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Effects of Lectin Activation on Sialyltransferase Activities in Human Lymphocytes†

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ABSTRACT: The effects of phytohemagglutinin (PHA) stimulation on the activities of sialyltransferase 1 (SAT-1), and sialyltransferase 3 (SAT-3), in human lymphocytes were investigated in vitro. For SAT-1 and SAT-3, respectively, the apparent K_m values with variable CMP-NeuAc concentrations were 0.19 and 0.015 mM and with variable LacCer were 0.075 and 0.17 mM. Progressive increases in the activities of SAT-1 and SAT-3 were detected in lymphocytes stimulated with PHA, whereas no increase was observed in control lymphocytes incubated in culture medium alone. These increased activities occurred within 18–36 h of incubation and preceded optimum lymphocyte proliferation. Intact lymphocytes were needed for the lectin-stimulated increase of sialyltransferase activities because neither concanavalin A nor phytohemagglutinin added to the broken cell preparation modulated SAT-1 activity. The glycolipid products formed as a result of these enzymatic reactions in the presence of endogenous and exogenous acceptors were tentatively identified by thin-layer chromatography and autofluorography. The addition of exogenous LacCer to the SAT-1 assay resulted in the radiolabeling of a small amount of ganglioside GM1b (3.4%), but GM3 was the major labeled product (96%). When $GgOse_4Cer$ was added to the SAT-3 assay, 32% GM3 and 24.6% GM1b were detected while 44% consisted of glycolipids not labeled in assays performed without exogenous acceptors. Of the radioactivity transferred to endogenous acceptors, 81.3% was in GM3 and 14.6% in GM1b. These results demonstrate that the modulation of sialyltransferase activity occurs earlier than cellular activation.

Both glycolipids and proteins exist in sialylated and nonsialylated forms, and the degree of sialylation of glycolipids may be of biological importance (Hakomori, 1981). Gangliosides are sialylated glycolipids that have been implicated generally in the regulation of cell division and specifically in cell surface contact inhibition of proliferation (Roseman, 1970; Hakomori, 1984). We previously reported that lectin stimulation of human peripheral blood lymphocytes results in a 9-fold increase of radiolabeled precursor incorporation into gangliosides of these cells (Yates et al., 1980). Although this finding indirectly suggests that the de novo synthesis of sialylated glycolipids is probably linked to cell activation, it is possible that the apparent sialyltransferase alteration could be explained by the altered cellular transport of the radiolabeled precursor glucosamine. One strategy to assess directly whether the sialylation of cell membrane constituents might be linked to cellular activation and division is to determine if sialyltransferase activities are modulated during cell proliferation.

The major ganglioside of human lymphocytes is GM3[1] with IV^3nLc_4 occurring in lesser amounts (Macher et al., 1981). The biosynthesis of GM3 is dependent on sialyltransferase 1 (SAT-1), and IV^3nLc_4 synthesis requires sialyltransferase 3 (SAT-3) activity (Basu & Basu, 1982; Basu et al., 1982). The experiments in this paper first determined the activities of these two sialyltransferases in normal human lymphocytes. Then, the activation and division of human lymphocytes triggered

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[1] Abbreviations: SAT-1, CMP-NeuAc:LacCer α-sialyltransferase; SAT-3, CMP-NeuAc:$nLcOse_4Cer$ α-sialyltransferase; GM3, sialyl(α2–3)lactosylceramide; GM1, sialyl(α2–3)gangliotetraosylceramide or $II^3NeuAcGg_4$; GM1b, sialyl(α2–3)gangliotetraosylceramide or $IV^3NeuAcGg_4$; IV^3nLc_4, sialyl(α2–3)neolactotetraosylceramide; LacCer, lactosylceramide; $GgOse_4Cer$, gangliotetraosylceramide; $nLcOse_4Cer$, neolactotetraosylceramide; NeuAc, *N*-acetylneuraminic acid; PBMC, peripheral blood mononuclear cells; SBSS, Seligman's balance salt solution; PHA, phytohemagglutinin; Con A, concanavalin A; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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by the lectin phytohemagglutin (PHA) were used as an in vitro model system to examine the question of whether the activities of these two enzymes might be linked to cell activation. Previously reported studies have examined the effects lymphocyte maturation on glycosyltransferases (Augener et al., 1980) and of lymphocyte activation on the incorporation of precursors into glycolipids (Hakomori, 1984). However, to our knowledge this is the first investigation to analyze possible alterations of glycosyltransferase activities toward glycolipid acceptors during lectin activation of lymphocytes from either humans or animals. Because glycolipids, integral components of most eukaryotic cell membranes, could have important roles during modulation of cellular activity, an evaluation of the effects of lymphocyte activation on the levels of these glycosyltransferases could yield information applicable to cellular growth in other systems. The experiments to be presented demonstrate that lymphocyte activation leads to early increases of sialyltransferase activities, which clearly precede the proliferative responses. Thus, these data are direct evidence that sialyltransferase activity is closely linked to early cell activation.

Materials and Methods

Isolation of Peripheral Blood Mononuclear Cells. Human peripheral blood mononuclear cells (PBMC) from normal donors were separated by density centrifugation over Ficoll–Hypaque gradients (Boyum, 1968). The PBMC were harvested from the gradient interfaces and rinsed 3 times in Seligman's balanced salt solution (SBSS, Grand Island Biologic Co., Grand Island, NY). PBMC were depleted of platelets by centrifugation through 4% bovine serum albumin at 400*g* for 10 min. The pellet was then resuspended in SBSS, and any contaminating red blood cells were removed by shock lysis with sterile double-distilled water for 20 s. The preparation was resuspended in SBSS and counted in a Coulter counter. By nonspecific esterase staining (Yam et al., 1971), the final preparation of PBMC contained 70–76% lymphocytes with the remainder of the cells being monocytes.

Cell Cultures. PBMC were suspended at a concentration of 2.0×10^6 cells/mL in RPMI-1640 media supplemented with 10% fetal bovine serum containing 100 units/mL penicillin, 100 μg/mL streptomycin, and 200 mM L-glutamine. The cells were cultured in 75-cm^2 tissue culture flasks (Corning Glassware, Corning, NY). An optimal concentration (10 μg/mL) of PHA (Gibco) was added to experimental cultures. Control cultures contained PBMC suspended in medium alone. Control and experimental cultures were incubated for various time intervals in 5% CO_2 and 95% air at 37 °C. After each incubation interval, the PBMC were washed twice with RPMI-1640, resuspended, and counted. An aliquot of PBMC was removed, and triplicate cultures were performed in flat-bottom microplates (no. 3040, Falcon Plastics, Oxnard, CA) for quantitation of lymphoblastic transformation. The degree of [^{3}H]thymidine incorporation observed after an 18-h pulse of 2.0×10^5 PBMC with 1 μCi of [^{3}H]thymidine (6.0 Ci/mmol, New England Nuclear, Boston, MA) was used as a quantitative index of cell proliferation. The [^{3}H]thymidine-pulsed PBMC were processed with a MASH cell harvestor, and the amount of incorporated radioactivity on filter pads was determined in a Beckman LS-7500 scintillation counter. The PBMC for analysis of sialyltransferase activities were washed 3 times in 0.32 M sucrose with 0.1% 2-mercaptoethanol and 0.001 M EDTA (pH 7.0) (SME). The cells were pelleted and stored at −80 °C until the sialyltransferase assays were performed.

Sialyltransferase Assays. PBMC pellets were homogenized in a Thomas tissue grinder size 0 with 2–3 volumes of SME solution, and the protein concentration of each sample was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The enzyme assays were prepared as two different series in 6 × 50 mm tubes (each in duplicate), which were performed in tandem. One series contained either LacCer or $GgOse_4Cer$, which served as exogenous acceptors for the sialyltransferases. The other series contained no exogenous acceptors, and thus the sialyltransferase activities were entirely dependent on endogenous acceptors. The tubes were dried under vacuum. The complete incubation mixture contained the following components (in micromoles unless stated otherwise) in a final volume of 0.065 mL: LacCer, 0.05, or $GgOse_4Cer$, 0.01; Triton CF-54 and Tween-80 (2:1) or Triton CF-54, 150–300 μg; cacodylate hydrochloride buffer, 5.0 (pH 6.2 for SAT-1 and pH 6.0 for SAT-3); $MgCl_2$, 0.05 M; cell hemogenate, 300–600 μg of protein (in a protein to detergent ratio of 2:1); CMP-[^{14}C]NeuAc, prepared by combining low specific activity of 1.6 mCi/mmol and high specific activity of 247.0–302.0 mCi/mmol in 1:1 ratio. The purity of the radioactive CMP-NeuAc was checked by descending chromatography on 1 in. × 23 in. Orange-C paper (Grade 589, Schleicher & Schuell Inc., Keene, NH) with ethanol–1.0 M ammonium acetate (7:3) as a solvent system. After incubation for 2 h at 37 °C, the reaction was stopped by adding 20 μL of methanol.

The samples were applied to Whatman 3MM paper and developed in a descending fashion with 1.0% sodium tetraborate (pH 9.0) for 5 h (Chein et al., 1973). The radioactive products remained at the origin whereas the free CMP-[^{14}C]NeuAc and [^{14}C]NeuAc migrated almost with the solvent front. The papers were air-dried, and the upper portion was developed in ascending fashion with the solvent system chloroform–methanol–H_2O (60:35:8). The areas of the paper containing each sample were cut into 2.5 cm × 1.5 cm pieces and radioactivity in these areas was quantitatively determined in Aquasol-2 scintillation system with a Beckman LS-7500 β scintillation counter.

Characterization of Sialyltransferase Products. The products of these enzymatic reactions were characterized by scaling up the enzyme assay 10-fold. Radiolabeled products were extracted from chromatographic papers with chloroform–methanol–H_2O (60:35:8) at 37 °C for 12 h and filtered through a scintered glass filter. Dried samples were dissolved in 0.2 mL of chloroform and applied to a 1.0-g silicic acid column. The columns were eluted with 20 mL each of the following solvents: fraction I, 100% chloroform; fraction II, chloroform–methanol, 4:1; fraction III, chloroform–methanol, 1:1; fraction IV, 100% methanol. Fractions III and IV were dried and applied to precoated silica gel 60 thin-layer plates (Merck, Darmstadt, West Germany) and developed in chloroform–methanol–0.2% $CaCl_2 \cdot 5H_2O$ (55:45:10). Autofluorograms of the plates were made by spraying them with EN^{3}HANCE (New England Nuclear, Boston, MA) 3 times and flashing briefly with a photographic light source at 3 ft. The plates were exposed to ortho-G X-ray film (Eastman Kodak Co., Rochester, NY) for 3 weeks at −70 °C. The bands corresponding to each spot in the X-ray film were scraped into scintillation vials, 10 mL of Aquasol-2 was added, and the amount of radioactivity was measured. Statistical analyses of SAT-1 and SAT-3 activities were performed by applying a regression model to the log of the values obtained from the sialyltransferase assays of control and PHA-activated PBMC.

Results

Kinetic Parameters of SAT-1 and SAT-3. Optimum reaction conditions were studied and determined to be those

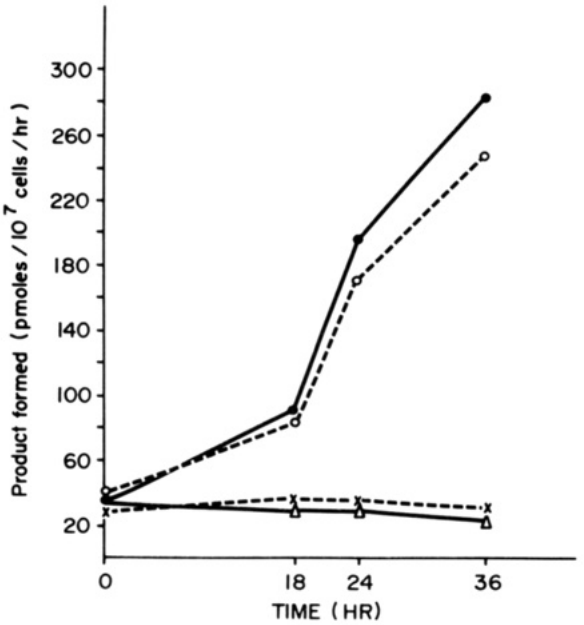

FIGURE 1: Effects of PHA on SAT-1 activity. PBMC were stimulated with PHA (10 μg/mL), and the enzyme assay was performed in the presence and absence of LacCer as described under Materials and Methods. Products were separated by a double chromatographic method, and radioactivity in the area containing the lipid product was determined. Each values is the mean of at least two separate determinations. The average error was 7.8%. Total enzyme activity in PHA-stimulated PBMC in the presence of exogenous acceptor (●); endogenous enzyme activity in PHA stimulated cells (○); total enzyme activity in unstimulated cells in the presence of exogenous acceptor (△); endogenous enzyme activity in unstimulated cells when no exogenous acceptor was added (×).

described under Materials and Methods. As determined by plotting [S]/[V] vs. [S], the apparent K_m and V_{max} with CMP-NeuAc as a variable substrate for SAT-1 were 0.19 mM and 101 pmol (mg of protein)$^{-1}$ h^{-1}, respectively, and for SAT-3 were 0.015 mM and 164.7 pmol (mg of protein)$^{-1}$ h^{-1}, respectively. With LacCer as the variable substrate, apparent K_m and V_{max} values for SAT-1 were 0.075 mM and 70.9 pmol (mg of protein)$^{-1}$ h^{-1}. The apparent K_m and V_{max} values for SAT-3 were 0.17 mM and 58.5 pmol (mg of protein)$^{-1}$ h^{-1} with $GgOse_4Cer$ as the variable substrate.

Effects of Lymphocyte Activation on SAT-1 and SAT-3 Activity. Lymphocyte activation and proliferation by PHA were estimated by the ratio of radioactivity incorporated into stimulated to nonstimulated cells (stimulation index). The stimulation indices of 1.1, 77.1, 88.2, and 92.7 were obtained at 0, 18, 24, and 36 h, respectively.

The activity of SAT-1 towards both total and endogenous acceptors increased progressively ($p < 0.001$) during the 36 h of PHA stimulation (Figure 1). Over the same time period, the activity of SAT-1 toward exogenous and endogenous acceptors in unstimulated cells did not change.

In unstimulated cells, the activity of SAT-3 was approximately 4 times higher than that of SAT-1, and the activity of SAT-3 toward both endogenous and exogenous acceptors increased progressively ($p < 0.001$) during the 36 h of PHA stimulation (Figure 2). SAT-3 activity toward exogenous $GgOse_4Cer$ in unstimulated cells increased during the first 18 h and decreased between 18 and 36 h of culture. The level of SAT-3 activity toward exogenous acceptor in PHA-stimulated cultures was significantly ($p < 0.001$) greater than that in control cultures.

Characterization of SAT-1 and SAT-3 Products. The major product (96%) formed in the presence of exogenous

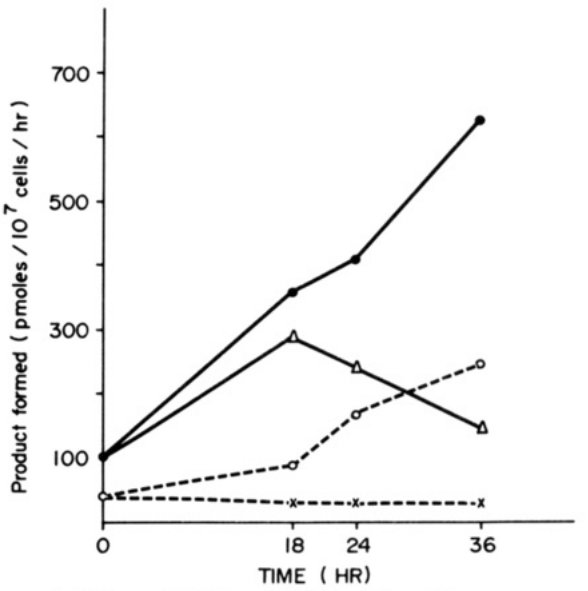

FIGURE 2: Effects of PHA on SAT-3 activity. The enzyme assay was performed in the presence and absence of exogenous acceptor $GgOse_4Cer$. Each value is the mean of at least two determinations. The average error was 10.4%. Data codes are the same as in Figure 1.

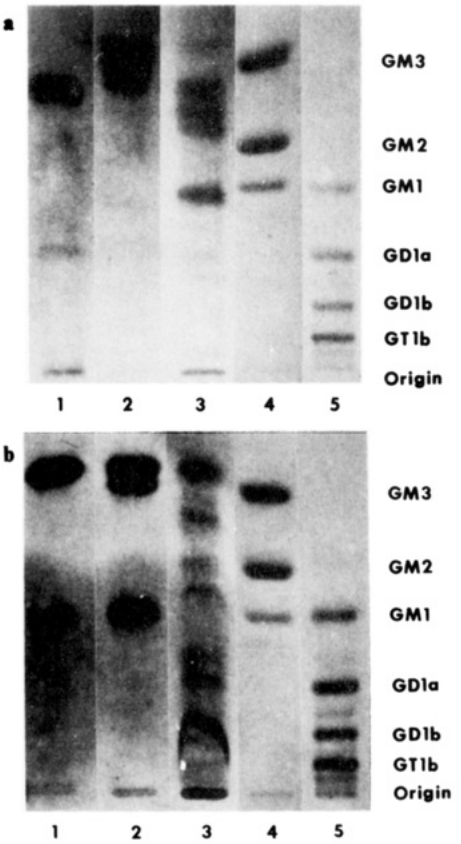

FIGURE 3: (a) Composite of thin-layer chromatograms of ganglioside fractions (lanes 1–3) eluted from 1.0-g Unisil column with 20 mL of chloroform–methanol (1:1) and detected by autofluorography. Standard gangliosides (lanes 4 and 5) were detected with resorcinol reagent. (Lane 1) Products obtained when LacCer was added to the SAT-1 assay system. (Lane 2) Products formed when no acceptor was added. (Lane 3) Products formed when $GgOse_4Cer$ was added to the SAT-3 assay system. (Lane 4) Nonradiolabeled standards GM3, GM2, and GM1. (Lane 5) Gangliosides isolated from normal human brain. (b) Thin-layer chromatograms similar to (a) except that samples in lanes 1–3 were eluted in the methanol fraction from Unisil columns.

LacCer was tentatively identified by migration on TLC as GM3 (Figure 3a). However, a small amount (3.4%) of

Table I: Effects of Mitogens on SAT-1 Activity in Broken Cell Preparation

mitogen (μg/mL)	[^{14}C]NeuAc incorporated[a]					
	phytohemagglutinin			concanavalin A		
	total	endogenous	exogenous	total	endogenous	exogenous
0	38.8	25.2	13.6	24.1	15.9	8.2
1.12	43.9	28.2	15.6	23.1	14.2	8.9
2.25	43.3	30.0	13.3	21.5	13.8	7.7
4.50	40.0	24.8	15.2	24.8	16.5	8.3
9.00	42.1	28.8	13.3	22.8	14.4	8.4
18.0	42.9	27.8	15.1	23.0	13.7	9.3

[a] Total refers to reaction rate with exogenous acceptor added. Endogenous refers to reaction rate with no exogenous acceptor added. Exogenous refers to values of total minus endogenous rates. All values are expressed as pmol (mg of protein)$^{-1}$ h^{-1} and represent the means of at least duplicate analyses. The average error was 9.4%.

product was formed that eluted in the methanol fraction and migrated close to the standard GM1 on TLC. This is probably GM1b (Figure 3b), but limited amounts of material precluded further structural characterization of either product. A radiolabeled compound that migrated with GM3 was also the major product (81.3%) that formed from endogenous acceptors. The major products from the SAT-3 assay (Figure 3), were a compound with a chromatographic mobility identical with that of GM3 (32.0%) and another (probably GM1b) that migrated close to GM1 (24.6%). However, several other radiolabeled bands eluting with both chloroform–methanol (1:1) and methanol were also seen (Figure 3). Radioactivity in the latter bands accounted for 43.6% of radiolabeled lipid products. None of these was present in any more than a trace amount from reactions with only endogenous acceptors (Figure 3) where GM3 (81.3%) and GM1b (14.6%) were the major products.

Effects of Lectin on SAT-1 Activity of Broken Cell Preparations. Different amounts of Con A and PHA were added directly to the assay reactants for SAT-1 activity. Up to a concentration of 18 μg of mitogen/mL of reaction mixture volume, neither Con A nor PHA had any effect on the amount of endogenous or exogenous product formed (Table I).

Discussion

The major new observations presented here are that activities of SAT-1 and SAT-3 can be detected in human lymphocytes and that the activities of these enzymes are closely linked to early events during cellular activation, which precede maximal proliferative responses. The apparent K_m values obtained in this study are for the most part comparable with those previously reported with other systems (Fishman et al., 1976; Simmons et al., 1975; Ng & Dain, 1977; Kemp & Stoolmiller, 1976). Differences between ours and some of the reported K_m values are probably due to differences in assay conditions and enzyme sources (Kaufman & Basu, 1966; Arce et al., 1966; Richardson et al., 1977).

Both SAT-1 and SAT-3 are involved in the synthesis of the two major lymphocyte gangliosides II^{3}NeuAc-LacCer (GM3) and IV^{3}nLc$_4$, respectively. Although GgOse$_4$Cer was used as the exogenous acceptor in the assay for SAT-3, convincing evidence indicates that both nLcOse$_4$Cer and GgOse$_4$Cer are equally effective acceptors for SAT-3 (Basu et al., 1982; Yip & Nguyen, 1981). Although the precise biological functions of glycosphingolipids remain to be determined, substantial evidence indicates important roles for them in a variety of cell surface phenomena including receptors for hormones, intercellular recognition and adhesion, growth control, and immunoregulation (Hakomori, 1981; Fishman & Brady, 1976). Lectin activation of lymphocytes has previously been used as an in vitro model system to study the biochemical mechanisms underlying cellular growth and immunoregulation (Yates et al., 1980), and several investigators have shown that some gangliosides inhibit the proliferative responses of lymphocytes to a variety of stimuli (Whisler & Yates, 1980; Sela, 1981). The relation of the present to the latter studies is unclear, but two possibilities are suggested. First, endogenously synthesized gangliosides may have different biological effects than exogenously added ones. Second, structurally different gangliosides could have opposing effects on lymphocyte activation.

Previous results show that lymphocyte activation increases the rate of incorporation of radiolabeled precursors into both neutral glycolipids and gangliosides (Narasimhan et al., 1976). The main question addressed in this study is whether early alterations in ganglioside biosynthesis represent increases in glycosyltransferase activities or might be explained by other factors, e.g., alterations in transport, pool size, appearance of an activator protein, or specific activities of the precursors. The present results demonstrate that lectin activation increases the activities of both SAT-1 and SAT-3. Within the first 18 h of incubation, both SAT-1 and SAT-3 activities more than double in PHA-stimulated cultures and continue to increase through to the end of the experiment (36 h). By 18 h of incubation, cell proliferation (estimated by thymidine incorporation) was quite active and continued unabated through to the end of the experiment. The parallel changes in sialyltransferase activities and lymphocyte proliferation suggest that sialylation of glycolipids may be an important aspect of lymphocyte activation, and/or cell division. The elevated levels of GM3 in leukemic cells (Westrick et al., 1983; Levis et al., 1976) and GM1b in other types of cancer (Matsumoto et al., 1981) are consistent with their being involved in the neoplastic process. In addition, the present findings indicate that the increased activity is not due to a direct effect of the lectin on the enzyme because the addition of PHA to the reaction mixture had no effect on the activity of either enzyme.

The addition of LacCer to assays of PHA-stimulated cells increased both the total amount of radiolabeled lipid product and the proportion of radiolabel in GM3 from 81 to 96% of the total radioactivity recovered in gangliosides. The addition of GgOse$_4$Cer to such assays considerably increased the total lipid labeling and increased the proportion of radioactive GM1b from 15 to 25%. GM3 and GM1b accounted for over 96% of the radiolabeled product when either LacCer or no exogenous acceptor was added to the reaction mixture, but these two gangliosides combined accounted for only 57% of the product when GgOse$_4$Cer was added. Several unidentified radioactive bands seen by autofluorography were isolated from the SAT-3 reaction mixture that were not isolated from either the SAT-1 or endogenous acceptor assay. Most of these compounds migrated on TLC more slowly than GM1b and may have been sialylated products of reactions in which GM1b was used as a substrate. Consistent with this is the evidence that biosynthesis of a disialoganglioside occurs during the

incubation of $GgOse_4Cer$ and CMP-NeuAc with embryonic chick brain (Kaufman & Basu, 1966) and NIE and CEF cells (Stoffyn & Stoffyn, 1980).

This study has demonstrated that in human PBMC the activities of two sialyltransferases are essential for the synthesis of the two major human lymphocyte gangliosides. PHA increases the activities of these enzymes in whole lymphocytes but not broken cell preparations. These changes in enzyme activities parallel the proliferation of lymphocytes and suggest that they may be of importance in the biochemical mechanisms of cell division. Studies of changes in related enzymes during lymphocyte activation should give a clearer picture of the role of glycolipids in immunoregulation, cell division, and neoplastic transformation.

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Registry No. SAT-1, 55071-95-9; SAT-3, 83745-06-6; GM3, 54827-14-4; GM1b, 57576-20-2.

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