A Ceramide Analogue Inhibits T Cell Proliferative Response through Inhibition of Glycosphingolipid Synthesis and Enhancement of *N,N*-Dimethylsphingosine Synthesis†

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Received January 22, 1990; Revised Manuscript Received April 2, 1990

ABSTRACT: The ceramide analogue 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol (PDMP) (particularly the D-threo isomer, D-PDMP) caused inhibition of cell growth in some types of cells, and this growth-inhibitory effect has been attributed to inhibition of UDP-Glc:Cer β-Glc transferase, resulting in reduced glycolipid synthesis and increased free ceramide [Inokuch, J., & Radin, N. S. (1987) *J. Lipid Res. 28*, 565-571; Okada, Y., et al. (1988) *FEBS Lett. 235*, 25-29]. In view of increasing evidence that the T cell proliferative immune response is modulated by glycosphingolipids (GSLs), the reagent D-PDMP was used to evaluate the role of GSLs in this respect. Con A induced or PHA-induced mitogenesis of C3H/HeJ mouse splenocytes, as well as IL2-dependent CTLL cell growth, were strongly inhibited in a dose-dependent manner when cells were preincubated in the presence of 5-10 μM D-PDMP, but not with its stereoisomer L-PDMP. Closely associated with this growth-inhibitory effect in the presence of D-PDMP, levels of essentially all GSLs, including GM3 and other gangliosides, were greatly reduced, whereas ceramide accumulated. Importantly, metabolically labeled radioactive bands, corresponding to free sphingosine and *N*-monomethylsphingosine, were found to be present in very small quantities (5-12%) relative to the band corresponding to *N,N*-dimethylsphingosine (DMS), which showed significant accumulation in D-PDMP-treated lymphocytes. The quantity of IL2 receptors and their affinity to IL2 on T cells did not change, but IL2-dependent tyrosine phosphorylation was greatly stimulated, following D-PDMP treatment. Thus, a decrease in ganglioside levels (including GM3) and increase in DMS may cooperate to enhance IL2-dependent tyrosine phosphorylation of several functionally unknown proteins (M_r 55K, 85K, and 100K), leading to inhibition of cell growth (possibly through exhaustion of intracellular ATP). Exogenous addition of subtoxic doses (1-5 μM) of DMS greatly inhibited IL2-dependent CTLL cell proliferation. Thus, a cell growth regulatory mechanism operating via DMS level must be equally important as that operating via GM3 level, and quantities of these two molecules may cooperatively regulate transmembrane signaling.

In view of increasing evidence that GSLs[1] and gangliosides modulate cell proliferation (Hakomori, 1981), particularly lymphoid cell response to mitogen (Marcus, 1984), the effect of ceramide analogue PDMP (particularly its D-threo isomer, D-PDMP) on proliferative immune response of T lymphocytes was investigated. PDMP specifically inhibited UDP-Glc:Cer β-Glc transferase, which is involved in the first step of synthesis of a series of GSLs (Inokuchi & Radin, 1987), and thus greatly reduced levels of all metabolically labeled GSLs and increased levels of labeled precursor ceramide in the same cells (Okada et al., 1988; see also Figure 7). Since free sphingosine has been implicated as a negative modulator of a transmembrane signaling mechanism through PK-C (Hannun & Bell, 1987; 1989), and in fact, *N,N*-dimethylsphingosine (DMS) but not parent sphingosine strongly inhibits PK-C in A431 cells (Igarashi et al., 1989), we studied the effect of D-PDMP on (1) IL2 receptor expression, (2) IL2 receptor binding affinity for IL2, (3) IL2 receptor internalization, and (4) IL2-dependent tyrosine kinase activity as related to metabolic changes of GSLs, ceramide, sphingosine, and DMS. D-PDMP treatment significantly reduced stimulation of T cell mitogenesis by Con A, PHA, and IL2, reduced synthesis and chemical quantity of GSLs, increased levels of DMS, and increased IL2-dependent tyrosine phosphorylation.

Materials and Methods

Materials. All glycosphinoglipids were prepared at The Biomembrane Institute by established procedures (Hakomori, 1983), and ceramide was prepared by the method of Carter et al. (1961). Sphingosine, *N*-monomethylsphingosine, DMS, and PDMP were prepared as previously described (Igarashi et al., 1989; Inokuchi & Radin, 1987). All other materials used were obtained commercially as described in the following sections.

† This study was supported by NIH Grant GM23100 and Outstanding Investigator Grant CA42505 (to S.H.) and funds from The Biomembrane Institute. B.F.-H. is the recipient of a fellowship (Fe25611) from the Deutsche Forschungsgemeinschaft.

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[1] Abbreviations: BSA, bovine serum albumin; Cer, ceramide; CM, chloroform-methanol; CMW, chloroform/methanol/water; Con A, concanavalin A; DMS, *N,N*-dimethylsphingosine; FCS, fetal calf serum; GlcCer, glucosylceramide; GSL, glycosphingolipid; HPTLC, high-performance thin-layer chromatography; IL2, interleukin 2; MAb, monoclonal antibody; PDMP, 1-phenyl-2-(decanoylamino)-3-morpholino-1-propanol; PHA, phytohemagglutinin; PK-C, protein kinase C; SDS, sodium dodecyl sulfate; Ser, serine; TLC, thin-layer chromatography.

0006-2960/90/0429-6314$02.50/0

Cell Culture. Splenocytes from 6 week old C3H/HeJ mice were depleted of red blood cells by 2-min incubation in Tris/NH_4Cl buffer (17 mM Tris-HCl containing 160 mM NH_4Cl, pH adjusted to 7.2 at room temperature), B cells were depleted on incubation with a nylon wool column at 37 °C, and T lymphocyte enriched samples were obtained by filtration through the column (Julius et al., 1973). T cells were cultured in serum-free RPMI 1640 (Gibco) containing 1 mM pyruvate, insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL) (CR-ITS Premix, Collaborative Research, Bedford, MA), and 2×10^{-5} M 2-mercaptoethanol (complete RPMI). Murine IL2-dependent T lymphocyte line CTLL (Gillis et al., 1978) was adapted to serum-reduced conditions and grown in HL-1 medium (Ventrex, Portland, ME), supplemented with 1% FCS and 10 units/mL human recombinant IL2 (Amgen, Thousand Oaks, CA).

Splenocytes and CTLL cells were treated with the D- and L-isomers of PDMP hydrochloride (maintained as sterile 4 mM aqueous stock solutions at 4 °C) to inhibit GSL biosynthesis. For mitogen stimulation assays, splenocytes were incubated with PDMP for 24 h at 37 °C in serum-free RPMI, washed three times, and seeded in mitogen-containing medium (in some cases PDMP and mitogen were added simultaneously). CTLL cells were incubated with PDMP in HL-1 medium (containing 1% FCS and 10 units/mL IL2) for 24 or 48 h. Cells received a fresh PDMP dose before assay periods unless otherwise indicated.

Murine T cell leukemia cell line L5178 clones AA12 and AV27 were established as previously described (Young et al., 1981b). Cells were cultured in HL-1 medium supplemented with 1% FCS. Growth was examined starting at a concentration of 10^4/mL in 24-well plates, in the presence or absence of L- or D-PDMP.

Characterization of GSLs in CTLL Cells. GSLs from the labeled cells were extracted by sonication for 20 min each in CMW, 10:10:1 (2×), 30:60:8, and 30:60:8 (0.8 N sodium acetate). Combined extracts were desalted by C18 reversed-phase chromatography and separated into neutral and acidic fractions by DEAE-Sephadex ion-exchange chromatography. Solvent systems for TLC analysis of neutral glycolipids and gangliosides were CMW 65:25:4 and 50:40:10 (0.2% $CaCl_2$), respectively. GSLs were characterized by comparison of TLC mobility or TLC immunostaining with specific MAb (Magnani et al., 1980). GlcCer was characterized by TLC analysis in borate system (Kean, 1966). Glycolipids were treated with jackbean β-galactosidase (Sigma Chemical Co.), fig α-galactosidase (Hakomori et al., 1971), or sialidase. Acid hydrolysis in 1% AcOH for 1 h at 98 °C was also used for desialylation. Enzymatic and chemical hydrolysis combined with immunostaining with various MAbs was applied as outlined in Table II. MAb 1A4E10 (IgM) directed to Gb3 (Galα1→4Gal) was established in this laboratory (unpublished data). MAbs 2D4 directed to Gg3Cer (Young et al., 1981a), 1B2 directed to Galβ1→4GlcNAc (Young et al., 1981a), anti-SSEA-3 directed to Gal-globoside (Kannagi et al., 1983), and TKH7 directed to Gg4Cer (T. Kjeldsen, H. Clausen, and S. Hakomori unpublished data) were prepared in this laboratory. MAb VESP2.6 directed to sulfatide was donated by Dr. Klaus Petry (Fred Hutchinson Cancer Research Center, Seattle, WA). MAb M2590 directed to GM3 was donated by Dr. M. Taniguchi (Chiba University, Chiba 280, Japan). MAb directed to Forssman antigen was donated by Dr. Karl-Eric Hellstrom (Oncogen Corp., Seattle, WA).

Assays for Biosynthesis of GSLs, Sphingomyelin, and Ceramide in CTLL Cells. To monitor the effect of PDMP on biosynthesis of ceramide-derived lipids in lymphocytes, CTLL cells were cultured for 24 h in the presence of D- or L-PDMP. The medium was then replaced, and cells received fresh PDMP and radiolabeled precursors (see below). Cells were then pelleted and washed three times, lipids were extracted, purified, and analyzed by separation on silica gel HPTLC plates (Merck, Darmstadt, FRG), and metabolically labeled lipids were visualized by autoradiofluorography. Components were quantitated by scraping off corresponding silica gel areas and measuring bound radioactivity by liquid scintillation counting.

Precursors for GSL analyses were D-[6-^{3}H]galactose and D-[6-^{3}H]glucosamine with specific activities of 25 and 30 Ci/mmol, respectively (ICN, Costa Mesa, CA), applied at 6 μCi/mL of cell suspension for 24 h in glucose-free RPMI containing 10 mM pyruvate and 5% FCS. The precursor for sphingomyelin with [*methyl*-^{3}H]choline (Amersham, Arlington Heights, IL), specific activity 75.4 Ci/mmol, applied at 6 μCi/mL over 14 h. The precursor for ceramide was [9,10-^{3}H]palmitic acid (Amersham), specific activity 54 Ci/mmol, applied at 10 μCi/mL over 12 h.

Sphingomyelin was isolated by extracting the cells four times with CM 2:1, partitioning between CMW (Folch-Pi et al., 1951), and two-dimensional TLC with CM/NH_4OH (7 M), 60:60:5 (two runs with intermediate drying), and CMW/AcOH, 50:30:4:8. Ceramide was isolated by similar extraction and partitioning, methanolysis of the ester lipids with 0.2 N NaOH in MeOH for 48 h at 37 °C, liquid–liquid partitioning, and TLC with dichloroethane/methanol/water, 180:40:1.

Metabolic Labeling of Sphingosine and Its N-Methyl Derivatives by [^{3}H]Ser in CTLL Cells. The precursor used for sphingosine biosynthesis was [^{3}H]Ser (Amersham; 30 Ci/mmol). CTLL cells were cultured in serine-deprived medium supplemented with [2,3-^{3}H]Ser (Amersham, 30 Ci/mmol, 2.5 μC/mL) with or without 10 μM PDMP (D- or L-isomer) for various durations up to 4 days. Lipids were extracted from the cells by sonication in CM, 2:1, and fractionated by CMW partitioning as above. The lower phase, containing sphingosine and its methyl derivatives, was treated with NaOH as described for ceramide. TLC was run with CM/7 M NH_4OH, 80:20:2, and lipid fractions were visualized by autoradiofluorography using Kodak X-OMAT AR5 film (Eastman Kodak, Rochester, NY). Specific lipid bands were quantitated by scraping off the silica gel portions. Individual silica gel samples were dispersed in 0.5 mL of water by sonication followed by mixing with 2 mL of scintillation cocktail, and the radioactivity was determined in a scintillation counter. A blank silica gel area was treated in the same way for determination of background and quenching effect. Further details are presented in the legend to Figure 4.

Quantitative GSL Assay. To quantitate GSLs present in CTLL cells before and after PDMP treatment, cells were cultured for 48 h in the absence or presence of 5 μM D- or L-PDMP. Total glycolipid fraction was extracted in CM and isolated from phospholipids, and free ceramide was isolated by Florisil chromatography (Sigma, St. Louis, MO) of peracetylated compounds as previously described (Laine et al., 1974) and tested for purity by TLC and specific chemical tests. Glycolipids were quantitated on the basis of sphingosine content, and aldehydes derived from sphingosine base by periodate oxidation were characterized and quantitated by gas chromatography (Gaver & Sweeley, 1965).

Mitogen Stimulation Assay. Splenocytes were stimulated with PHA (Difco, Detroit, MI) or Con A (Sigma) at 10^6 cells/mL and incubated in 96-well plates (200 μL/well) for

72 h in a humidified 5% CO_2–air atmosphere at 37 °C. The absolute concentration of Difco's PHA solution is not published; the original solution was therefore diluted to various degrees (1:100 to 1:1000) and used for mitogenesis. Con A concentration was also varied from 1 to 8 μg/mL. DNA synthesis was measured by incorporation of [^{3}H]thymidine (6.7 Ci/mmol; ICN). Following labeling for 8 h with 0.5 μCi/well, quadruplicate cultures were harvested onto glass fiber filters fitted on a PHD cell harvester (Cambridge Technology Inc., Cambridge, MA) and assayed by liquid scintillation counting.

DNA Synthesis and IL2 Production Assays. For measurement of IL2-induced DNA synthesis in T cells, CTLL cells were washed three times to remove residual IL2. Cells (10^5/mL) were seeded into 96-well plates with HL-1 medium containing 1% FCS and IL2 (0–1 units/mL). Quadruplicate cultures were kept for 24 h (5% CO_2–air, 37 °C) and labeled with [^{3}H]thymidine (0.5 μCi/well) 4 h prior to harvesting, and radioactivity was counted as described above. For study of the effect of exogenously added sphingosine and its derivatives on DNA synthesis, CTLL cells were washed extensively and seeded at a density of 1.5×10^4 cells/well. Various concentrations of sphingosine, ceramide, *N*-monomethylsphinogsine, or DMS (solubilized in ethanol) were added, together with IL2 (0–5 units/mL). Plates were cultured for a total of 24 h and labeled with [^{3}H]thymidine (1 μCi/well) during the last 6 h. [^{3}H]Thymidine incorporation was determined as described above.

For measurement of IL2 production in mitogen-stimulated splenocytes, 5×10^6 cells were cultured in the presence of Con A (1 μg/mL) or PHA (diluted 1:1000) for 24 h. Supernatants were harvested, filtered, and added in serial dilutions to microtiter wells containing 10^4 CTLL cells (Gillis et al., 1978). Growth factor induced DNA synthesis in the CTLL cells was assayed as above.

IL2 Receptor Expression. Mitogen-induced IL2 receptor expression was analyzed in C3H/HeJ splenic T cells. Cells (10^7/mL) were seeded in the presence or absence of 5 μM PDMP, stimulated with 2.5 μg/mL Con A for 48 h, harvested, and stained with rat anti-mouse IL2 receptor MAb AMT-13 (Osawa & Diamantstein, 1984) and mouse anti-rat IgG fluorescein conjugate (both antibodies from Boehringer Mannheim, Indianapolis, IN). Fixed cells (in 1% paraformaldehyde) were examined by flow cytofluorometry with an EPICS profile analyzer.

IL2 Binding Assay. For measurement of IL2 binding to its surface receptor, human recombinant IL2 was ^{125}I-labeled as described (Cosman et al., 1984), using Enzymobead reagent (Bio-Rad). CTLL cells (2.6×10^7) were washed three times, resuspended in 650 μL of RPMI containing 2% BSA, 20 mM Hepes buffer, and 0.2% sodium azide, pH 7.2 (binding medium), and incubated at room temperature for 10 min. Cells (2×10^6/well) were then seeded into 2-fold serial dilutions of ^{125}I-IL2 (3.21×10^9 cpm/pmol) in 96-well plates and incubated at 37 °C for 30 min on a mini orbital shaker. Duplicate 65-μL aliquots of the incubation mixtures were transferred to precooled 400-μL polyethylene centrifuge tubes containing 200 μL of a phthalate oil mixture (Dower et al., 1984). Cell-bound ^{125}I-IL2 was separated from unbound material by 1.5-min centrifugation in an Eppendorf microfuge, and radioactivity was measured in a γ counter. Association constants (K_a) and binding site numbers were calculated from Scatchard plots. Nonspecific binding was measured in the presence of a 1000-fold molar excess of unlabeled IL2.

IL2 Internalization Assay. The procedure of Fujii et al. (1986) was adapted for the murine CTLL cell system. CTLL cells were washed three times and taken up (2×10^7 cells/mL) in precooled binding medium without sodium azide. ^{125}I-IL2 (3.76×10^9 cpm/pmol) was added for a final concentration of 500 pM, and cells were incubated at 4 °C for 90 min. Kinetic experiments showed that these time and temperature values were optimal for IL2 binding equilibrium without internalization. Following the binding step, cells were washed three times in ice-cold phosphate-buffered saline containing 2% BSA. For IL2 internalization, the cells were taken up in 37 °C binding medium (no azide) and incubated at this temperature in a water bath. At intervals, 2×10^6 cell aliquots were taken in duplicate and pelleted. To release surface-bound IL2, cells were taken up in precooled 0.2 M glycine hydrochloride buffer (pH 3.8, containing 0.25 M NaCl) and kept on ice for 15 min. Control experiments using buffers at various pH values and protease solutions for the releasing step showed that these conditions were optimal to remove surface-bound IL2 and maintain cell viability. Surface-bound and internalized radioactivity were subsequently measured in the supernatant and cell pellet, respectively, after centrifugation with phthalate oil mixture. As a negative control, the internalization step was carried out at 0 °C, which completely blocked IL2 uptake.

Tyrosine Phosphorylation Assay. CTLL cells were used to detect IL2-dependent protein phosphorylation on tyrosine residues by modification of the assay system of Saltzman et al. (1988). Cells were washed three times, resuspended [(2–5) $\times 10^6$ cells/mL] in HL-1 medium containing 1% FCS and 50 μM sodium orthovanadate, and starved of IL2 for 2.5 h (37 °C, 5% CO_2 atmosphere). Cells were loaded with 100 units/mL IL2 (some were used as negative controls). IL2 treatment was terminated after 10 min by centrifugation for 1 min at 1000*g*. Cell pellets were resuspended in RPMI, SDS sample buffer was added, and samples were boiled in a water bath for 5 min. After spinning in a microfuge (12 000 rpm, 5 min), samples were analyzed by electrophoresis on 8% polyacrylamide gels followed by electroblotting onto nitrocellulose. The nitrocellulose blots were blocked in 10 mM Tris-buffered saline (with 0.05% Tween 20 and 5% BSA, pH 7.2 at 37 °C) and incubated with anti-phosphotyrosine MAb Py-20 (ICN). Immune complexes were detected with ^{125}I protein A (ICN) and visualized by autoradiography.

Results

Effect of PDMP on Mitogen-Induced T Cell Responses. Splenocytes from C3H/HeJ mice were stimulated with Con A or PHA and cultured for 72 h in the presence or absence of 5 or 10 μM D-PDMP, as described under Materials and Methods. Mitogen-induced DNA synthesis was greatly reduced when splenocytes were cultured in the presence of D-PDMP (Figure 1). This reduction was not due to an inhibition of early T cell activation steps, since the production of IL2 was not impaired in D-PDMP-treated splenocytes (data not shown). Con A induced or PHA-induced supernatants of drug-treated and control cells stimulated DNA synthesis in CTLL cells. A minor reduction of CTLL cell growth in D-PDMP containing supernatants vs controls was caused by the presence of D-PDMP, as shown by testing D-PDMP-containing supernatants of unstimulated splenocytes supplemented with defined IL2 concentrations.

Effect of PDMP on IL2-Dependent T Cell Growth. We next examined proliferative responses of CTLL cells to limiting IL2 concentrations in the presence of PDMP. As shown in Figure 2, IL2-dependent mitogenic response was inhibited by D-PDMP in a dose-dependent manner, but less so by L-PDMP. When CTLL cells were treated for 24 h in the presence of

Table I: Effect of PDMP on Biosynthesis of GSLs, Sphingomyelin, and Ceramide in CTLL Cells[a]

	(A) Glycolipid Biosynthesis [^{3}H]Gal and [^{3}H]GlcNH$_2$ incorporation [cpm/(1 × 10^7 cells)]		
	total GSL	neutral GSL	acidic GSL
control	1350000 ± 6000	1300000 ± 34000	50400 ± 2800
+D-PDMP	221000 ± 17000 (16)	193000 ± 15000 (15)	28000 ± 2000 (56)
+L-PDMP	646000 ± 23000 (48)	606000 ± 19000 (47)	40000 ± 2000 (79)

	(B) Phospholipid Biosynthesis [^{3}H]choline incorporation [cpm/(2 × 10^5 cells)]
	sphingomyelin
control	902 ± 98
+D-PDMP	720 ± 83 (80)
+L-PDMP	840 ± 74 (93)

	(C) Lipid Biosynthesis [^{3}H]palmitic acid incorporation [cpm/(5 × 10^4 cells)]	
	free ceramide	glucosylceramide
control	39600 ± 3600	8950 ± 690
+D-PDMP	67200 ± 5100 (170)	1580 ± 230 (18)
+L-PDMP	43100 ± 3900 (109)	5710 ± 470 (64)

[a] Values in parentheses are percent of control. CTLL cells were cultivated in the absence (control) or presence of 5 μM D- or L-PDMP for 48 h. For GSL labeling cells, were labeled with [^{3}H]Gal and [^{3}H]GlcNH$_2$ (6 μCi/mL each) for 24 h. For sphingomyelin labeling cells were labeled with [^{3}H]choline (6 μCi/mL) for 14 h. For ceramide labeling, cells were labeled with [^{3}H]palmitic acid (10 μCi/mL) for 12 h. Metabolically labeled lipids were extracted, analyzed by TLC, and quantitated as described under Materials and Methods. Results represent mean cpm ± SD of triplicate samples.

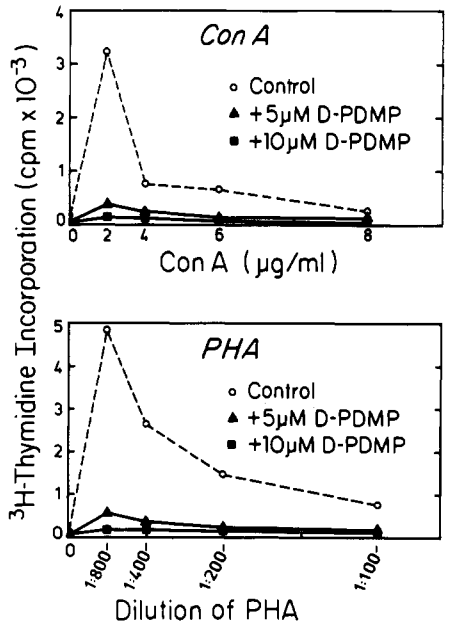

FIGURE 1: Effect of D-PDMP on mitogen-induced DNA synthesis in murine splenic T cells. A total of 1 × 10^6 cells/mL were stimulated with Con A (upper panel) or PHA (lower panel) in the presence of 5 or 10 μM D-PDMP. DNA synthesis was measured by [^{3}H]thymidine uptake during an 8-h labeling period at the end of 72-h culture. All cpm values shown are means of quadruplicate cultures. Similar results were obtained in several independent experiments.

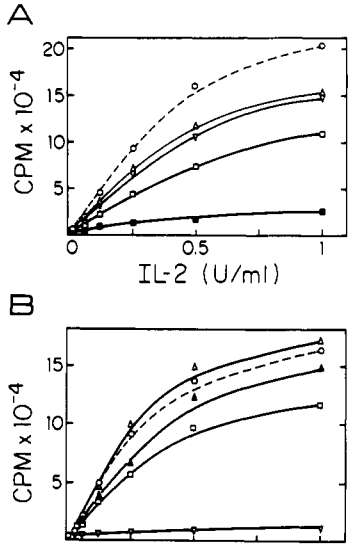

FIGURE 2: Effect of PDMP on IL2-induced DNA synthesis in CTLL cells. (A) Dose-dependent effect of D-PDMP on CTLL growth in limiting IL2 concentrations. Cells were pretreated with the indicated D-PDMP concentrations for 24 h in the presence of 10 units/mL IL2 and seeded into limiting IL2 concentrations at 1 × 10^5 cells/mL, receiving fresh doses of D-PDMP at the same concentrations. DNA synthesis was measured by [^{3}H]thymidine uptake during a 4-h labeling period at the end of 24-h culture. Values represent means (cpm) of quadruplicate cultures. (○) Control; (△) 1 μM; (▽) 2 μM; (□) 4 μM; (■) 5 μM concentration of D-PDMP in medium. (B) Effect of L-PDMP on CTLL IL2 response and recovery of CTLL IL2 response from D-PDMP treatment. Cells were preincubated with 5 μM PDMP for 24 h in the presence of 10 units/mL IL2 and then subjected to IL2 assay after receiving fresh PDMP [total of 48 h; (△) for L-PDMP, (▽) for D-PDMP; (○) control]. For recovery, D-PDMP-pretreated cells were assayed in limiting IL2 concentrations in the absence of D-PDMP [(□) for 24-h recovery period; (▲) for 48-h recovery period]. Similar results were obtained by several independent experiments.

D-PDMP, followed by culture in the absence of D-PDMP, IL2 response returned to control levels. This result indicates that sufficient recovery in amounts of GSL occurred and that the effect of PDMP does not depend on toxicity. The doubling time of CTLL cells in the presence of IL2 (10 units/mL) was prolonged from 14 to 22 h by D-PDMP (5 μM). As a control, growth factor independent mouse lymphoma cells (L5178Y) (Okada et al., 1985) were not affected (10 μM D-PDMP), indicating that D-PDMP is not cytostatic in general. The growth of cell line L5178Y is highly dependent on insulin and transferrin and may not be affected by D-PDMP.

Effect of PDMP on Metabolism of GSL, Sphingomyelin, and Ceramide in T Lymphocyte Line. Murine T lymphocytes (CTLL cell line) were cultured for 48 h in the presence of 5 μM D- or L-PDMP, followed by labeling in medium containing ^{3}H-labeled galactose or glucosamine. In another set of experiments, cells were metabolically labeled with [^{3}H]palmitic acid or [^{3}H]choline in the presence or absence of D- or L-PDMP, as described under Materials and Methods and in footnote *a* of Table I.

Incorporation of [^{3}H]Gal and [^{3}H]GlcNH$_2$ into both neutral and acidic GSLs was greatly reduced in the presence of PDMP, particularly D-PDMP (Table IA). This is in agree-

Table II: Neutral GSLs and Gangliosides of CTLL Cells, Their Relative Compositions Based on Metabolic Labeling, and Effects of D- and L-PDMP[a]

band	identified structure	basis of identification	cpm/10^7 cells (% of control)		
			control	D-PDMP	L-PDMP
		Neutral GSLs			
1	GlcCer	TLC mobility on borate-impregnated plate	213 000	11 600 (5)	121 500 (44)
2	LacCer a	β-galactosidase treatment gave GlcCer; not susceptible to fig α-galactosidase	61 900	17 000 (27)	30 100 (61)
	LacCer b		76 290	20 400 (26)	41 700 (54)
	LacCer c		41 900	6 700 (16)	21 300 (51)
3	Gb3Cer	positive with anti-Gb3 MAb 1A4E10	434 200	44 600 (10)	198 300 (45)
4	Gg3Cer	positive with anti-Gg3 MAb 2D4	248 600	42 300 (17)	99 350 (39)
5	unidentified	negative with anti-SSEA-3 MAb (directed to Gal-globoside), MAb to Forssman, MAb 1B2 to Galβ1→4GlcNAc, and MAb TKH7 to Gg4	27 600	21 000 (76)	9 500 (34)
		Gangliosides			
1	GM4	negative with anti-sulfatide MAb VESP2.6; gave GalCer when treated with 1% AcOH, 98 °C, 1 h	8 350	6 600 (79)	7 200 (86)
2	GM3	positive with anti-GM3 MAb M2590; gave LacCer when treated with 1% AcOH, 98 °C, 1 h	18 100	11 100 (61)	15 500 (85)
3	GM2	TLC mobility identical with that of GM2; no further information available	2 750	1 500 (54)	1 700 (61)
4	GM1	TLC mobility identical with that of GM1; positive with MAb TKH7; gave Gg4Cer when treated with 1% AcOH, 98 °C, 1 h	13 600	3 800 (27)	9 500 (69)

[a] Metabolically labeled neutral glycolipids extracted and separated by TLC for 24 h as described under Materials and Methods. Bands revealed on autoradiography of TLC were scraped, and radioactivity was measured by scintillation counter.

ment with the previous finding that D-PDMP inhibits UDP-Glc:Cer β-Glc transferase (Inokuchi & Radin, 1987). Corresponding to the PDMP-induced decrease in GSL biosynthesis, the total chemical quantity of GSL, as determined by sphingosine content in gas chromatography, was greatly reduced (63%) in the presence of D-PDMP and moderately reduced (20%) in the presence of L-PDMP. On the other hand, biosynthesis of sphingomyelin, as measured by [^{3}H]choline incorporation, was not significantly affected[2] (Table IB). However, incorporation of [^{3}H]palmitic acid into free ceramide was significantly increased, while that into glucosylceramide was greatly reduced in the presence of D-PDMP (Table IC). Four neutral GSLs were identified as GlcCer, LacCer (consisting of three bands, Table II, a–c), Gb3Cer, and Gg3Cer. Band 5, which had the same mobility as tetra- to pentahexoside, was not identified. Four ganglioside bands (Figure 3) were identified as GM4, GM3, GM2, and GM1. The chemical and immunochemical basis of these identifications, the relative activity of these GSL components based on metabolic labeling, and components affected by treatment with D- and L-PDMP are shown in Table II. The greatest reduction was seen in GlcCer in the presence of D-PDMP, followed by similar reduction of LacCer, Gb3, and Gg3. All gangliosides except GM4 were significantly reduced by the presence of D-PDMP but were less affected by L-PDMP. The reduction in mass of GM3 was demonstrated independently by immunoblotting with the GM3-specific MAb M2590. A reduction of GM3 concentration in B16 melanoma cells induced by D-PDMP has been reported previously (Inokuchi et al., 1989).

Accumulation of DMS in CTLL Cells Caused by D-PDMP Treatment. Since significant accumulation of ceramidase was observed in PDMP-treated CTLL cells, detectability of free sphingosine might be expected since ceramide could be degraded into sphingosine and fatty acid by ceramide (Spence et al., 1986). However, TLC analysis of the sphingosine fraction after [^{14}C]palmitic acid labeling did not reveal the presence of free sphingosine in either PDMP-treated or -untreated CTLL cells. However, a weak radioactive band corresponding to DMS was detected in PDMP-treated cells (data not shown). A stronger and much clearer band corresponding to DMS was observed when cells were labeled with [^{3}H]Ser [serine is the precursor of the $-CH(NH_2)CH_2OH$ backbone of sphingosine] and analyzed as described under Materials and Methods. Significant accumulation of a radioactive band corresponding to DMS was observed during the 4-day incubation period, in a manner similar to that of ceramide accumulation. Relative to control value, the accumulation was increased over 300% by D-PDMP treatment, but less than 50% by L-PDMP treatment (Figure 4). A radioactive band corresponding to unsubstituted sphingosine and/or *N*-monomethylsphingosine (these two compounds overlap under various TLC conditions) was enhanced in a similar manner by D-PDMP treatment, but the quantity was only 12–20% that of DMS. These findings suggest that sphingosine, which is produced from accumulated ceramide, is converted directly

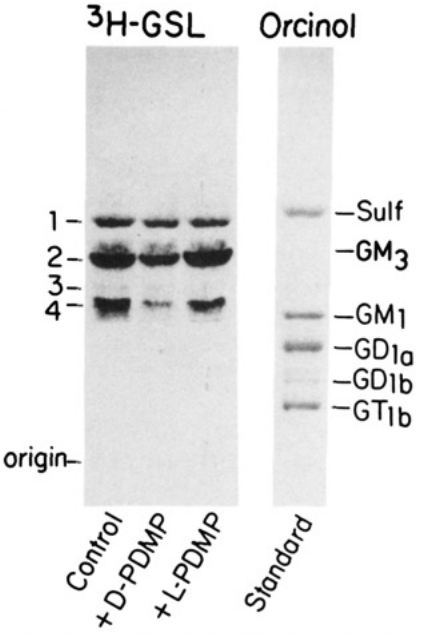

FIGURE 3: Profile of metabolically labeled acidic GSLs in the presence of PDMP. CTLL cells were incubated for 48 h in the presence or absence of 5 μM PDMP and were labeled by using [^{3}H]galactose and [^{3}H]glucosamine during the last 24 h as described under Materials and Methods. The purified acidic GSL fractions were subjected to TLC autoradiography.

[2] This finding indicates that the rate-limiting step in regulation of sphingomyelin concentration in CTLL cells may depend not on phosphocholine transferase from lecithin to ceramide, but rather on an earlier step.

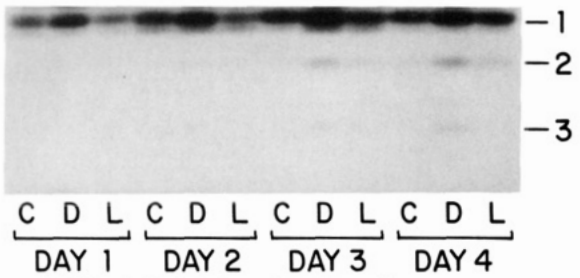

FIGURE 4: Metabolic labeling of DMS by [^{3}H]Ser in CTLL cells. CTLL cells (3×10^7) were suspended in 90 mL of a 1:1 mixture of serine-free RPMI and HL-1 medium (each containing 1% FCS), 10 units/mL IL2, and 450 μCi of [^{3}H]Ser. Three aliquots (30 mL each) of this suspension were cultured separately: one supplemented with 37.1 μL of 4 mM D-PDMP, the second with the same amount of L-PDMP, and the third serving as untreated control. From each culture, the same numbers of cells were removed at days 1, 2, 3, and 4, lipid extracted, and subjected to basic methanolysis to eliminate all ester phospholipids. The sphingolipid fraction (ceramide and sphingosine) was then subjected to TLC autoradiography. Each lane of lipid applied on TLC was equivalent to the lipid derived from 2×10^6 cells. Migration positions of standard sphingolipids are indicated as follows: (1) ceramide; (2) DMS; (3) free sphingosine or *N*-monomethylsphingosine. (C) Control; (D) treated with D-PDMP (10 μM); (L) treated with L-PDMP (10 μM).

Table III: Characterization of IL2 Receptors on CTLL Cells after PDMP Treatment[a]

	high affinity		low affinity	
cell type	K_a	site number	K_a	site number
control	5.3×10^9	2200	4.5×10^7	16 540
+D-PDMP	3.2×10^9	2500	5.7×10^7	19 130
+L-PDMP	2.3×10^9	2800	6.4×10^7	17 190

[a] CTLL cells were cultivated for 48 h in the presence of 5 μM D- or L-PDMP and 10 units/mL IL2 and then submitted to ^{125}I-IL2 equilibrium binding as described under Materials and Methods. The data presented were calculated after Scatchard plot analysis.

to DMS in D-PDMP-treated CTLL cells and does not accumulate in significant amounts (Figure 4).

Effect of PDMP on IL2-Dependent Receptor Expression. Since mitogen stimulation in T cells is accompanied by IL2 receptor expression (Robb et al., 1981; Cantrell & Smith, 1983), the effect of PDMP on these T cell responses was studied following Con A or PHA stimulation as described under Materials and Methods. The degree of IL2 receptor expression was determined with an anti-mouse IL2 receptor MAb. Mitogen-induced receptor expression was unchanged in PDMP-treated splenocytes (data not shown).

Effect of PDMP on IL2 Receptor Affinity, Internalization, and IL2-Induced Tyrosine Phosphorylation. Since the IL2 response of CTLL cells was inhibited by D-PDMP in a dose-dependent manner, and the inhibition of IL2-dependent proliferation by D-PDMP was reversible, the effect of PDMP on IL2 receptor functions was analyzed in D- or L-PDMP-treated CTLL cells. The results (Table III) show that IL2 bound equally well to PDMP-treated (48 h) vs -untreated cells, and therefore, the inhibitory effect of PDMP on IL2-dependent cell growth was not a function of IL2 receptor affinity or number of binding sites per cell. Scatchard plot analysis of IL2 binding to D-PDMP-treated CTLL cells clearly showed that PDMP treatment did not change IL2 receptor affinity (data not shown).

In order to determine the effect of PDMP on IL2 internalization, CTLL cells were incubated with IL2 in the presence or absence of PDMP for 48 h, and IL2 internalization was assayed as described under Materials and Methods. IL2 internalization was slightly increased in comparison to that in control cells (data not shown). There was no significant

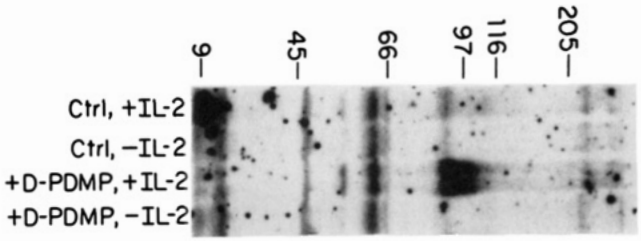

FIGURE 5: Effect of D-PDMP on IL2-induced tyrosine phosphorylation of CTLL proteins. CTLL cells were preincubated in 5 μM D-PDMP and 10 units/mL IL2 for 48 h prior to a 2.5-h IL2 starvation period. Cells were stimulated with 100 units/mL IL2 for 10 min, pelleted at 1000*g* for 1 min, resuspended in RPMI, and boiled in the presence of SDS sample buffer. Equivalents of 4×10^6 cells were submitted to SDS–PAGE on 8% polyacrylamide gels. The figure shows the tyrosine-phosphorylated proteins after Western blotting and detection with anti-phosphotyrosine MAb Py-20 and ^{125}I protein A.

differences in those cells treated with PDMP. These data indicate clearly that PDMP treatment did not affect primary IL2 receptor functions in CTLL cells.

Since tyrosine phosphorylation of various uncharacterized proteins (M_r 120K, 100K, 85K, 59K, and 38K) in T cells is closely correlated with IL2-dependent T cell stimulation (Saltzman et al., 1988; Ogawa et al., 1987; Benedict et al., 1987; Mills et al., 1990), CTLL cells were cultured with IL2 in the presence or absence of D-PDMP for 48 h, starved of IL2, and then stimulated with IL2. Tyrosine phosphorylation pattern was determined by using an anti-phosphotyrosine MAb as described under Materials and Methods. In agreement with previous reports, IL2-induced tyrosine phosphorylation was detected in proteins with M_r's of approximately 100K, 85K, and 55K (Figure 5). Pretreatment of cells with 5 μM D-PDMP for 48 h caused an increase in IL2-dependent tyrosine phosphorylation of these three bands. When cells were cultured in the presence of D-PDMP for only 2.5 h during the IL2 starvation period, there was no change in tyrosine phosphorylation bands as compared to untreated control cells (data not shown).

Effect of DMS on CTLL Cell Growth. The effect of PDMP may be correlated with increased synthesis of DMS, a possible modulator of transmembrane signaling. We therefore examined the direct effect of DMS on CTLL cell growth (Figure 6). At low doses (1–5 μM), this compound strongly inhibited cell growth. On incubation with 0.5 unit/mL IL2, the concentration of DMS producing 50% inhibition was 3.5 μM. Cell growth returned to normal when DMS was removed. D-*erythro*-Sphingosine showed a similar but weaker inhibitory effect on DNA synthesis in CTLL cells ($KI_{50} = 6$ μM) in the presence of the same (0.5 unit/mL) concentration of IL2. However, when cells were stimulated by high, nonlimiting concentrations (1–5 units/mL) of IL2, the inhibitory effect of DMS (5 μM) was unchanged, whereas that of unsubstituted sphingosine was abolished (Table IV). Ceramide produced no effect on DNA synthesis regardless of IL2 concentration, possibly because it could not be absorbed rapidly enough. The effect on cell growth of *N*-monomethylsphingosine with 1.0 unit/mL IL2 was significantly higher than that of free sphingosine.

In addition, DMS (3.5 μM) completely inhibited Con A stimulated or PHA-stimulated splenocyte growth (data not shown).

Discussion

Cell proliferation is controlled by transmembrane transduction mechanisms via membrane-associated receptor kinase, PK-C, and receptor–G protein–adenylate cyclase systems.

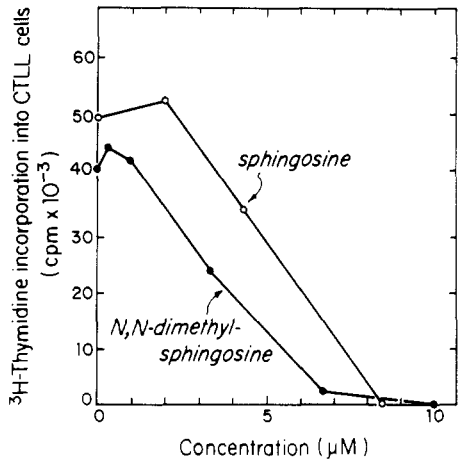

FIGURE 6: Dose-dependent inhibitory effect of DMS and sphingosine on IL2-dependent CTLL cell proliferative response. CTLL cells were seeded at a density of 1.5×10^4 cells/well (96-well dish) and cultured in the presence of various concentrations of sphingosine and DMS, together with IL2 (0.5 unit/mL), for a total of 24 h and labeled with [^{3}H]thymidine (1 μCi/well) during the last 6 h. [^{3}H]Thymidine incorporation was determined as described under Materials and Methods. Each point represents the arithmetic mean of incorporated radioactivity in triplicate cultures. Standard deviations were below 5%. In the absence of IL2 (control), incorporation of [^{3}H] radioactivity by CTLL cells was approximately 200 cpm.

Table IV: Inhibitory Effect of Sphingosine and Its Analogues (5 μM Concentration) on IL2-Dependent [^{3}H]Thymidine Incorporation in CTLL Cells

additive	IL2 (units/mL)		
	0.5	1.0	5.0
ceramide	NI[a]	NI	NI
sphingosine	51[b]	15	5
N-monomethylsphingosine	43	41	ND
N,*N*-dimethylsphingosine	99	98	92

[a] NI, no inhibition or slight activation. ND, not determined. [b] Values indicate inhibition of [^{3}H]thymidine incorporation (%). For experimental details, see Materials and Methods and Figure 6 legend.

Although increasing numbers of membrane components have been identified, our knowledge of the exact mechanisms involved (particularly coordination between the various components within a system) is extremely fragmentary. Since each of these systems (receptor kinase, PK-C, receptor–G protein–adenylate cyclase) is closely associated with the cell membrane and is susceptible to specific environmental lipid components, current research is focused in part on the effects of GSLs and their breakdown products on growth factor receptor kinase and PK-C. GSLs, particularly gangliosides, strongly inhibit receptor-associated kinases (Bremer et al., 1984, 1986; Hanai et al., 1988), and their breakdown products, particularly lyso-GM3, inhibit PK-C (Igarashi et al., unpublished results). Sphingosine was found to inhibit PK-C, in contrast, to 1,2-diacylglycerol, which stimulates PK-C activity and has been implicated as a negative modulator of transmembrane signaling through PK-C (Hannun et al., 1986, 1987). Recently, DMS was found to be a much stronger inhibitor of PK-C activity than unsubstituted sphingosine (Igarashi et al., 1989), as well as an enhancer of tyrosine kinases such as src-kinase (Igarashi et al., 1989) and EGF receptor kinase (Igarashi et al., unpublished results).

Immune responses occur basically via control of proliferative responses of immunocytes, particularly lymphocytes. A number of previous studies indicated that lymphoid cell proliferation can be inhibited by exogenous addition of gangliosides (Lengle et al., 1979; Ryan & Shinitzky, 1979; Whisler & Yates, 1980; Ladisch et al., 1983; Marcus et al., 1987). More recent studies indicate that exogenously added ganglioside modulates expression of the immunoregulatory antigen CD4 (Offner et al., 1987; Kawaguchi et al., 1989). As an alternative to the approach focusing on the effect of exogenously added ganglioside on immune function, the approach using PDMP to reduce levels of GSLs should be useful in elucidating the role of GSLs in T lymphocyte proliferative response, since PDMP greatly modifies the entire pattern of endogenous GSLs (see the introduction). The present study was therefore focused on the following: (1) the effect of PDMP on proliferative T cell response and analysis of cellular response parameters, particularly IL2 receptor function and subsequent changes in tyrosine phosphorylation patterns of membrane components; and (2) associated metabolic changes of sphingolipids in general, particularly synthesis of sphingosine and its derivatives.

Results of this study clearly indicate that the proliferative response of T lymphocytes is inhibited specifically by D-PDMP (but not L-PDMP). This inhibitory effect was reversible and did not involve number of IL2 receptors, affinity of receptors to IL2, or IL2 internalization, although PDMP strongly enhanced tyrosine phosphorylation in CTLL cells. In association with these changes in proliferative response, PDMP treatment greatly reduced levels of all GSLs, particularly GM3 ganglioside, which has been known to inhibit tyrosine phosphorylation. On the other hand, PDMP-treated cells induced accumulation of metabolically labeled ceramide. Interestingly, free sphingosine (expected to be yielded from ceramide) showed no apparent accumulation. In contrast, a [^{3}H]Ser-derived substance corresponding to DMS accumulated significantly in PDMP-treated cells. Since DMS has an enhancing effect on tyrosine kinase, a reduction of GM3 and accumulation of DMS may cooperate to enhance IL2-dependent tyrosine phosphorylation of membrane components, leading in turn to cell growth inhibition, probably via exhaustion of intracellular ATP. The same mechanism has been observed in A431 mutant cells in which EGF receptor tyrosine phosphorylation stimulated by EGF leads to growth inhibition (Kawamoto et al., 1983; Hanai et al., 1988). On the other hand, PK-C, was well as tyrosine kinases, has been suggested to be involved in T lymphocyte proliferative response (Alexander & Cantrell, 1989). Since GM3 (particularly lyso-GM3) and DMS are strong inhibitors of PK-C, a great reduction of GM3 and increase of DMS may work cooperatively to reduce a PK-C-dependent transduction pathway, although this hypothesis remains to be investigated in CTLL cells.

Since PDMP treatment not only blocks all GSL synthesis but also enhances DMS synthesis (see Figure 7 and its legend), the cell biological effects of PDMP as described in previous studies (Inokuchi et al., 1987, 1989; Okada et al., 1988) must be considered in light of the accumulation of DMS, which is a strong modulator of both PK-C and tyrosine kinases. Interestingly, PDMP treatment of cells did not cause accumulation of metabolically labeled sphingosine, or perhaps sphingosine is absent in untreated CTLL cells. On the contrary, catabolism of ceramide is closely associated with *N*-methyltransferase (Igarashi & Hakomori, 1989) which converts free sphingosine to DMS[3] (Figure 4). In addition, exogenously added subtoxic doses of DMS inhibited IL2-de-

[3] There is a possibility that a synthetic pathway to DMS from ceramide is present at a nonlysosomal site where free sphingosine is converted to DMS by a specific transmethylase. Hydrolysis of ceramide by ceramidase takes place at the lysosomal membrane, where ceramide is immediately resynthesized by the reverse reaction (Gatt et al., 1966).

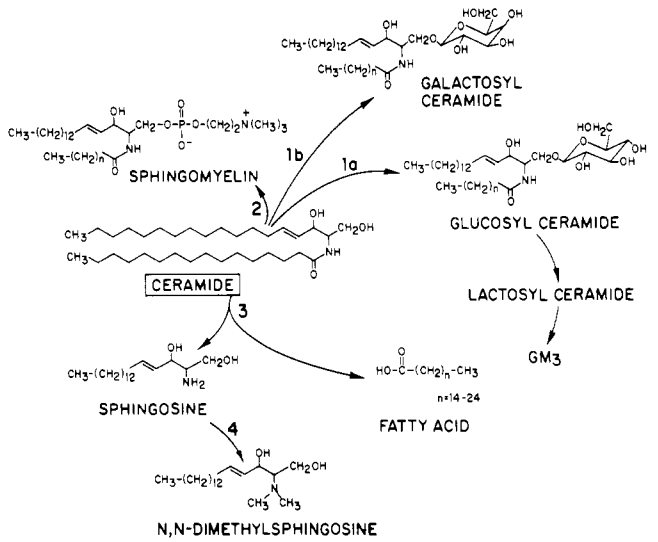

FIGURE 7: Schematic relationship between various sphingolipid components. Sphingosine synthesized from palmitoyl-CoA and serine is immediately assembled into ceramide with fatty acyl-CoA at the endoplasmic reticulum and is then converted to either GSL (route 1) or sphingomyelin (route 2). In the majority of extraneural cells, ceramide is converted to glucosylceramide (route 1a) followed by addition of a large variety of sugar residues to form a complex pattern of GSL species. In some cells, particularly neural cells, galactosylceramide is formed (route 1b), resulting in comparatively few GSL species. Conversion of ceramide to glucosylceramide is blocked strongly by D-PDMP, resulting in accumulation of ceramide which is then internally degraded by ceramidase into sphingosine and fatty acid (route 3). Immediate conversion of free unsubstituted sphingosine into DMS (route 4) apparently occurs in CTLL cells (present study), in brain cells (Igarashi & Hakomori, 1989), and possibly in other neuroimmunological cells. PDMP treatment of cells therefore results in (1) phenotypic changes associated with reduction of GSLs, (2) accumulation of ceramide and DMS, and (3) no effect on sphingomyelin level.

pendent CTLL cell growth (Figure 6; Table IV), in a similar manner to D-PDMP treatment. Since activity of this enzyme (sphingosine *N*-methyltransferase) has been detected in rat brain (Igarashi & Hakomori, 1989), and in view of the close association between the nervous and immune systems, DMS may play an important role in modulating neuroimmunologic responses. Further studies along this line are in progress.

ACKNOWLEDGMENTS

We thank Carl Roberts for carrying out the GC/MS analysis of sphingenine and Dr. Stephen Anderson for editing the manuscript.

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