN and Neu5Acα2-6′LNβ1-3′Lexβ1-3′Lex synthesized in the present experiments play any essential roles in the generation of immunoprotection.

4.5. Conclusions

Taken together, our present data describe properties inherent in HL-60 cell sialyltransferases, showing that α3-sialylation per se may precede or follow internal, Fuc-TIV-catalyzed α3-fucosylation of polylactosamine backbones. However, our data do not show whether internal α3-fucosylation actually precedes, follows or intermingles with the α3-sialylation reactions in the Golgi or *trans*-Golgi network of living HL-60 cells. The data also suggest that α3-fucosylation of polylactosamine backbones at the peridistal LN units, intrinsically, will inhibit α6-sialylation.

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