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# Biosynthesis *in vitro* of fucose-containing glycosphingolipids in human neuroblastoma IMR-32 cells

**(5′-bromodeoxyuridine/blood group active glycosphingolipids/fucosyltransferases/galactosyltransferases)**

KATHLEEN A. PRESPER, MANJU BASU, AND SUBHASH BASU

Department of Chemistry, Biochemistry and Biophysics Program, University of Notre Dame, Notre Dame, Indiana 46556

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**ABSTRACT** Two different glycolipid:fucosyltransferase activities involved in the biosynthesis *in vitro* of blood group-related glycosphingolipids have been detected in a membrane preparation isolated from a human neuroblastoma-derived clonal cell line, IMR-32. The membrane preparation contains an α (1, 2)-fucosyltransferase (EC 2.4.1.89) that catalyzes the transfer of fucose from GDP-[$^{14}$C]fucose to neolactotetraosylceramide or neolactopentaosylceramide to form types H-I and B-I glycolipids, respectively. The second fucosyltransferase catalyzes the transfer of fucose to lactotriaosylceramide [GlcNAc(β1-3)Gal(β1-4)Glc-Cer] to form a tetraglycosylceramide intermediate of the novel Le$^a$-type glycolipid. UDP-galactose: lactotriaosylceramide β-galactosyltransferase (EC 2.4.1.86) had 4 times the activity of UDP-galactose:α-galactosyltransferase (EC 2.4.1.87) when tested under similar conditions. α-Fucosyltransferase activities and the incorporation of [$^{14}$C]fucose into glycoproteins and glycolipids were also compared in cells differentiated in the presence of 4 μM BrdUrd and 6-mercaptoguanosine.

The metabolism of neutral fucose-containing glycosphingolipids has become an increasingly important area of investigation because of their presence in erythrocytes (3–10), dog small intestine (11), hog gastric mucosa (12), bovine liver (13), and plasma membranes of primate cell surfaces (14). Fucolipids containing sialic acid have been isolated from boar testis (15) and human kidney (16). Accumulation of short-chain fucolipids and an associated deletion of long-chain fucolipids in oncornavirus-transformed baboon and human embryonic cell lines were observed by Steiner *et al.* (17). Recently a blood group H-I active fucolipid was isolated from rat ascites hepatoma AH7974F (18). Isolation of α-L-fucosylceramide (19) and a novel Le$^a$-type fucolipid (20) from human colon tumor and adenocarcinoma, respectively, suggests the occurrence of metabolic control of neutral fucolipids during malignancy or differentiation of higher primate cells (21).

Because specific glycosyltransferases are involved in the biosynthesis *in vitro* of fucose-containing glycolipids (14, 21–26) and glycoproteins (27–29), changes in the level of activities of these glycolipid:glycosyltransferases may be correlated during tumorigenesis or chemically induced differentiation of tumor cells of higher primate origin. It has been reported recently (30, 31) that glycoprotein:fucosyltransferase activities in human plasma increase substantially during tumor progression and the development of metastases. Knowledge of the biosynthesis *in vitro* of specific fucolipids in cultured tumor or differentiated cells of human origin has been limited until now (21).

The present report is concerned primarily with the stepwise biosynthesis *in vitro* of blood groups H-I and B-I and an intermediate of the novel Le$^a$-type fucolipid in undifferentiated and chemically [BrdUrd or 6-mercaptoguanosine (sGuo)] differentiated human neuroblastoma (IMR-32) cells (32). Specific clones of mouse neuroblastoma cells are known to undergo significant morphological changes (i.e., neurite formation) in the absence of serum (33) or in the presence of dibutyryl cyclic AMP [(But)$_2$cAMP] (34, 35) and BrdUrd (36, 37). Biochemical changes relative to cell surface glycoconjugates are expected to accompany these observed morphological changes.

## MATERIALS AND METHODS

**Materials.** Unlabeled GDP-L-fucose was prepared according to Schachter *et al.* (38). Unlabeled UDP-D-galactose was purchased from Calbiochem. GDP-L-[$^{14}$C]fucose (174 mCi/mmol), UDP-D-[$^{14}$C]galactose (274 mCi/mmol), and L-[$^{14}$C]fucose (53.2 mCi/mmol) were purchased from New England Nuclear. Lactosylceramide [Gal(β1-4)Glc-Cer] (LcOse$_1$-Cer) was isolated from bovine spleen (14). The B-active pentaglycosylceramide, lactopentaosylceramide [Gal(α1-3) Gal(β1-4)GlcNAc(β1-3)-Gal(β1-4)Glc-Cer], was isolated from rabbit erythrocytes (22). Both lactotriaosylceramide [GlcNAc(β1-3)Gal(β1-4)Glc-Cer] (LcOse$_3$-Cer) and neolactotetraosylceramide [Gal(β1-4)-GlcNAc(β1-3)Gal(β1-4)Glc-Cer] (nLcOse$_4$-Cer) were prepared from the rabbit lactopentaosylceramide by sequential degradation of terminal galactose units with purified fig α-galactosidase and papaya β-galactosidase. The glycosidases were purifed according to described methods (14). The purified glycosphingolipids were analyzed by gas/liquid chromatography (22) and mass spectrometry. Mass spectral data were interpreted according to Bjorndal *et al.* (39). BrdUrd and sGuo were purchased from Sigma Chemical Co.

**Cell Culture.** Control cultures of human neuroblastoma clone IMR-32 (purchased from American Type Culture Collection) were maintained at 37° in 250-ml (75 cm$^2$) Falcon plastic flasks containing 15 ml of Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum and 10% horse serum (Gibco), in a humidified atmosphere of 90% air/10% $CO_2$. The medium was changed three times a week. When monolayers reached confluence, the cells were subcultured by using $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (7.0 mM potassium phosphate/0.14 M NaCl) containing 0.1% EDTA, pH 7.2. Cells were harvested by using this same mixture, and membrane fractions for glycolipid:glycosyltransferase assays were isolated at the junction of 0.32 M and 1.2 M on a discontinuous sucrose density gradient (14, 40).

**Glycolipid Glycosyltransferase Assays.** Complete incubation mixtures for the glycolipid α-fucosyltransferase assay

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Abbreviations: sGuo, 6-mercaptoguanosine; (But)$_2$cAMP, $N^6$,$O^{2'}$-dibutyryladenosine cyclic-3′,5′-monophosphoric acid; LcOse$_2$-Cer, lactosylceramide; LcOse$_3$-Cer, lactotriaosylceramide; LcOse$_4$-Cer, lactotetraosylceramide; nLcOse$_4$-Cer, neolactotetraosylceramide; nLcOse$_5$-Cer, neolactopentaosylceramide.

Table 1. GDP-fucose:glycolipid fucosyltransferase activities

| Acceptor | | [$^{14}$C]Fucose incorporated, pmol/mg protein/2 hr | | | |
|---|---|---|---|---|---|
| Symbol | Structure | IMR-32 | N-18 | 103 | 104Cl |
| $LcOse_3$-Cer | GlcNAc-Gal-Glc-Cer | 320 (3,350) | 0 | 0 | 0 |
| $nLcOse_4$-Cer | Gal-GlcNAc-Gal-Glc-Cer | 5,580 (14,340) | 0 | 910 | 1420 |
| $nLcOse_5$-Cer | Gal<br>\|<br>Gal-GlcNAc-Gal-Glc-Cer | 6,630 (20,490) | 0 | 885 | 1110 |

The complete incubation mixtures contained the same components as described for glycolipid glycosyltransferase assays except that the indicated substrates were used and the values in parentheses (pmol/mg of protein per 45 min) were obtained with the membrane fraction as enzyme source. The rates of the reactions were proportional to the given ranges of protein concentration and remained constant with time of incubation up to 2 hr.

contained the following components (in micromoles) in final volumes of 0.025–0.04 ml: acceptor glycosphingolipid, 0.025; detergent G-3634-A (Atlas Chem.), 0.05 mg; $MgCl_2$, 0.13; cacodylate·HCl buffer (pH 6.4), 10; GDP-L-[$^{14}$C]fucose, 0.014 ($3.2 \times 10^6$ dpm/$\mu$mol); and enzyme fractions consisting of homogenates or membranes isolated from IMR-32 neuroblastoma cells, 0.1 to 0.14 mg of protein [estimated by the method of Lowry *et al.* (41)]. The mixtures were incubated for 2 hr at 37° and the reaction was stopped by addition of 2.5 $\mu$mol of EDTA. The whole liquid content and a 100-$\mu$l chloroform/methanol (2:1) wash of the tube were spotted on Whatman 3MM paper and assayed by a double chromatographic method (24). The appropriate areas of each chromatogram were determined quantitatively by liquid scintillation techniques with a Beckman scintillation counter (model LS-3133T).

Incubation conditions for glycolipid galactosyltransferase assays were the same as described above, except that the incubation mixture contained the following (in micromoles): detergent Triton X-100; 0.1 mg; cacodylate·HCl buffer (pH 7.3), 10; $MnCl_2$, 0.125; and UDP-[$^{14}$C]galactose, 0.02 ($2.3 \times 10^6$ dpm/$\mu$mol).

## RESULTS

**Comparison of Glycolpid $\alpha$-Fucosyltransferase Activities in Cultured Tumor Cell Lines.** GDP-L-[$^{14}$C]fucose:glycosphingolipid $\alpha$-fucosyltransferase activities were compared in homogenates of human (IMR-32) and mouse (N-18) neuroblastoma clones (obtained from Shraga Makover of Roche, Inc.) and in the guinea pig embryonic 103 cell line and its chemically transformed 104Cl variant (obtained from C. H. Evans of the National Cancer Institute) (Table 1). The highest activity of $\alpha$(1,2)fucosyltransferase (EC 2.4.1.89) (24) was observed in human neuroblastoma (IMR-32) cells with neolactotetraosylceramide and neolactopentaosylceramide as acceptors, whereas mouse neuroblastoma clone N-18 had little or no activity with any acceptor. Relatively high $\alpha$(1,3) [or $\alpha$(1,4)] fucosyltransferase activity was also present in the IMR-32 cell homogenate. Both $\alpha$(1,2) and $\alpha$(1,3) fucosyltransferase activities were highest in the membrane preparation isolated from the IMR-32 cells (values given in parentheses, Table 1). However, only glycolipid:$\alpha$(1,2) fucosyltransferase activity was detected in homogenates of guinea pig embryonic 103 cells or benzo[*a*]pyrene-transformed 104Cl cells (42) under similar conditions.

Table 2. UDP-galactose:glycolipid galactosyltransferase activities

| Acceptor | | [$^{14}$C]Galactose incorporated, pmol/mg protein/2 hr | | | |
|---|---|---|---|---|---|
| Symbol | Structure | IMR-32 | N-18 | 103 | 104Cl |
| $LcOse_2$-Cer | Gal-Glc-Cer | 50 | 30 | 390 | 520 |
| $LcOse_3$-Cer | GlcNAc-Gal-Glc-Cer | 1830 | 1750 | 16,000 | 20,120 |
| $nLcOse_4$-Cer | Gal-GlcNAc-Gal-Glc-Cer | 450 | 430 | 820 | 1,680 |
| GM2 | GalNac-Gal-Glc-Cer<br>\|<br>NeuAc | ND | ND | 180 | 120 |

The complete incubation mixture contained the same components as described for glycolipid glycosyltransferase assays except that the indicated substrates were used. The rates of the reactions remained constant with time of incubation up to 2 hr and were proportional to the given ranges of protein concentration. ND, not detectable under the assay conditions.

After chemical transformation, 25% and 55% higher activities were observed with neolactopentaosyl- and neolactotetraosylceramide, respectively.

**Comparison of Glycolipid Galactosyltransferase Activities in Cultured Tumor Cells.** UDP-[$^{14}$C]galactose:glycosphingolipid $\alpha$- and $\beta$-galactosyltransferase activities were compared in homogenates of tumor clones of human, mouse, and guinea pig origin (Table 2). The activity of UDP-galactose:lactotriaosylceramide $\beta$-galactosyltransferase (EC 2.4.1.86) (43) was highest in guinea pig embryonic cells and increased after chemical transformation with benzo[*a*]pyrene (i.e., in 104Cl cells). However, the activities of UDP-glactose:lactotriaosylceramide $\beta$-galactosyltransferase (EC 2.4.1.86) and UDP-galactose:neolactoteraosylceramide $\alpha$-galactosyltransferase (EC 2.4.1.87) (22) were the same in both human and murine neuroblastoma clones. It is interesting that, under our experimental conditions, either little or almost no activity was seen with UDP-galactose:lactosylceramide $\alpha$-galactosyltransferase or UDP-galactose:GM1 $\beta$-galactosyltransferase (EC 2.4.1.62) (44) in neuroblastoma clones.

**Incorporation of L-[$^{14}$C]Fucose in Chemically Differentiated IMR-32 Cells.** Significant morphological changes occurred within 48–72 hr in IMR-32 cells grown in the presence of 4 $\mu$M BrdUrd (Fig. 1). As shown in Table 3, the incorporation of L-[$^{14}$C]fucose into glycoprotein was inhibited 40% during

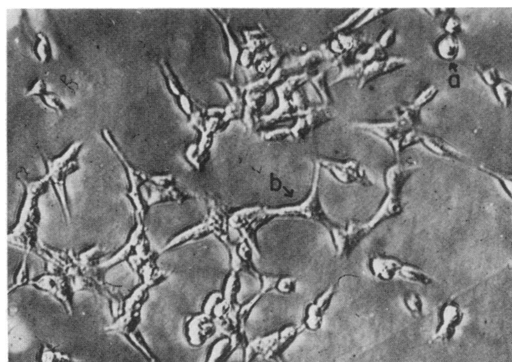

FIG. 1. Phase contrast micrographs of nondifferentiated (a) and differentiated (b) IMR-32 cells. The cells, in the logarithmic phase, were maintained on Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 4 $\mu$M sGuo for 72 hr for maximum differenetiation without cytotoxic effect. Cultures were grown in Falcon T flasks (75 cm$^2$). (×150.)

Table 3. Effect of BrdUrd treatment on [$^{14}$C]fucose incorporation into glycoconjugates of IMR-32 cells

| Incubation time, hr | [$^{14}$C]Fucose incorporated, % of control | |
|---|---|---|
| | Glycoprotein | Glycolipid |
| 30 | 60 | 60 |
| 48 | 63 | 23 |
| 72 | 54 | 14 |

Human neuroblastoma IMR-32 clone was maintained on Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10% horse serum (Gibco). Cultures were grown in Falcon T flasks (75 cm$^2$). At half-confluence, the medium was changed and the cells were maintained for the indicated periods on a new medium containing 4 $\mu$M BrdUrd and L-[$^{14}$C]fucose (20,000 dpm/ml). The cells were washed with cold $Ca^{2+}$- and $Mg^{2+}$-free phosphate-buffered saline (pH 7.2) and harvested with the same buffer containing 0.1% EDTA. An aliquot was filtered through Whatman borosilicate paper (GF/A; 2.4 cm; 1 $\mu$m porosity) on a Millipore apparatus. The cells that adhered to the discs were washed with 15 ml of cold 5% trichloroacetic acid or 5% trichloroacetic acid followed by 15 ml of chloroform/methanol (2:1) for determination of [$^{14}$C]fucose incorporation into glycoprotein (retained on GF/A paper) or glycolipid, respectively. The glass fiber discs were dried at 100°, and the radioactivity was assayed in a toluene liquid scintillator system with a Beckman LS-3133T scintillation counter. Each value is the mean of duplicate experiments.

a 30-hr incubation and to a maximum of 46% by 72 hr of incubation in the presence of 4 $\mu$M BrdUrd. A significant decrease (85%) in glycolipid biosynthesis was observed under similar conditions. There was almost a 2-fold increase in both glycoprotein and glycolipid when the cells were differentiated in the presence of sGuo for 72 hr (Fig. 2).

The levels of glycolipid $\alpha$-fucosyltransferase activity were also compared in cells treated with sGuo, with neolactotetraosylceramide and lactopentaosylceramide as acceptors (Table 4). The $\alpha$(1,2)fucosyltransferase level did not change significantly, with respect to control values, during differentiation of the human IMR-32 clone in the presnece of sGuo.

**Isolation and Characterization of $^{14}$C-Labeled Products.** The labeled products were isolated from large-scale (40- to 100-fold) incubation mixtures with lactopentaosylceramide and lactotriaosylceramide as substrates. The radioactive products were first separated by a double chromatographic method as

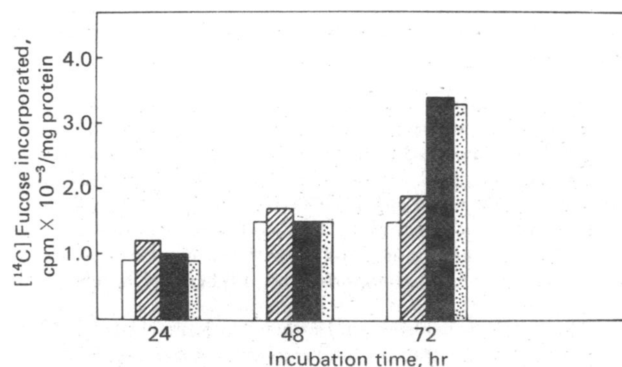


FIG. 2. Effect of sGuo treatment on [$^{14}$C]fucose incorporation into glycoconjugates of IMR-32 cells. The cells, in the logarithmic phase, were maintained on 15 ml of complete Dulbecco's modified Eagle's medium containing 4 $\mu$M sGuo and L-[$^{14}$C]fucose (20,000 dpm/ml) for the indicated times. The incorporation of [$^{14}$C]fucose into glycolipid and glycoprotein was quantitated by the method described in Table 3. Untreated cells: ▨, trichloroacetic acid wash; □, trichloroacetic acid followed by chloroform/methanol wash. Treated cells: ■, trichloroacetic acid wash, ▤, trichloroacetic acid followed by chloroform/methanol wash.

Table 4. Effect of sGuo on glycolipid $\alpha$(1,2)fucosyltransferase of IMR-32 cells

| Acceptor | Activity, pmol/mg protein/2 hr | |
|---|---|---|
| | Control | sGuo |
| nLcOse$_4$-Cer | 2520 | 2330 |
| nLcOse$_5$-Cer | 2930 | 3170 |

The complete incubation mixtures contained the same components as described for glycolipid glycosyltransferase assays except that the indicated substrates were used in the incubation mixtures and cells treated with 4 $\mu$M sGuo for 72 hr were used as enzyme source for the values given in the last column.

described for glycolipid glycosyltransferase assays. The radioactive product was eluted from Whatman 3MM paper with chloroform/methanol/water, 60:35:8 (vol/vol), dried, and applied to a Unisil column. $^{14}$C-Labeled products were eluted with increasing concentrations of methanol as described (24). The thin-layer chromatographic migrations of the two enzymatic products obtained with lactotriaosylceramide and lactopentaosylceramide as acceptors indicated that these products were [$^{14}$C]fucose-containing tetraglycosylceramide (lane 2, Fig. 3) and a [$^{14}$C]fucose-containing hexaglycosylceramide (lane 5, Fig. 3), respectively. The distribution of the major radioactive bands indicates that the [$^{14}$C]fucose-containing tetraglycosylceramide, [$^{14}$C]Fuc$\alpha$-GlcNAc($\beta$1-3)Gal($\beta$1-4)Glc-Cer, migrated just behind neolactotetraosylceramide, Gal($\beta$1-4)-GlcNAc($\beta$1-3)Gal($\beta$1-4)Glc-Cer, and that the [$^{14}$C]fucose-containing hexaglycosylceramide migrated with human erythrocyte B-I type hexaglycosylceramide, Gal($\alpha$1-3)-Gal[($\alpha$1-2)Fuc]($\beta$1-4)GlcNAc($\beta$1-3)Gal($\beta$1-4)Glc-Cer. The [$^{14}$C]fucose-containing tetraglycosylceramide was hydrolyzed 62–66% by both *Charonia lampas* (45) and *Venus mercenaria*

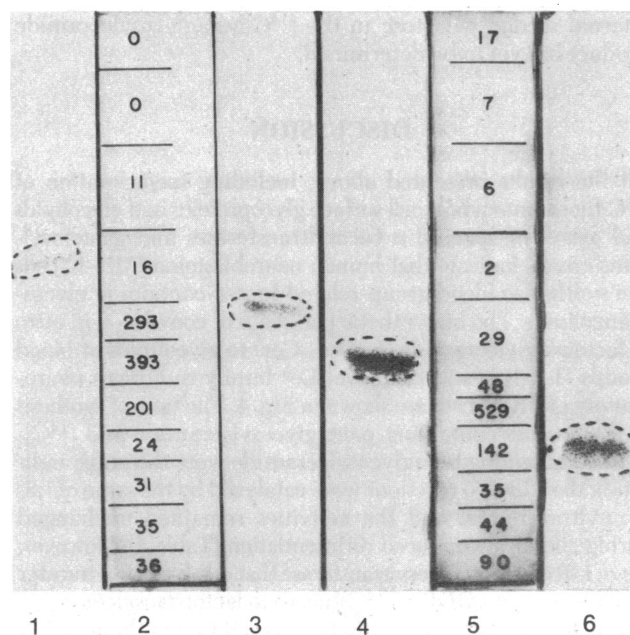


FIG. 3. Migration of $^{14}$C-labeled products on thin-layer chromatography plate. The purified [$^{14}$C]fucose-labeled products (1000–1500 dpm) were applied to a silica gel G plate (250 $\mu$m) and developed in ascending fashion. Lanes: 1, LcOse$_3$-Cer; 2, [$^{14}$C]fucose-LcOse$_3$-Cer; 3, nLcOse$_4$-Cer; 4, nLcOse$_5$-Cer (rabbit erythrocyte); 5, [$^{14}$C]fucose-nLcOse$_5$-Cer; 6. blood group B-active glycosphingolipid (human erythrocyte). Lanes 2 and 5 were scraped and radioactivity was assayed by Beckman LS-3133T liquid scintillation spectrometry; results are shown by numbers. The spots in lanes 1, 3, 4, and 6 were visualized by spraying with diphenylamine reagent (22).

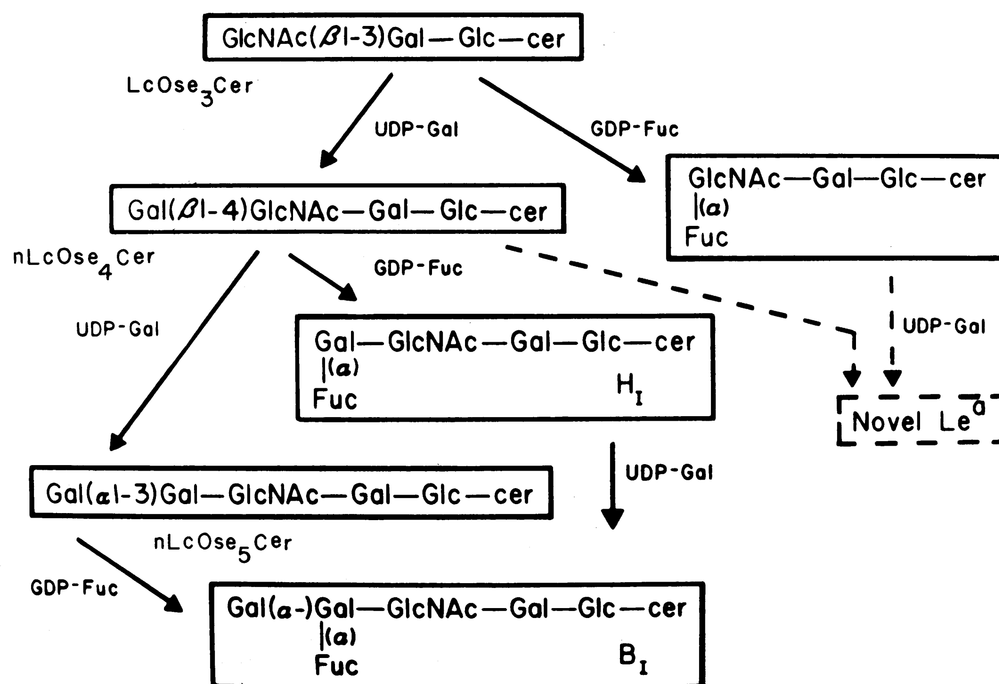


FIG. 4. Biosynthetic pathways of fucose-containing glycosphingolipids in human neuroblastoma IMR-32 clone.

(46) fucosidases and 94% by 0.1 M trichloroacetic acid (2 hr, 100°). Less extensive hydrolysis was observed with the [$^{14}$C]-fucose-containing hexaglycosylceramide when treated with α-fucosidases of mollusc origin, and 83% hydrolysis was observed with 0.1 M trichloroacetic acid (2 hr, 100°). Whether the [$^{14}$C]fucose is linked to the terminal *N*-acetylglucosamine in the former [$^{14}$C]tetraglycosylceramide product and to the internal second galactose in the [$^{14}$C]hexaglycosylceramide product has yet to be determined.

## DISCUSSION

All the results presented above, including incorporation of [$^{14}$C]fucose into whole cell surface glycoproteins and glycolipids and assays of specific α-fucosyltransferases and galactosyltransferases, indicate that human neuroblastoma IMR-32 cells can synthesize blood group-related fucose-containing glycosphingolipids. The biosynthetic pathways of conversion *in vitro* of lactotriaosylceramide ($LcOse_3$-Cer) to glycolipids of blood groups H-I, B-I, and the novel Le$^a$ family in human neuroblastoma IMR-32 cells are shown in Fig. 4. The rates of synthesis of [$^{14}$C]fucose-containing pentaglycosylceramide and [$^{14}$C]-fucose-containing hexaglycosylceramide were the same, indicating that the two reactions were catalyzed by the same α(1,2) fucosyltransferase, and the activities remained unchanged during chemically induced differentiation (Table 4). However, the α(1,3) or α(1,4) fucosyltransferase that catalyzed the transfer of L-fucose from GDP-L-[$^{14}$C]fucose to lactotriaosylceramide was different from the one that catalyzed the synthesis of H-I or B-I glycosphingolipids, as shown by the 10-fold increase in its activity in isolated membranes of IMR-32 cells (Table 1).

Species-specificity studies revealed (Table 1) that the α(1,3) or α(1,4) fucosyltransferase was completely absent from guinea pig embryonic 103 and chemically transformed 104Cl cells, although both the latter cell types displayed virtually identical α(1,2)fucosyltransferase activities with $nLcOse_4$-Cer and $nLcOse_5$-Cer as acceptors. The absence of any α-fucosyltransferase and the presence of glycolipid:galactosyltransferases in mouse N-18 cells suggest that in tumor cells of lower primates a non-fucose-containing blood group glycosphingolipid (i.e., neolactopentaosylceramide) is probably predominant on the cell surfaces. It is also evident from our recent *Bandeiraea simplicifolia* $^{125}$I-labeled lectin binding studies that mouse neuroblastoma NS-20 clones contained more of an α-linked terminal galactose-containing compound than did N1E-115 cells (35). The number of binding sites also did not change when the cells were grown in the presence of 4 μM BrdUrd or sGuo (47). Recently, Sundsmo and Hakomori (48) reported on the relatively high content of neolactotetraosylceramide in DNA virally transformed NIL hamster cell surfaces compared with nontransformed cells. The evidence presented in this paper suggests that in lower animals (e.g., mouse neuroblastoma) the lactotriaosylceramide is converted to non-fucose-containing pentaglycosylceramide (Fig. 4) whereas in higher primate cells (e.g., human neuroblastoma) it can be converted to either H-I or B-I type fucose-containing glycosphingolipids.

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