Neutral and Sialosyl Glycosphingolipid Composition and Metabolism of Human T-Lymphoblastic Cell Line MOLT-3 Cells: Distinctive Changes as Markers Specific for Their Differentiation

By Makoto Akashi, Fumimaro Takaku, Hisao Nojiri, Yasusada Miura, Yashitaka Nagai, and Masaki Saito

Changes in the composition and metabolism of glycosphingolipid (GSL), which is one of the cell surface constituents, during cell differentiation of human T-lymphoblastic leukemia cell line MOLT-3 cells were examined with special reference to their alterations in E rosette-forming capacity and expression of surface antigens specific for T-cell lineage. Three molecular species of neutral GSL and \( \geq 13 \) molecular species of acidic sialosyl-GSL (ganglioside) were detectable on high-performance thin-layer chromatography (HPTLC) in untreated MOLT-3 cells. The major components were ceramide monohexoside and gangliosides GM3 and GD1a. When the cells were induced by 12-O-tetradecanoyl phorbol 13-acetate (TPA) to differentiate into more mature T cells, the ganglioside composition changed distinctly, and the total ganglioside content increased considerably; mono-, di-, and tri-sialosyl gangliosides concurrently showed significant increase, but no new molecular species of GSL specific for the differentiation were detected. The activity of one sialyltransferases, CMP-sialic acid:CDH sialyltransferase, which synthesizes ganglioside GM3 and the total sialic acid content of the cell surface, paralleled the extent of cell differentiation. Examination of another human T-lymphoblastic leukemia cell line, HPB-ALL, indicated that TPA could also induce the cells to differentiate along T-cell lineage and that changes in the ganglioside pattern during differentiation are similar to those of MOLT-3 cells. The results indicate that human T-lymphoid cell differentiation intimately involves elongation of neutral oligosaccharide-moieties and the addition of sialic acid residues to gangliosides, resulting in more mature T cells containing higher gangliosides. Both the sialyltransferase activity and the sialic acid content, as well as the ganglioside pattern, might be new biochemical markers specific for human T-lymphoblastic cell differentiation.

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469
MATERIALS AND METHODS

Chemicals. TPA was obtained from Chugai Pharmaceutical, Tokyo, dissolved in dimethylsulfoxide (DMSO), and stored at −20°C until use. Sialidase [EC 3.2.1.18; N-acetylneuraminosyl glycohydrolase] from Vibrio cholerae was purchased from Behringwerke AG, Marburg, FRG. Sialic acid and a bovine brain ganglioside mixture (Sigma, Type II), consisting of 7.5% GM1, 2.6% GD3, 5.6% GD1α, 11.5% GD1b, and 21.7% GT1b,7 were obtained from Sigma Chemical, St Louis. An anti-GD3 monoclonal antibody, 5H6, was a gift from Dr. Yoshitaka Nagai (Professor, Faculty of Medicine, University of Tokyo). All other reagents were of the best grade available and were from Wako Pure Chemicals, Osaka, Japan.

Cells, cell culture, and growth measurement. Human T-lymphoblastic cell lines, MOLT-3, and HPB-ALL cells, exhibit common T-lymphoblastic characteristics such as the expression of common thymocyte antigens.8,9 They were grown in Falcon tissue culture flasks (Becton Dickinson Labware, Oxnard, CA) in RPMI 1640 (Flow Laboratories, McLean, VA), supplemented with 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS, Flow Laboratories), at 37°C in a humidified atmosphere of 5% CO2. The cell number and viability were determined in a hemocytometer in 0.1% erythrosin B by the dye exclusion method.

E Rosette assay and establishment of a MOLT-3 or HPB-ALL subpopulation having a low E rosette-forming capacity. The ability of MOLT-3 or HPB-ALL cells to bind to sheep RBCs (SRBCs) was determined according to the E rosette assay method, as previously described.10,11 Normal human peripheral blood lymphocytes were used as positive controls. The parent MOLT-3 cells (E rosette-forming capacity: 50% to 60%) or HPB-ALL cells (90% to 95%) were allowed to bind 2-aminoethyl-isothiouronium bromide (AET)-treated SRBCs (E) at 4°C for 60 minutes; E rosette-positive [E(+)] and E rosette-negative [E(−)] cells were separated by centrifugation on a Ficoll-Hypaque gradient (sp gr 1.007, Pharmacia, University of Tokyo). All other reagents were of the best grade available and were from Wako Pure Chemicals, Osaka, Japan.

Differentiation-induction by TPA in the subpopulation having a low E rosette-forming capacity. MOLT-3 cells were seeded at an initial concentration of 2 × 10⁶ cells/mL and then incubated with three different concentrations (1.62, 16.2, and 162 nmol/L) TPA. HPB-ALL cells were treated with 16.2 nmol/L TPA. Duplicate cultures were carried out for each of the experimental culture-day points. To examine effects of the time of exposure to TPA, ie, the “commitment time,” MOLT-3 cells were incubated with 16.2 nmol/L TPA for 5, 15, 30, and 60 minutes, respectively, washed, and then transferred to fresh medium without TPA. The E rosette assay was performed at 72 or 96 hours after initiation of the culture, and E rosette-forming capacities were compared with those observed in the continuous presence of TPA for 72 or 96 hours, respectively. DMSO, which was used to dissolve TPA, had no detectable effect on either MOLT-3 or HPB-ALL cells.

Cell surface antigens. To assess quantitative and qualitative changes in surface antigens of the TPA-treated cells, OKT-series monoclonal antibodies, such as OKT4, OKT8, OKT11 (Ortho Diagnostic Systems, Raritan, NJ), were used for fluorescence-activated flow cytometry (Spectrum III, Ortho).

Lipid extraction. For lipid analysis, cells were collected, washed twice with phosphate-buffered saline (PBS), lyophilized, and kept at −80°C until use. Total lipids were extracted from the lyophilized materials, equivalent to 1 to 3 × 10⁶ cells, with a mixture of chloroform-methanol (C:M) (1:1, vol/vol) containing 5% (vol/vol) water, and with C-M (1:2), successively. The extracts were combined and then separated into the acidic and neutral lipid fractions according to the method described by Ando and co-workers.18 The pellet was assayed for protein by the method of Lowry and colleagues.19

Analysis of neutral GSLs. Neutral GSLs were purified from the lipid fraction according to the acetylation method of Saito and Hakomori.20 They were separated on high-performance thin-layer chromatography (HPTLC) plates with a solvent system of C-M:water (56:25:4), sprayed with orcinol-H₂SO₄ reagent, and then visualized with a dual-wavelength TLC scanner (Shimadzu CS-910) at 540 nm. The hexose content of neutral GSLs was determined by the anthrone-H₂SO₄ method (Shimadzu CS-910) at 540 nm. The hexose content of neutral GSLs was determined by the anthrone-H₂SO₄ method of Shichida and colleagues.21

Analysis of gangliosides. The acidic lipid fraction was subjected to mild alkaline treatment to hydrolyze containing phospholipids and then desalted by gel filtration on a Sephadex G-50 column. The recovered ganglioside fraction was separated on a HPTLC plate with a solvent system of C-M:0.5% CaCl₂·2H₂O (55:45:10) or C-M:2.5 N ammonium hydroxide (60:35:8). The gangliosides were sprayed with resorcinol-HCI reagent, visualized by heating the HPTLC plate at 95°C, and then determined quantitatively by the densitometric scanning method.22 Lipid-bound sialic acid in the total gangliosides fraction was estimated by the resorcinol-HCl method as modified by Suzuki.23

Structural determination of gangliosides. For structural analysis of gangliosides, enzymatic hydrolysis with sialidase and the TLC-immunostaining were done. Gangliosides were dissolved in 90 μL distilled water, mixed with 10 μL Vibrio cholerae sialidase, and then incubated at 37°C for 16 hours. After incubation, 2 mL C-M (1:1) was added and the products were immediately evaporated under N₂ stream at 42°C and examined by TLC. Resorcinal-HCl was used for detection of the material.

For TLC-immunostaining, gangliosides were developed on silica gel-coated plastic plate (Polygram; Macherey-Nagel, Duren, FRG) with C-M:3 N ammonia (60:40:9) and incubated with the blocking buffer (1% polyvinylpyrrolidone and 0.2% NaCl) in PBS at 37°C for 1 hour. This was followed by incubation for 16 hours with anti-GD3 monoclonal antibody 5H6, which reacts with gangliosides having the NeuAc residue linked to the terminal galactose through the α2-3 linkage (Y. Nagai et al, unpublished observations, January 1988). After the plate was washed five times with the washing buffer (0.1% Tween 20 in PBS), it was reacted with the blocking buffer at 37°C for 15 minutes and then incubated with the peroxidase-conjugated anti-rabbit Ig G, A, and M antisera, diluted to 1:1,000, for 1 hour at room temperature. The substrate of peroxidase was 0.003% 4-chloro-l-naphthol in 50 mmol/L Tris-HCl buffer (pH 7.4) containing 200 mmol/L NaCl. The spots were quantitatively determined with a dual-wavelength TLC densitometer at the analytic wavelength of 580 nm and the control wave length of 710 nm.

Assay of CMP-sialic acid:CDH sialyltransferase (EC 2.4.99.9; N-acetylneuraminic-acid-lactosylceramide sialyltransferase) activity. MOLT-3 cells were washed twice with PBS, suspended and sonicated at 0°C in 25 mmol/L sodium cacodylate buffer (pH 6.3), and then used as enzyme preparations. The sialyltransferase activity was assayed according to the method described by Hashimoto and co-workers.24 A typical incubation mixture (100 μL) containing the enzyme protein (30 to 120 μg) and CMP-[¹⁴C] NeuAc (10 nmol) was incubated with or without CDH (10 nmol) at 37°C in a shaking water bath for 60 minutes, and the reaction was terminated by the addition of 40 μL ice-cold 0.5 mol/L KCl-0.25 mmol/L EDTA solution. Then, 0.1 mmol/L KCl solution (5 mL), containing 0.3 mg egg lecithin was added, and the reaction mixture was applied to a SEP-PAK C18 cartridge. The lipid fraction, which was trapped by the SEP-PAK on washing with water, was eluted with methanol, and the residue was dried in vacuo. The dried residue was reconstituted in 0.1 mol/L NaH₂PO₄-0.1 mol/L NaOH buffer (pH 7.4), containing 0.1 mmol/L KCl and 0.5 mmol/L MgCl₂, and then applied to the Sepharose 6B column. The enzyme activity was determined in the eluate with a sensitive radioassay using [¹⁴C] NeuAc-labeled lactosylceramide.24
followed by elution with C-M (1:1) according to the method of Kundu and Suzuki. The radioactivity incorporated into the lipid fraction was determined with a liquid scintillation counter. The experiment was performed in triplicate, and the endogenous value determined on omission of the lipid acceptor from the reaction mixture was subtracted from the experimental data.

Assay of sialidase-susceptible sialic acid residues. Sialidase-susceptible sialic acid residues were assayed as follows: MOLT-3 cells were washed twice with PBS and then incubated with 0.1 U Vibrio cholerae sialidase in 0.1 mL PBS (pH 6.5) at 37°C for 60 minutes. After centrifugation, the supernatant was collected and assayed for released sialic acid by the method of Roboz and colleagues and the pellet was assayed for protein by the method of Lowry and colleagues.

RESULTS

Heterogeneity of MOLT-3 or HPB-ALL cells in terms of E rosette-forming capacity. We investigated cellular heterogeneity of a freshly subcultured preparation of leukemic T-cell line MOLT-3 cells and divided them into three subpopulations on the basis of capacity to form E rosettes. Cells showed a very low (~10%), an intermediate (50% to 60%), and a very high (80% to 90%) E rosette-forming capacity, respectively. We could also establish a subpopulation of HPB-ALL cells showing an E rosette-forming capacity of ~40% from the parental cells of very high E rosette-forming capacity (~100%). The ability of each subpopulation to form E rosettes remained constant for at least 3 months. To obtain unambiguous results as to the differentiation-induction of MOLT-3 or HPB-ALL cells with TPA, we used the first MOLT-3 subpopulation, of which only ~10% exhibited E rosette formation (Table 1) and the HPB-ALL subpopulation of relatively lower E rosette-forming capacity (~40%) (Table 2).

Effect of TPA on cell proliferation and cell volume. When MOLT-3 cells were seeded at an initial concentration of 2 x 10^6 cells/mL, they grew at a similar rate to the control culture for the first 24 hours in the presence of TPA, and then the growth rate remarkably decreased and leveled off after culture day 4 (Fig 1A). The growth inhibition with TPA was almost completely dose dependent and, simultaneously, the cell volume was significantly decreased over the first 4 days after treatment with 16.2 nmol/L TPA. The proliferation of HPB-ALL cells was also significantly inhibited and the cell volume slightly decreased by the TPA treatment. The cell viability was constantly >95% throughout the experiments.

Fig 1. Inhibition of the growth and induction of E rosette-forming capacity by TPA of the very low E rosette-forming MOLT-3 subline cells. The cells at the logarithmic growing phase were recultured at an initial cell concentration of 2 x 10^6 cells/mL with three different concentrations of TPA. E rosette formation was assayed at various times after the incubation was started as described in the text. Each value represents the mean of duplicate quantitations for two separate experiments. SD was <10%. Viability was >90% in each group throughout the culture periods. Without TPA (control) (○), with 16.2 nmol/L TPA (△), with 16.2 nmol/L TPA (◇), with 162 nmol/L TPA (◇), and with 162 nmol/L TPA (◇). (A) Effect of TPA on growth of MOLT-3 subline cells. (B) Effect of TPA on kinetics of induction of E rosette-positive cells.

### Table 1. Surface Antigen Profile of MOLT-3 Subpopulation Having Very Low E Rosette-Forming Capacity

<table>
<thead>
<tr>
<th>Monoclonal Antibody</th>
<th>Positive Cells (%)</th>
</tr>
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<tbody>
<tr>
<td>OKT 4</td>
<td>47.8</td>
</tr>
<tr>
<td>6</td>
<td>88.1</td>
</tr>
<tr>
<td>8</td>
<td>88.1</td>
</tr>
<tr>
<td>9</td>
<td>70.8</td>
</tr>
<tr>
<td>11</td>
<td>12.8</td>
</tr>
</tbody>
</table>

The surface antigen profile of the very low (~10%) E rosette-forming MOLT-3 subline cells was determined by indirect immunofluorescent assay using OKT series monoclonal antibodies. The cells were analyzed in an Ortho Spectrum III fluorescence-activated flow cytometer.

### Table 2. Effect of TPA Treatment on Cell Surface Antigens in HPB-ALL Cells

<table>
<thead>
<tr>
<th>Surface Antigen</th>
<th>Untreated HPB-ALL Cells (%)</th>
<th>TPA-Treated HPB-ALL Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E rosette</td>
<td>46.5</td>
<td>87.0</td>
</tr>
<tr>
<td>OKT 4</td>
<td>95.2</td>
<td>32.0</td>
</tr>
<tr>
<td>6</td>
<td>65.6</td>
<td>59.6</td>
</tr>
<tr>
<td>8</td>
<td>80.3</td>
<td>82.6</td>
</tr>
<tr>
<td>9</td>
<td>88.1</td>
<td>12.4</td>
</tr>
<tr>
<td>(4/8 ratio)</td>
<td>1.2</td>
<td>0.4</td>
</tr>
<tr>
<td>11</td>
<td>42.0</td>
<td>92.0</td>
</tr>
</tbody>
</table>

The cell surface antigen(s) that reacted with OKT 4, 6, 8, 9, and 11 monoclonal antibodies were determined by indirect immunofluorescent assay on culture day 5 after the incubation was started with or without 16.2 nmol/L TPA at 37°C. The ratio of OKT4/8 was calculated. Each value represents the mean of duplicate quantitations in two different experiments. SD was <6%. Viability was >90% throughout the culture periods.

Effect of TPA on E rosette-forming capacity. The ability of MOLT-3 cells to form E rosettes increased markedly with increasing concentrations of TPA in a time-dependent manner, and a significant increase in the percentage of E rosette-positive cells was observed at a concentration as low as 1.62 nmol/L with the extent of increase ranging approximately from three to four times the control level after culture day 4 (Fig 1B). TPA could also increase the E rosette-forming capacity on another T-cell line, HPB-ALL cells (Table 2).

Effect of varying time of exposure to TPA on differentiation-induction. To examine the culture time required for eliciting differentiation-induction, MOLT-3 cells were incubated with 16.2 nmol/L TPA for 5, 30, and 60 minutes, respectively, washed, and then transferred to fresh medium without TPA. The growth inhibition almost paralleled the
exposure time (Fig 2A), but the continual presence of TPA throughout the incubation caused considerably higher growth inhibition. The relationship between the time of exposure to TPA and the induction of E-positive cells is shown in Fig 2B. Even with an exposure time of as short as 5 minutes, an increasing tendency of the percentage of E-positive cells was already observable, and considerable induction of E-positive cells was observed on 15-minute exposure to TPA. After this exposure time, the induction of E rosette-forming capacity leveled off, and a longer exposure time caused no further increase in induction (Fig 2B). The results indicate that exposure to TPA for only a short time is required for induction of this differentiation marker. Alternatively, the short exposure time required may be a result of very rapid and irreversible binding of TPA to these cells.

Cell surface antigens of MOLT-3 cells that reacted with OKT-series monoclonal antibodies. Table 1 shows the surface antigen profile of untreated MOLT-3 cells as to reactivity of OKT-series monoclonal antibodies. This profile was different from that for mature peripheral T cells, in that MOLT-3 cells expressed surface antigens that reacted with OKT6 and OKT9 monoclonal antibodies, which have been generally assumed to react with immature T lymphocytes (thymocytes). Approximately 90% of the cells expressed the surface antigen that reacted with OKT8 monoclonal antibody, which is usually dominant in suppressor-cytotoxic T cells, whereas ~50% of the cells were positive for OKT4, which is known to be specific for inducer-helper cells. The results indicate that some MOLT-3 cells express both OKT4 and OKT8 markers.

Changes in reactivity of monoclonal antibodies OKT4, OKT6, OKT8, OKT9, and OKT11 with MOLT-3 cells during differentiation induced by TPA. Kinetics of changes in the cell surface markers of MOLT-3 cells during exposure to TPA are shown in Fig 3. Induction with TPA resulted in a significant increase in the number of OKT11...
positive cells, which was shown to be time dependent. This increase almost paralleled that in the E rosette-forming capacity. In contrast, a remarkable decrease in the number of OKT4-, OKT6- and OKT8-positive cells was observed during culture with TPA, and concomitantly, a significant decrease in OKT4/8 ratio occurred. The proportion of cells expressing OKT9 antigen decreased rapidly after exposure to TPA and they were almost undetectable on day 3 or 4.

Effect of TPA on the expression of cell surface antigens of HPB-ALL subline cells. Treatment of HPB-ALL cells with TPA resulted in a significant increase in the number of OKT11-positive cells with a concomitant increase of E rosette-forming capacity (Table 2). The OKT4-positive cells remarkably decreased although no remarkable changes could be seen in the number of OKT8-positive cells, which resulted in a remarkable decrease in OKT4/8 ratio. The OKT6-positive cells showed a slight tendency to decrease.

Glycosphingolipid (GSL) compositions of MOLT-3 and HPB-ALL cells. Both neutral GSL and ganglioside compositions of MOLT-3 cells were quantitatively analyzed by HPTLC, and the structure of gangliosides was examined in more detail by TLC-immunostaining procedure and enzymatic hydrolysis. The results are presented in Table 3. Neutral GSL of the cells was found to be composed of three components, ie, ceramide monohexoside (CMH), ceramide dihexoside (CDH) and paragloboside (PG) (Fig 4). The major component was CMH, and no bands were seen in ceramide trihexoside (CTH) or globoside (globotetraosyl ceramide) regions. Gangliosides of MOLT-3 cells consisted of ≥13 molecular species (Figs 5A and 6A). Bands 1 and 2, which were the major component and comprised ~50% of the total amount of gangliosides, had the mobility identical to that of GM3, and reacted with the monoclonal antibody 5H6 on TLC-immunostaining (Fig 7), indicating that this component is GM3. The trace of band 5, which was located near ganglioside GM2 (Tay-Sachs ganglioside) on HPTLC, was identified as sialylparagloboside, since this band also reacted with the antibody 5H6. Two thin bands that migrated to the vicinity of GM1 were observed (bands 6 and 7). Bands 8 and 9, which migrated below GM1 in the neutral solvent containing calcium (Fig 5A) and were located ahead of GM1 in the alkaline solvent containing ammonium hydroxide, were identified as GD3 since they strongly reacted with the monoclonal antibody 5H6 (Fig 7). The Rf values of gangliosides assigned for bands 10 and 11, and 13 were similar to those of GD1a doublet, and GT1b, respectively. Treatment of these gangliosides with Vibrio cholerae sialidase increased the amount of two bands corresponded to GM1, and then bands 10 and 11 were considered to be GD1a.

Gangliosides of HPB-ALL cells consisted of 11 or more molecular species, and the major component was ganglioside GM3 (bands 1 and 2), which comprised >90% of the total amount of gangliosides (Figs 5B and 6C, D).

Changes in GSL patterns during differentiation of MOLT-3 and HPB-ALL cells. When differentiation was induced with TPA, no new molecular species of GSLs appeared in the differentiation-induced cells, but characteristic changes in the GSL composition were observed (Figs 4 through 6). During such in vitro MOLT-3 cell differentiation, the total amount of neutral GSLs showed a remarkable increase, with CMH predominantly increasing and CDH showing a slight tendency to increase when calculated on the
The second disialosyl ganglioside GD3, which became one of the major showing the most remarkable increase was found to be 6).

The neolacto-type monosialosyl ganglioside, SPG, remained with TPA when calculated on the basis of nanograms lipid-

More remarkable changes in both the amount and composition of gangliosides were observed during differentiation with TPA when calculated on the basis of nanograms lipid-bound sialic acid/mg protein (Fig 4). More remarkable changes in both the amount and composition of gangliosides were observed during differentiation with TPA when calculated on the basis of nanograms lipid-bound sialic acid per milligram of protein than on the basis of micrograms lipid-bound sialic acid per 10^8 viable cells (Fig 6). Monosialosyl ganglioside GM3, the major component of MOLT-3 cells, considerably increased, but the component showing the most remarkable increase was found to be disialosyl glycoside GD3, which became one of the major components after induction with TPA (Fig 6A). The second major disialosyl ganglioside, GD1a, and a minor component, trisialosyl ganglioside GT1b, also significantly increased. The neolacto-type monosialosyl ganglioside, SPG, remained as a minor component even after induction with TPA (Fig 6A and B).

Treatment of HPB-ALL cells with TPA significantly increased the total amount of gangliosides and no new molecular species could be seen in the differentiation-induced cells (Figs 5B and 6C and D), which was in good accordance with the result concerning TPA-induced MOLT-3 cells. GM3 and GD1a (bands 10 and 11) significantly increased, and GD3 (bands 8 and 9) showed a slight but significant increase. The results were in the tendency of change of the ganglioside pattern similar to those with TPA-treated MOLT-3 cells.

Changes in sialyltransferase [EC 2.4.99.9; CMP-sialic acid:CDH sialyltransferase] activity during differentiation of MOLT-3 cells with TPA. As shown in Table 4, the CMP-sialic acid:CDH sialyltransferase activity responsible for biosynthesis of ganglioside GM3 was significantly enhanced during differentiation of MOLT-3 cells with TPA. The activity on day 3 after induction with 16.2 nmol/L TPA was about twice the control level and that on day 5 was three times higher than the control, which well explained the parallel increase in the product, ganglioside GM3. The results indicate that the remarkable increase in ganglioside GM3 of MOLT-3 cells after treatment with TPA was owing to significant activation of the sialyltransferase responsible for biosynthesis of the ganglioside.

Sialidase [EC 3.2.1.18; N-acetyleneuraminosyl glycohydrolase]-susceptible sialic acid residues on the surface of TPA-treated MOLT-3 cells. To determine the total amount of sialic acid residues of sialoglycoconjugates located on the surface of MOLT-3 cells during differentiation with TPA, relatively mild sialidase-treatment was performed to avoid cell damage. Under the conditions used for the present experiment, >90% of the starting cells were recovered as viable cells after the sialidase treatment. As shown in Table 5, sialidase-releasable sialic acid residues increased significantly during induction with 16.2 nmol/L TPA, and their content was more than two-fold higher on day 5 than on day 3. The results indicate that the increase in sialic acid residues on the cell surface is intimately correlated with the differentiation of MOLT-3 cells, as demonstrated by the remarkable increase in gangliosides, which represent lipid-bound sialic acid residues located on the surface.

DISCUSSION

We investigated characteristic changes in GSL profiles during differentiation of human T-lymphoblastic cell lines MOLT-3 and HPB-ALL induced by TPA, inasmuch as we had found that particular molecular species of sialosyl GSLs, gangliosides, specifically increased depending on the direction and stage of differentiation of human myeloid leukemic cells, ganglioside profile becoming more complex in more mature granulocyte cells8,9,26, furthermore, these specific molecules themselves exhibited remarkable differentiation-inducing activities toward the myeloid cells.9,12,23 In addition, we reported previously that the ganglioside composition of human T-cell lines were generally more complex than that of B-cell lines, the content being greater for T-cell lineage, and that E rosette-positive T-cell lines showed a relatively higher content and a more complex profile of gangliosides compared with E rosette-negative counterparts.21 It has been reported that certain human T-lymphoblastic cell lines, notably MOLT-3 and Jurkat, can be induced to differentiate in culture by TPA,5,6 and that a freshly cloned culture of MOLT-3 cells showed cellular heterogeneity with respect to size, proliferative activity, and presence and quantities of biochemical and immunologic markers, suggesting that MOLT-3 cultures may contain a heterogenous population of
Fig 6. Changes in the ganglioside composition during differentiation of the MOLT-3 (A, B) and HPB-ALL (C, D) subline cells induced by TPA. The total amount of gangliosides was determined by measuring the lipid-bound sialic acid contents as described in the text. Each ganglioside component was determined quantitatively by the densitometric scanning method. Each value represents the mean of duplicate quantitations in two different experiments. SD was <10%. Numbers correspond to those in Fig 5.

We have also found heterogeneity of T-cell lines, both MOLT-3 and HPB-ALL cells, in terms of E rosette-forming capacity, and succeeded in establishing a subline from the parental MOLT-3 cells, which stably exhibited a very low E rosette-forming capacity, ie, only ~10% of the cells were able to form E rosettes (Fig 1B) and to react with monoclonal OKT11 antibody (Fig 3A), whereas ~60% of the parental cells constantly showed an E rosette-forming capacity. When this subline was treated with TPA, its growth was remarkably inhibited; simultaneously, differentiation was induced in terms of a considerable enhancement of E rosette-forming capacity and a significant increase in the surface antigen that reacted with OKT11. Concomitantly, the ganglioside pattern remarkably changed with significant increases in almost all the ganglio-series molecular species including mono-, di-, and trisialosyl gangliosides, making it more complex. Moreover, the band 8 and 9 components increased most predominantly and were identified as disialosyl ganglioside GD3 doublet since they reacted with anti-GD3 antibody on a thin-layer plate, migrated below GM1 with the neutral solvent with calcium, were located ahead of GM1 with the alkaline solvent, and their precursor molecule, monosialosyl ganglioside GM3, was considerably increased, especially when calculated on the basis of milligram of protein instead of the cell number. This may be attributed to the fact that TPA can induce, in the cell line MOLT-3, physical and morphological changes consistent with the differentiation of the cells, ie, after treatment with TPA, the cell line contracted in cell volume, to about half the volume of untreated controls, and analysis of the cell volume indicated that the cells on the whole decreased in volume and became more homogenous in volume than untreated cells.

The sialyltransferase, CMP-sialic acid:CDH sialyltransferase, which synthesizes ganglioside GM3 and is a key enzyme in biosynthesis of ganglio-series gangliosides, significantly increased during MOLT-3 cell differentiation.
Each value for the sialyltransferase activity and the content of gangliosides represents the mean ± SD for two and three experiments, respectively.

Fig 7. HPTLC chromatograms showing hydrolytic cleavage of the sialyl residue from gangliosides of MOLT-3 cells by *Vibrio cholerae* sialidase. The gangliosides were incubated with 10mU *Vibrio cholerae* sialidase at 37°C for 16 hours. After incubation, the reaction product was processed as described in the text. Resorcinol-HCl was used for detection of the materials. Lane 1, ganglioside GM1; lane 2, ganglioside GD3; lane 3, ganglioside GD1a; lane 4, gangliosides of TPA-treated MOLT-3 cells; lane 5, gangliosides of TPA-treated MOLT-3 cells hydrolyzed with *Vibrio cholerae* sialidase; lane 6, ganglioside GD1a hydrolyzed with *Vibrio cholerae* sialidase.

Fig 8. TLC chromatogram immunostained with monoclonal antibody 5H6 and its densitometric scanning pattern of the gangliosides of MOLT-3 cells. Gangliosides were separated on silica gel-coated plastic plate with chloroform-methanol-3N ammonia (60:40:9). After blocking, the plate was incubated with the monoclonal antibody 5H6 and then reacted with peroxidase-conjugated anti-rabbit Ig G as described in the text.

induced by TPA. This explained our present finding of remarkable changes in the ganglioside profile during differentiation. Normal human peripheral blood lymphocytes were previously found to be richer in total gangliosides than thymocytes and tonsil lymphocytes and to contain ganglioside GM3 as the major component, suggesting that gangliosides increased as normal T-cell differentiation proceeded. Iwamori and colleagues recently reported that cortisone-sensitive and cortisone-resistant thymocytes in rabbits expressed different ganglioside patterns and that the major ganglioside GM3 content in the former cells (immature T cells) was much higher than that in the latter (mature T cells). Together, these results suggest that the amount of gangliosides, especially ganglioside GM3, might characterize increase during differentiation along T-cell lineage, regardless of whether the cells are normal or malignant. Furthermore, sialidase-susceptible sialic acid residues on the surface of MOLT-3 cells also increased significantly after induction with TPA, which indicates that sialoglycoproteins as well as sialoglycolipids might be augmented during differentiation stage-associated changes, as previously shown in both human lymphoid leukemia cells and murine T lymphocytes. We also showed that TPA is capable of inducing another T cell-type leukemic cell line (HPB-ALL cells) to differentiate into more mature cells, and that changes in the ganglioside pattern during differentiation are similar to those of MOLT-3 cells. These results evidently

<table>
<thead>
<tr>
<th>Culture</th>
<th>CMP-NeuAc:CDH Sialyl Transferase Activity</th>
<th>GM3</th>
<th>Total Gangliosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>66.4 ± 5.2†</td>
<td>69.6 ± 0.5</td>
<td>122.4 ± 1.0</td>
</tr>
<tr>
<td>Day 3</td>
<td>108.3 ± 16.7</td>
<td>127.7 ± 2.5</td>
<td>303.7 ± 6.1</td>
</tr>
<tr>
<td>Day 5</td>
<td>210.7 ± 9.7</td>
<td>276.2 ± 3.3</td>
<td>601.6 ± 7.4</td>
</tr>
</tbody>
</table>

MOLT-3 subline cells were treated with 16.2 nmol/L TPA.

*Sialyltransferase activity is expressed at the amount of [14C] NeuAc incorporated into the lipid fraction per milligram of protein of the cell lysate in the presence of the acceptor, CDH.

†Each value for the sialyltransferase activity and the content of gangliosides represents the mean ± SD for two and three experiments, respectively.
support our hypothesis that the ganglioside pattern is a specific marker for the differentiation of human T-cell lineage.

Recently Kiguchi and co-workers reported that treatment of CEM cells, a T-cell leukemia cell line, and TPA resulted in a significant increase in the amount of a lacto-series ganglioside, SPG. However, we showed in the present study that this ganglioside molecule was a minor component in untreated MOLT-3 cells and remained so even after induction of the cells with TPA. Considering such discrepancies and the previous report by Nagasawa and colleagues that two leukemic T-cell lines, CCRF-CEM and CCRF-HSB-2, were unresponsive to the inducer, TPA, the responsiveness to TPA may vary, depending on the kind of cell line, and dramatic changes in the ganglioside profile during differentiation with TPA may not be a general phenomenon for all leukemic T-cell lines. Kuriyama and co-workers investigated GSLs of adult T-cell leukemia-lymphoma (ATLL) cells and showed the characteristic presence of ganglioside GD3, associating its appearance with the malignant transformation of T lymphocytes into ATLL cells, although their identification of GD3 was dependent only on HPTLC analyses.

Our current results demonstrate that short time exposure (only 15 minutes) to TPA is sufficient to induce a significant increase in the level of E rosette-forming capacity, suggesting that the commitment time required for differentiation-induction of the leukemic T lymphoblasts may be ≤30 minutes. Why the exposure time needed for TPA to induce the cells is so short remains to be elucidated, but the short time may be owing to the very rapid and strong binding of TPA to the cells. This explanation is supported by reports that TPA can bind tightly and rapidly to many cells, including human blood cells. In studies on Friend erythroleukemia and murine myeloid leukemia cells, inducers had to be present for only a short initial period for induction to occur.

We have also analyzed differentiation-associated changes in MOLT-3 and HPB-ALL cells using monoclonal antibodies to the cell surface antigens of human T cells. Judging from staining patterns with OKT surface markers, MOLT-3 cells are more similar to thymocytes than they are to more mature peripheral blood T cells, which is in good agreement with the results reported by Nagasawa et al. Among OKT surface markers, antigen(s) that reacts with OKT11 monoclonal antibody has been reported to be expressed throughout human T-lymphocyte ontogeny and to play an important physiologic role in T-cell activation, probably as a component(s) of the E rosette receptor, since OKT11 monoclonal antibody can block E rosette formation and the E rosette-forming capacity appeared to be dependent on OKT11 antigen density. After induction of MOLT-3 cells with TPA, very different staining patterns were observed in the cells (ie, there was a significantly increased level of the more mature marker, OKT11), whereas there were considerable decreases in the level of the less mature markers, OKT4, OKT6, and OKT8. Thus, TPA may be capable of inducing the appearance of more mature markers in the T-lymphoblastic cells, which might result in a significant increase in the OKT11 antigen density. After treatment with TPA, E rosette-forming capacity increased with concomitant decrease of OKT 4/8 ratio on both cell lines. Thus, TPA may induce the differentiation of cells resembling the suppressor T-cell subset, which may be autosuppressive through the effect of gangliosides. The present finding that a high percentage of untreated MOLT-3 cells expressed OKT9 antigen may reflect their high proliferative activity since the antigen is abundant in highly dividing cells. The reactivity of OKT9 monoclonal antibody rapidly decreased during the exposure to TPA, paralleling the growth inhibition. Both a reduction in OKT9 reactivity and inhibition of cell proliferation have also been observed simultaneously in HL-60 cells exposed to phorbol esters.

Closely inspection of the GSL profiles revealed some discrepancies between the induced MOLT-3 cells and normal T lymphocytes with fully mature characteristics, ie, in human normal peripheral lymphocytes or T lymphocytes, gangliosides GD3 could not be detected by us or other researchers on chemical analysis, with CDH being the predominant neutral GSL, whereas ganglioside GD3 and neutral CMH increased significantly during differentiation of MOLT-3 cells. The results suggest that the in vitro process of differentiation of MOLT-3 cells may be defective owing to their malignant property or that TPA may not be sufficiently potent to induce terminal differentiation of MOLT-3 cells. These problems remain to be clarified.

ACKNOWLEDGMENT

We thank Dr Masao Iwamori, Department of Biochemistry, Faculty of Medicine, University of Tokyo, for instruction in the TLC-immunoassaying method.

REFERENCES


3. Rovera G, Santoli D, Damski C: Human promyelocytic leukemia cells in culture differentiated into macrophage-like cells when

<table>
<thead>
<tr>
<th>Table 5. Sialidase-Susceptible Sialic Acid Residues on Cell Surface of TPA-Treated MOLT-3 Cells</th>
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<tbody>
<tr>
<td>Culture Time (day)</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Day 3</td>
</tr>
<tr>
<td>Day 5</td>
</tr>
</tbody>
</table>

MOLT-3 subline cells were incubated with 0.1 U Vibrio cholerae sialidase in 1.0 mL 10 mmol/L sodium phosphate buffer (pH 6.5) containing 0.15 mol/L NaCl at 37°C for 60 minutes; the released sialic acid was then measured as described in the Materials and Methods section.

*Each value represents mean ± SD for two experiments.
treated with a phorbol diesters. Proc Natl Acad Sci USA 76:2779, 1979


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Neutral and sialosyl glycosphingolipid composition and metabolism of human T-lymphoblastic cell line MOLT-3 cells: distinctive changes as markers specific for their differentiation

M Akashi, F Takaku, H Nojiri, Y Miura, Y Nagai and M Saito

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