Th2 Suppressor Cells Are More Susceptible to Sphingosine Than Th1 Cells in Murine Contact Photosensitivity

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Murine contact photosensitivity (CPS) to 3,3′,4′,5-tetrachlorosalicylanilide (TCSA) is a cutaneous delayed-type hypersensitivity reaction in which both positive and negative regulatory pathways exist. The latter pathway is mediated by antigen-specific, CD4+ suppressor T cells (CPS-Ts) that are Th2 cells. We examined the effects of sphingosine and synthetic cell-permeable analogs of ceramide on the cellular kinetics of CPS-Ts and immune lymph node cells from TCSA-photosensitized mice (CPS-LNC), along with other murine T-cell populations. The addition of sphingosine at 10 or 3 μM to *in vitro* cultures suppressed DNA synthesis of CPS-Ts and Th2 clones, including D10 cells and 24-2 cells, but not that of CPS-LNC or Th1 clones, including 23-1-8 and 28-4 cells. This suggested that sphingosine exerts its inhibitory effects preferentially on the proliferation of Th2 cells. Although suppressing DNA synthesis, sphingosine augmented the production and mRNA expression of interleukin-4 (IL-4) and enhanced the expression of the IL-4 receptor in CPS-Ts. In addition, the ability of sphingosine to induce signal transduction of CPS-Ts was confirmed by elevation of the intracellular free Ca^{++} concentration. Because CPS-Ts exposed to sphingosine exhibited a lower G_2M/G_1 ratio than control, these seemingly ambivalent phenomena may be caused by retardation of the G_1 to S phase progression, a cell-cycle dysregulation known to augment cytokine production. In contrast to sphingosine, cell-permeable ceramide did not affect the proliferation of these cells when stimulated with mitogen/antigen and did not augment IL-4 production by CPS-Ts. Our study suggests that sphingosine modifies the Th1/Th2 balance by preferentially affecting the cellular kinetics of Th2. ***Key words: sphingolipids/T lymphocytes/DNA synthesis/cytokines. J Invest Dermatol 107:34–40, 1996***

Murine contact photosensitivity (CPS) is a highly specific delayed-type hypersensitivity reaction (Takigawa and Miyachi, 1982) in which both positive (Takigawa and Miyachi, 1982) and negative (Takigawa *et al*, 1984; Tokura *et al*, 1987, 1990) regulatory pathways exist. This cutaneous allergic reaction is induced and elicited by skin application of photohapten plus ultraviolet A (UVA) irradiation of skin (reviewed in Tokura and Takigawa, 1993). The studies on both ultraviolet B light (UVB)-induced local immunosuppression (Takigawa *et al*, 1984; Tokura *et al*, 1987; Yagi *et al*, 1996) and unresponsiveness of mice possessing the H-2^k haplotype (Tokura *et al*, 1990) demonstrate that CPS to a strong photohapten, 3,3′,4′,5-tetrachlorosalicylanilide (TCSA), is downregulated by antigen-specific, afferent limb-acting, CD4+CD8− suppressor T cells (Ts). Murine CD4+ helper T cells are divided into Th1 cells, which release interleukin-2 (IL-2) and interferon-γ (IFN-γ) and mediate delayed-type hypersensitivity, and Th2 cells, which produce IL-4, IL-5, and IL-10 and are involved in the production of immunoglobulins (Mosmann and Coffman, 1989). Our recent study has shown that T cells in contact photosensitivity to TCSA (CPS-Ts) belong to the Th2 cell class with regard to production and mRNA expression of cytokine (Yagi *et al*, 1996).

Sphingomyelin turnover is a novel pathway for transmembrane signal transduction in which sphingolipids, such as ceramide and its breakdown product sphingosine, participate in cell growth and differentiation, oncogenic transformation, and immune recognition and responsiveness (Hannun and Bell, 1989; Hakomori, 1990; Merrill and Jones, 1990; Ballou, 1992). In this process, ceramide produced by hydrolysis of sphingomyelin at the plasma membrane initiates a cascade of multiple signal-transduction pathways (Okazaki *et al*, 1989; Kim *et al*, 1991; Wiels *et al*, 1991; Dressler *et al*, 1992). Sphingosine commonly derived by deacylation of ceramide inhibits the activity of protein kinase C (Hannun and Bell, 1989; Imboden *et al*, 1985). In several types of cells, including fibroblasts (Olivera *et al*, 1992), HL-60 cells (Okazaki *et al*, 1989; Kim *et al*, 1991), and B-lymphocytes (Wiels *et al*, 1991), sphingomyelin-derived lipids act as endogenous modulators, but little is known

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Abbreviations: C2, *N*-acetylsphingosine; CPS, contact photosensitivity; CPS-LNC, lymph node cells from mice contact-photosensitized with TCSA painting plus UVA irradiation; CPS-Ts, suppressor T cells specific for suppression of contact photosensitivity to TCSA; IL-4R, interleukin-4 receptor; KLH, keyhole limpet hemocyanin; LC, Langerhans cells; TCSA, 3,3′,4′,5-tetrachlorosalicylanilide; TNP, trinitrophenyl; TNP-LNC, TNP-primed lymph node cells.

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concerning the participation of sphingolipids in the cellular kinetics of T cells.

Recent observations have demonstrated that signal transduction of Th2 cells is unique in that participation of protein kinase C is not essential for events associated with cell growth and cytokine production (Gajewski *et al*, 1990; Munoz *et al*, 1990; Kawakami and Parker, 1992; Tamura *et al*, 1993). Furthermore, our preliminary study suggested that the inhibitory effect of sphingosine on T-cell proliferation was specific for Th2 cells. Because a large amount of free sphingosine exists in the stratum corneum of epidermis (Wertz and Downing, 1990; Wakita *et al*, 1992), it is thought that sphingosine diffuses backwards *in vivo* and affects infiltrating T cells in some skin disorders, such as contact dermatitis. Whether sphingolipids up- or downregulate cutaneous sensitivity is an issue to be explored. The effect of synthetic cell-permeable ceramide on cellular functions is mimicked by exogenous addition of sphingomyelinase (Kim *et al*, 1991; Dressler *et al*, 1992; Wakita *et al*, 1994), and thus cell-permeable sphingolipids are widely used for analysis of the sphingomyelin pathway in signal transduction. In this study, therefore, we investigated the modulatory effects of the cell-permeable sphingolipids on the kinetics of T cells concerned with positive and negative pathways of CPS. The results show that exogenous sphingosine and ceramide affect the proliferation of T cells depending on the cell type, and that sphingosine inhibits DNA synthesis but augments IL-4 production, with retardation of G_1 to S phase progression in CPS-Ts.

MATERIALS AND METHODS

Animals Female BALB/c and AKR mice were obtained from Japan SLC (Hamamatsu, Japan). BALB/c mice, 8–12 wk old, were used unless otherwise mentioned.

Ceramides, Sphingosine, and H-7 *Trans*-D-erythro-2-amino-4-octadecene-1,3-diol (sphingosine) was purchased from Sigma Chemical Co. (St. Louis, MO). Short-chain ceramides, including *N*-acetylsphingosine (C2), *N*-hexanoylsphingosine (C6), and *N*-octanoylsphingosine (C8), were prepared by *N*-acylation of D-erythro-sphingosine using anhydrotic acetic acid, hexanoylchloride, and octanoylchloride (Aldrich Chemicals, Milwaukee, WI) (molar ratio, 1:10), as reported previously (Gaver and Sweeley, 1966). After removing residuals on a diethylaminoethyl-Sephadex column (Sigma), we incubated the eluate in 1 N NaOH to dissect esterified bonds. The products were purified by preparative thin-layer chromatography, and purity greater than 95% was verified by high-performance liquid chromatography analysis (Wakita *et al*, 1992).

Ceramides and sphingosine were conjugated with fatty acid–free bovine serum albumin (BSA; Boehringer Mannheim, Indianapolis, IN) (Merrill *et al*, 1989). Briefly, the aliquot of sphingolipid dissolved in absolute ethanol (100 mM) was added to 2 mM fatty acid–free BSA dissolved in phosphate-buffered saline (PBS, pH 7.4) to form stock solutions of sphingosine- or ceramide-albumin complex at a molar ratio of 1:1. The same concentration of BSA without sphingolipid was used as control.

A protein kinase C inhibitor, H-7 (Seikagaku Co., Tokyo, Japan) was used at final concentrations of 5 and 25 μM.

Culture Medium RPMI-1640 (Gibco Laboratories, Grand Island, NY) was supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 5×10^5 M 2-mercaptoethanol, 10^{-5} M sodium pyruvate, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, 1% nonessential amino acids (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Preparation of Langerhans Cell (LC)–Enriched Epidermal Cells LC were prepared and used as antigen-presenting cells, as described previously (Tokura *et al*, 1994b). The LC-enriched cell population contained approximately 15% of I-A^+ cells, representing LC, and had strong antigen-presenting ability for hapten, protein antigen, and superantigen (Tokura *et al*, 1994a, 1994b).

Spleen Cells Erythrocyte-lysed BALB/c spleen cells (3×10^5 cells/well) (Tokura *et al*, 1994b) were stimulated with concanavalin A (Con A; Sigma Chemical Co.) at a final concentration of 3 μg/ml.

Lymph Node Cells Primed by Contact Photosensitization With TCSA Plus UVA (CPS-LNC) Lymph node cells were taken from BALB/c mice that were sensitized 7 d before with 1% TCSA painting plus UVA irradiation, as reported previously (Takigawa and Miyachi, 1982). Th1 cells in these lymph node cells were responsible for TCSA photosensitization in H-2^d mice (Takigawa and Miyachi, 1982; Tokura and Takigawa, 1993). As assessed by enzyme-linked immunosorbent assay, the 3-d culture supernatants from CPS-LNC (3×10^6/ml) of BALB/c mice contained substantial amounts of IFN-γ (295 pg/ml) and IL-2 (1.3 U/ml), whereas IL-10 and IL-4 (less than 0.1 U/ml) were undetectable. Thus, we used BALB/c CPS-LNC as a representative of Th1 cells that counteract CPS-Th2, as mentioned later. In stimulatory experiments, CPS-LNC (2×10^5 cells/well) were cultured with 5 U/ml recombinant IL-2 (rIL-2; Genzyme Co., Boston, MA).

CPS-Ts To induce CD4+ CPS-Ts, the shaved abdomen of BALB/c mice was preirradiated with UVB and sensitized with TCSA/UVA, as described previously (Tokura *et al*, 1987). Spleen cells taken from these mice were cultured by pulsing with TCSA-photomodified spleen cells (Tokura *et al*, 1988) plus rIL-4 (Genzyme Co.) every 2 wk for 8-12 wk. The cells thus propagated had a CD4+CD8− phenotype, suppressed the *in vivo* and *in vitro* afferent limbs of contact photosensitivity to TCSA, and exclusively produced IL-4 and IL-10, but not IL-2, IFN-γ, or transforming growth factor–β, together with transcription of mRNA for the corresponding cytokines—indicative of a Th2 nature (Yagi *et al*, 1996). CPS-Ts consisted of two populations, 50–60% Vβ7+ cells and 40–50% Vβ13+ cells, as assessed by flow cytometry using T-cell receptor Vβ-specific monoclonal antibodies (Yagi *et al*, 1996). For stimulation assay, CPS-Ts (10^4 cells/well) were cultured with TCSA-photomodified spleen cells in the presence of rIL-4 (20 U/ml).

CTLL-2 A murine cytotoxic T-cell line, CTLL-2, was kindly provided by Dr. J. Yagi (Tokyo Women's Medical College). The cells were maintained by subculture every 3 d in the culture medium containing Con A–stimulated rat spleen cell culture supernatants (Collaborative Research Inc., Bedford, MA) at 10%. For stimulation assay, 10^4 cells/well were cultured for 24 h in the presence of 10% Con A supernatant.

D10 Cells D10.G4.1 (D10) (Kaye *et al*, 1984), a murine Th2 clone derived from AKR mice that reacts specifically with conalbumin, was provided by Dr. C. Janeway (Yale University School of Medicine) through the courtesy of Drs. J. Yagi (Tokyo Women's Medical College) and K. Takagi (University of Shizuoka). D10 cells were used at least 3 wk after feeding with conalbumin (Sigma Chemical Co.; final concentration, 100 μg/ml), feeder cells, and Con A–stimulated rat spleen cell supernatants. In proliferation experiments, D10 cells (2×10^4 cells/well) were stimulated with 5% Con A supernatants or with conalbumin plus LC-enriched epidermal cells (10^5 cells/well) prepared from AKR mice.

Keyhole Limpet Hemocyanin (KLH)–Specific T-Cell Clones 24-2, 23-1-8, and 28-4 Three KLH+I-A^k–specific T-cell clones—24-2 (Th2 type), 23-1-8 (Th1 type), and 28-4 (Th1 type) (Nakayama *et al*, 1988, 1989)—were kindly provided by Dr. Y. Asano (Department of Immunology, Faculty of Medicine, Tokyo University). These cell clones were used 3 wk after feeding with KLH (Sigma Chemical Co.; final concentration, 10 μg/ml), feeder cells, and Con A–stimulated rat spleen cell supernatants. In the present experiments, these cell clones (4×10^4 cells/well) were stimulated with 5% Con A supernatants.

Lymph Node Cells Primed by Contact Sensitization With Trinitrophenyl (TNP) (TNP-LNC) BALB/c mice were sensitized with picryl chloride (2,4,6-trinitrochlorobenzene; Tokyo Kasei Co., Tokyo, Japan) by painting 50 μl of 5% picryl chloride in ethanol:acetone (3:1) onto the clipped abdomens. Inguinal and axillary lymph node cells (3×10^5 cells/well) taken 7 d after sensitization were stimulated with TNP-modified LC-enriched epidermal cells (10^5 cells/well). For TNP modification, LC-enriched epidermal cells (10^7/ml) in PBS were incubated with an equal volume of 10 mM 2,4,6-trinitrobenzene sulfonic acid (Tokyo Kasei Co.) in PBS at room temperature for 30 min (Greene *et al*, 1978).

Assay for T-Cell Responses and Calculation of Percentage Enhancement by Sphingosine and C2 Various cell types were cultured in triplicate in the presence or absence of relevant stimulants in 96-well microtiter plates (Corning Glass Works, Corning, NY) at 37°C under 5% CO_2 in air for 66–72 h. Sphingosine, ceramides, and BSA as a control were added at the start of culture. Methyl tritiated thymidine ([^{3}H]TdR) (Amersham Intl., Arlington, IL; 1 μCi/well) was added to the culture 14–16 h before harvest. The cells were harvested on glass fiber filters using a cell harvester (Cambridge Technologies, Watertown, MA), and their radiouptake was measured in a scintillation counter.

The percentage enhancement of the response was calculated as follows: (cpm of sphingosine or ceramide-addition group − cpm of BSA-addition group)/(cpm of BSA-addition group − cpm of nonstimulated group) × 100. When stimulant(s) was not added, percentage enhancement represented: (cpm of sphingosine or ceramide-addition group − cpm of BSA-addition group)/(cpm of BSA-addition group).

Table I. Effects of Sphingosine (Sph), C2, and H-7 on the Proliferation of Various T-Cell Populations

			Percentage Enhancement of Response (Mean ± SD)[a]					
			Sph (μM)		C2 (μM)		H-7 (μM)	
Cells	Type	Stimulation With:	1	10 (or 3[b])	1	10	5	25
CPS-Ts	Th2	Photo TCSA-SC[c] + rIL-4	−36 ± 2	−56 ± 3	−3 ± 2	−10 ± 4	−31 ± 5	−91 ± 2
CPS-LNC	Mainly Th1	rIL-2	5 ± 4	2 ± 3	4 ± 3	−3 ± 5	−29 ± 4	−93 ± 3
		None	4 ± 2	8 ± 4	5 ± 3	30 ± 5	NT	NT
D10	Th2	Conalbumin + LC	−23 ± 5	−46 ± 6	−3 ± 1	−3 ± 1	−5 ± 6	−73 ± 7
		Con A supernatant	−20 ± 6	−48 ± 9	7 ± 3	8 ± 3	NT	NT
		None	−8 ± 3	−15 ± 5	−3 ± 2	−3 ± 3	NT	NT
24-2[b]	Th2	Con A supernatant	−29 ± 3	−97 ± 2	−12 ± 5	−13 ± 3	NT	NT
23-1-8[b]	Th1	Con A supernatant	−3 ± 3	−31 ± 6	2 ± 4	−24 ± 5	NT	NT
28-4[b]	Th1	Con A supernatant	−5 ± 4	−30 ± 7	1 ± 3	−20 ± 4	NT	NT
TNP-LNC	Mainly Th1	TNP-LC	5 ± 4	4 ± 2	7 ± 3	−2 ± 3	−32 ± 6	−92 ± 2
		None	−5 ± 4	15 ± 8	3 ± 5	58 ± 7	NT	NT
Spleen cells	Mixed	Con A	−10 ± 3	−12 ± 5	−5 ± 4	2 ± 3	NT	NT
		None	2 ± 3	10 ± 5	35 ± 7	50 ± 5	NT	NT

[a] The mean ± SD of percentage augmentation was obtained from three independent experiments. The underlined values represent more than 25% or less than −25% enhancement. NT, not tested.
[b] In KLH-specific cell clones 24-2, 23-1-8, and 28-4, Sphingosine was used at 3 μM instead of 10 μM.
[c] Photo TCSA-SC, TCSA-photomodified spleen cells.

Assay for IL-4 CPS-Ts (2×10^4/150 μl) were cultured for 48 or 72 h in a 96-well microtiter plate in the presence of sphingosine or C2. The culture supernatants were subjected to the IL-4 assay using CTLL-2. The CTLL-2 used in this study were sensitive to IL-4 as well as IL-2. Because CPS-Ts produce exclusively IL-4, but not IL-2, at both the protein and mRNA levels when stimulated with Con A (Yagi *et al*, 1996), the proliferative response of CTLL-2 to the supernatants reflected the amount of IL-4. Neither sphingosine nor C2 alone in the supernatant samples affected the proliferation of CTLL-2.

As described previously (Yagi *et al*, 1996), CTLL-2 were cultured in the presence of serially 4-fold diluted supernatants. [^{3}H]TdR (1 μCi/well) was added for the last 4 h. Murine recombinant IL-4 (Genzyme Co.) was used as a standard for all assays. Units of IL-4 in the test samples were calculated using the following equation: Units of test sample = (reciprocal titer of test sample at 20% of maximal cpm of standard/reciprocal titer of standard at 20% of maximal cpm) × activity of standard preparation.

Assay for IFN-γ CPS-LNC (2×10^5/150 μl) were cultured for 72 h in a 96-well microtiter plate in the presence of sphingosine or C2. A commercially available enzyme-linked immunosorbent assay kit (Endogen Inc., Boston, MA) was used for measuring IFN-γ in the culture supernatants according to the manufacturer's directions.

Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) CPS-Ts (10^7/ml) were cultured in the presence of sphingosine or BSA (control) at 10 μM. After 3 h, total RNA was prepared from an equal number of viable cells, as described previously (Chomczynski and Sacchi, 1987). First-strand cDNA was reverse transcribed using each RNA sample and was amplified by PCR with an RNA PCR kit (GeneAmp RNA PCR Kit; Takara Biomedicals, Osaka, Japan) according to the manufacturer's directions. All pairs of primers for β-actin, IL-2, IL-4, IL-10, and IFN-γ and PCR conditions were described previously (Yagi *et al*, 1996). The PCR products and DNA molecular-weight marker VI (Boehringer Mannheim GmbH, Mannheim, Germany) were separated in 2% agarose gels and stained with 1 μg/ml ethidium bromide. The origin of amplified DNA bands was confirmed by nonisotopic Southern blot hybridization, as described previously (Yagi *et al*, 1996).

Determination of IL-4 Receptor (IL-4R) Expression Hanks' balanced salt solution containing 0.1% NaN_3 and 1% fetal bovine serum was used for staining and washing. CPS-Ts were first incubated with rat anti–IL-4R monoclonal antibody (IgG2a, κ chain, Genzyme Co.) for 30 min at 4°C, then washed three times and stained with an excess amount of purified fluorescein-conjugated goat anti-rat κ chain (Cosmo Bio Co. Ltd, Tokyo, Japan). The stained cells were analyzed on a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA). To determine the background level of fluorescence, a monoclonal immunoglobulin isotype standard for rat IgG2a, κ (PharMingen, San Diego, CA) was used as the first antibody.

Measurement of Intracellular Free Ca^{++} Concentration ($[Ca^{++}]_i$) CPS-Ts and CPS-LNC were enriched for T cells with nylon wool–column passage (Julius *et al*, 1983). As described previously (Wakita *et al*, 1995), cells (10^6 cells/ml) were incubated in the dark for 1 h at 37°C with 20 μM acetoxymethylester of Fura-2 (Dojin Laboratories, Kumamoto, Japan) in phenol red–free minimal essential medium. The cells were washed five times and resuspended in the same medium before the addition of sphingosine. The fluorescence intensity of approximately 40 cells in each group was monitored with computerized color change on a display using a Digital Image Processing System (Argus-100/CA; designed by Hamamatsu Photonics Co., Hamamatsu, Japan). The data were expressed as the mean of relative fluorescence intensity of four cells.

Cell Cycle Analysis CPS-Ts were fixed with 70% ethanol in PBS, incubated with 1 mg/ml ribonuclease A (Sigma Chemical Co.) for 30 min at 37°C and then with 50 μg/ml propidium iodide for 20 min at room temperature, and analyzed by flow cytometry using a computed DNA cell cycle program (SFIT; Becton Dickinson). Each reaction step was followed by washing in PBS.

Statistical Analyses Student's t test was used to determine the statistical differences between means.

RESULTS

Effects of Sphingosine and Ceramides on the Proliferation of Various T Cells In a preliminary experiment, the proliferative response of spleen cells to Con A was not inhibited by C2, C6, or C8 at concentrations less than 10 μM. At 100 μM, both C6 and C8 abolished DNA synthesis by T cells, whereas C2 did not affect the response (data not shown). Because the proliferation of keratinocytes is not influenced by the addition of C6 or C8 at this concentration (Wakita *et al*, 1994), T cells were considered to be more susceptible to C6 and C8 than were keratinocytes. Thus, we used C2 to investigate the biologic activity of ceramides in the following experiments.

Sphingosine and C2 were added at concentrations of 1 and 10 μM to the cultures of eight types of T cells, which were stimulated to variable degrees **(Table I)**. Because sphingosine is known to inhibit protein kinase C activity, its effect was also compared with that of a protein kinase C inhibitor, H-7, at 5 and 25 μM. **Figure 1** shows the typical effects of sphingosine and C2 on the [^{3}H]TdR incorporation of six critical T-cell types, and **Table I** summarizes the mean values of their percentage augmentation obtained from three independent experiments.

The addition of sphingosine did not affect the proliferation of CPS-LNC, TNP-LNC, or spleen cells. On the other hand, sphingosine at 1 or 10 μM suppressed the DNA synthesis of CPS-Ts in response to TCSA-photomodified spleen cells plus IL-4. Sphingosine at 10 μM also inhibited the proliferation of D10 cells in response to Con A supernatant and to conalbumin plus LC. Because

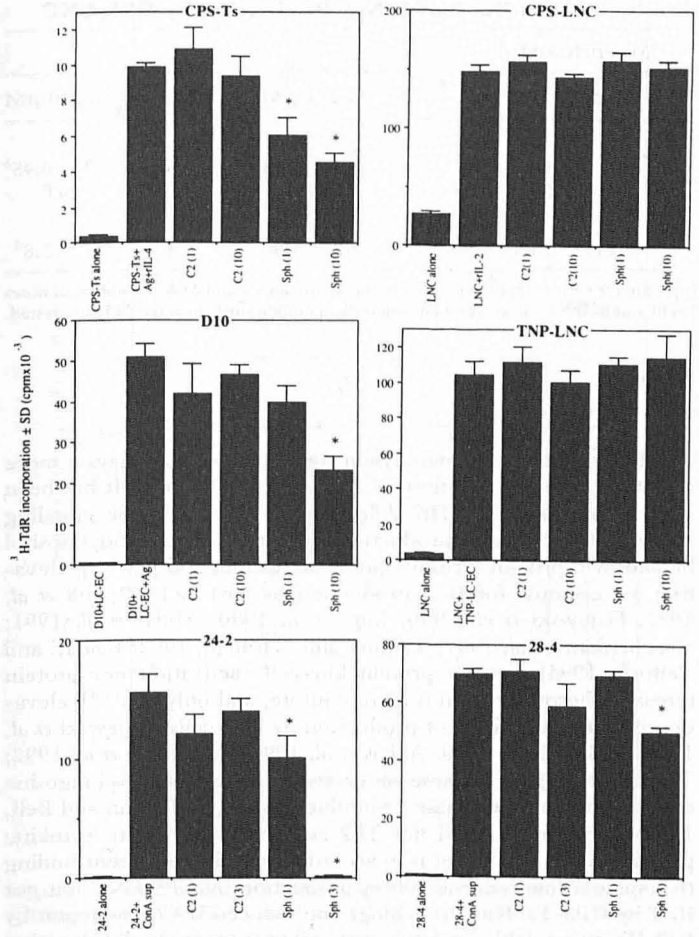

Figure 1. Sphingosine-induced suppression of DNA synthesis in Th2-type but not Th1-type cells. The six types of T cells were cultured for 72 h with C2 or sphingosine at the indicated concentrations (*in parentheses*) in the presence of the following stimulants: CPS-Ts, TCSA-photomodified spleen cells plus rIL-4; D10, Langerhans cell–enriched epidermal cells (LC-EC) plus conalbumin; 24-2, con A supernatants; TCSA-LNC, recombinant IL-2; TNP-LNC, TNP-modified LC-EC; and 28-4, con A supernatants. *Error bars*, SD. *$p < 0.05$, compared with corresponding stimulant-addition group (*second column*). Sph, sphingosine.

CPS-LNC contain Th1 with a minimal number of Th2 cells (Tokura *et al*, 1990) (see *Materials and Methods*), these data suggest that the proliferation of Th2 cells, but not Th1 cells, was susceptible to exogenous addition of sphingosine. Because sphingosine at 10 μM suppressed the DNA synthesis of all the three KLH-specific T-cell clones by 95–100%, its effect was tested at concentrations of 3 and 1 μM. Whereas sphingosine inhibited the proliferation of Th2 clone 24-2 by 97% at 3 μM and by 29% at 1 μM, Th1 clones 23-1-8 and 28-4 were suppressed by approximately 30% at 3 μM and were not significantly inhibited at 1 μM, confirming the susceptibility of Th2 cells to sphingosine. The inhibition seemed to be due to the direct effect of sphingosine on the T cells and not to perturbation of LC function, because the response of D10 to Con A supernatant as well as antigen plus LC was suppressed by sphingosine.

In contrast to sphingosine, no significant effect of C2 was observed in any types of cells when stimulated with mitogen, cytokine, or antigen/LC. The DNA synthesis of nonstimulated spleen cells, TNP-LNC, or CPS-LNC was only marginally augmented by C2 at 10 μM.

H-7 inhibited proliferation not only of CPS-Ts and D10 cells, but also of CPS-LNC and TNP-LNC. These results suggest that sphingosine did not suppress the DNA synthesis of CPS-Ts and D10 cells exclusively by inhibiting protein kinase C activity.

Augmentation of Cytokine Production by Sphingosine in CPS-Ts But Not in CPS-LNC CPS-Ts were cultured with sphingosine or C2 at 1 and 10 μM for 48 or 72 h, and the amount of IL-4 in the supernatants was assayed. As shown in **Table II**, CPS-Ts cultured with 10 μM sphingosine for 48 h (experiment 1) and those exposed to sphingosine at 1 and 10 μM for 72 h (experiment 2) released significantly higher amounts of IL-4 than did the control groups. The IL-4 production was unchanged with C2 (experiment 1). The augmenting effect of sphingosine on IL-4 production was specific for CPS-Ts, because sphingosine did not enhance, but rather suppressed, IFN-γ production by CPS-LNC. C2 was more suppressible than sphingosine for CPS-LNC (experiment 3).

To exclude the possibility that CPS-Ts are changed to produce Th1-type cytokines after exposure to sphingosine, we performed RT-PCR to detect mRNA of CPS-Ts that were incubated with sphingosine or BSA (control) using primers specific for IL-2, IL-4, IL-10, and IFN-γ. As shown in **Fig 2**, IL-4 and IL-10 mRNA were clearly present in both sphingosine-addition and BSA-addition samples. The expression of IL-4 mRNA was augmented by exposure to sphingosine as compared with the sample with BSA. On the other hand, mRNA for neither IL-2 nor IFN-γ was detected in these two samples, whereas control PCR product from 28-4 cells exhibited these messages. These findings indicate that the Th2-type cytokine pattern is unchanged by sphingosine in CPS-Ts and confirm that sphingosine has an augmenting effect on IL-4 production by CPS-Ts.

Augmentation of IL-4R Expression on CPS-Ts by Sphingosine and C2 CPS-Ts were cultured for 72 h in the presence of sphingosine or C2, and IL-4R expression on the cell surface was assessed by flow cytometry. As shown in **Fig 3**, both sphingosine and C2 increased IL-4R expression, as monitored by both peak fluorescence intensity and mean fluorescent value.

Elevation of $[Ca^{++}]_i$ by Sphingosine in CPS-Ts and CPS-LNC We next examined whether sphingosine induces signal transduction in CPS-Ts or CPS-LNC by monitoring $[Ca^{++}]_i$. **Figure 4** illustrates the pattern of $[Ca^{++}]_i$ change in these two types of cells. The addition of sphingosine promptly increased cytoplasmic levels of Ca^{++} in CPS-Ts and CPS-LNC, suggesting that signaling was inducible by sphingosine. The two cell types differed from each other, however, in that CPS-Ts showed a more sustained elevation of $[Ca^{++}]_i$ than CPS-LNC. C2 did not elevate $[Ca^{++}]_i$ in either type of cells (data not shown).

Sphingosine-Induced Retardation of G_1 to S Phase Progression in CPS-Ts After cultivation for 96 h with sphingosine or C2, the cell cycle of CPS-Ts was examined by computed flow cytometric analysis. As shown in **Table III**, the percentage of cells at G_2+M phase and the G_2M/G_1 ratio were substantially decreased by incubation with sphingosine but not with C2, as compared with the control group. This suggests that sphingosine retarded the progression from G_1 to S phase. Such retardation of the cell cycle was not observed in Th1 cell clone 28-4 exposed to sphingosine at 3 μM, as the G_2M/G_1 ratios of the sphingosine-added group and of the BSA-added control group were 2.15 and 2.08, respectively.

DISCUSSION

This study showed that the addition of exogenous sphingolipids, in particular sphingosine, differentially modulated T-cell proliferation and cytokine production, depending on the T-cell type. This indicates that in addition to the phosphatidylinositol pathway, sphingomyelin turnover participates profoundly in the biologic events of certain types of T cells. Sphingosine preferentially inhibited DNA synthesis in CPS-Ts but stimulated the cells to produce IL-4 and to enhance IL-4R expression, whereas this sphingolipid did not affect the DNA synthesis and inhibited the cytokine production in CPS-LNC. Because CPS-Ts belong to Th2 cells, and

Table II. Sphingosine (Sph) Augmentation of IL-4 Production by CPS-Ts But Not IFN-γ Production by CPS-LNC

	Materials Added				
	BSA, 10 μM	Sph, 1 μM	Sph, 10 μM	C2, 1 μM	C2, 10 μM
IL-4 concentration in supernatants of CPS-Ts (± SD, U/ml)[a]					
Experiment 1	4.8 ± 0.34	4.6 ± 0.17[b]	11.2 ± 1.25[c]	4.7 ± 0.28[b]	5.2 ± 0.45[b]
Experiment 2	3.6 ± 0.46	7.2 ± 0.29[c]	9.8 ± 0.83[d]	NT	NT
INF-γ concentration in supernatants of CPS-LNC (± SD, pg/ml)[a]					
Experiment 3	253 ± 23.9	177 ± 18.8[d]	138 ± 3.8[d]	52 ± 5.3[d]	47 ± 5.8[d]

[a] CPS-Th2 (2×10^4/150 μl medium) and CPS-LNC (2×10^5/150 μl) were cultured in triplicate for 48 h (experiment 1) or 72 h (experiments 2 and 3) at the indicated doses of sphingosine and C2. The culture supernatants were subjected to IL-4 assay using CTLL-2 cells and to IFN-γ assay using enzyme-linked immunosorbent assay. NT, not tested.
[b] Statistically not significant compared with control (BSA) group.
[c] $p < 0.05$ compared with control (BSA) group.
[d] $p < 0.01$ compared with control (BSA) group.

the proliferating cells in response to antigen in CPS-LNC are Th1 cells, sphingosine seems to inhibit DNA synthesis in Th2 but not Th1 cells. This notion was supported by the finding that D10 and 24-2 cells were more susceptible to sphingosine than were 23-1-8, 28-4 cells, or TNP-LNC. The elevation of $[Ca^{++}]_i$, a known marker for signal transduction, further showed the ability of sphingosine to promote cell kinetics of CPS-Ts. In addition, sphingosine retarded the cell cycle of CPS-Ts by blocking the G_1 to S phase progression, which seems to be associated with the reduction in DNA synthesis. CPS-Ts used in this study consisted of a comparable number of Vβ7- and Vβ13-bearing T-cell populations (Yagi *et al*, 1996), both of which are reactive with antigen plus IL-4 and have a Th2 cytokine profile. Vβ7+ T cells have an *in vivo* and *in vitro* suppressive activity, whereas Vβ13+ cells are nonfunctional (Yagi *et al*, 1996). It remains unknown which population is predominantly responsible for these sphingosine-induced alterations of Th2 kinetics.

Th2 but not Th1 cells are activated by IL-1 (Solari *et al*, 1990) and tumor necrosis factor–α in a synergistic manner (Tokura *et al*, 1994b), and both cytokines are agonists of sphingomyelin turnover (Kim *et al*, 1991; Wiels *et al*, 1991; Dressler *et al*, 1992; Mathias *et al*, 1993). Our results together with these observations raise the possibility that the sphingomyelin signaling pathway plays a more important role in activation of Th2 than of Th1 cells. It has been reported recently that Th2 differ from Th1 cells in the signaling pathway for cytokine production. Whereas phosphatidylinositol breakdown, protein tyrosine kinase activation, and $[Ca^{++}]_i$ elevation are essential for IL-2 production by Th1 cells (Rosoff *et al*, 1987; Gajewski *et al*, 1990; June *et al*, 1990; Abbas *et al*, 1991; Glaichenhaus *et al*, 1991; Tamura and Nariuchi, 1992; Baldari and Telford, 1994), neither protein kinase C activation nor protein tyrosine phosphorylation is a prerequisite, and only $[Ca^{++}]_i$ elevation is mandatory for IL-4 production by Th2 cells (Gajewski *et al*, 1990; Munoz *et al*, 1990; Abbas *et al*, 1991; Kawakami *et al*, 1992; Tamura *et al*, 1993). These observations indicate that sphingosine can exert its protein kinase C–inhibiting ability (Hannun and Bell, 1989) only for Th1 and not Th2 cells, with respect to cytokine production. This concept is in accordance with the present finding that sphingosine reduced IFN-γ production by CPS-LNC but not IL-4 by CPS-Ts. Rather, sphingosine induced IL-4 production by CPS-Ts, presumably because of its ability to promote the elevation of $[Ca^{++}]_i$. $[Ca^{++}]_i$ elevation by sphingosine is due to Ca^{++} release from intracellular stores and not to the influx of extracellular Ca^{++} (Ghosh *et al*, 1990). In Th2 cells, the elevation of $[Ca^{++}]_i$ by anti-CD3 antibody is suggested to be caused by Ca^{++} influx (Tamura *et al*, 1993). $[Ca^{++}]_i$ was promptly elevated immediately after the addition of sphingosine, as observed in this study, whereas $[Ca^{++}]_i$ is increased slowly without a prompt rise after anti-CD3 stimulation (Tamura *et al*, 1993). Thus, sphingosine seems to differ from T-cell receptor/CD3-mediated signaling in both the elevation pattern and the source of $[Ca^{++}]_i$.

Sphingosine blocked the progression from G_1 to S phase in CPS-Ts, whereas C2 had no inhibitory effect on the cells. Sphingosine, but not C2, has been reported to bind DNA (Koiv and Kinnuen, 1994). Such a direct interaction between sphingosine and nucleic acid might be a key issue to elucidate the mechanism(s) of

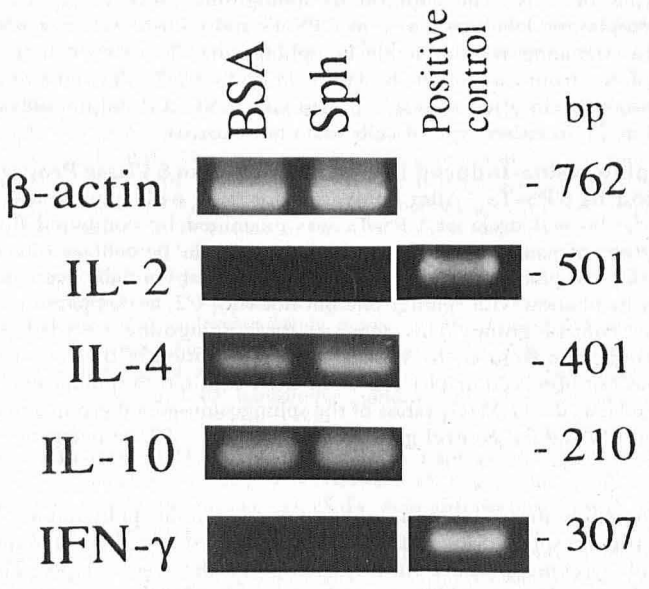

Figure 2. RT-PCR for detection of cytokine mRNA in CPS-Ts incubated with sphingosine. CPS-Ts were cultured with sphingosine or BSA (control) at 10 μM for 3 h. RT-PCR was performed with primers specific for each cytokine. The *numbers* indicate the size (base pairs) of amplified products. The positive control sample for IL-2 and IL-4 was obtained from 28-4 cells. Sph, sphingosine.

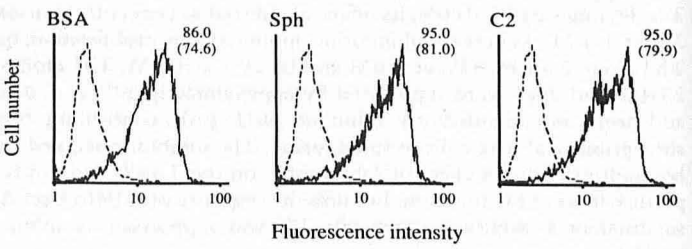

Figure 3. Augmentation of IL-4R expression on CPS-Ts by sphingosine and C2. CPS-Ts were cultured for 72 h in the presence of BSA (control), sphingosine (Sph), or C2 at 10 μM. After cultivation, the expression of IL-4R was analyzed by flow cytometry. *Dotted lines*, fluorescence intensity of isotype immunoglobulin control. *Numbers* and *numbers in parentheses*, peak fluorescence intensity and mean fluorescent value, respectively.

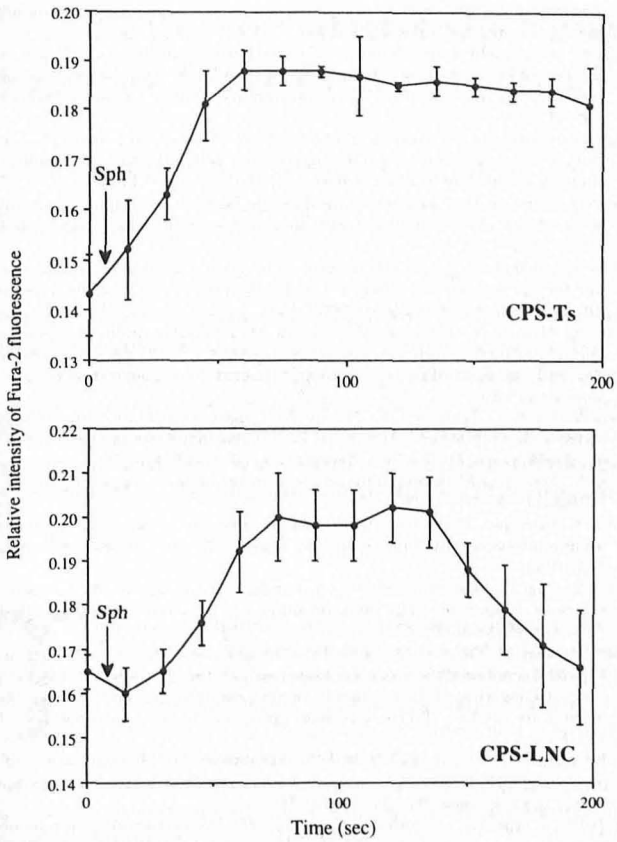

Figure 4. Induction of intracellular calcium mobilization by sphingosine in CPS-Ts and CPS-LNC. Fluorescence of Fura-2 was monitored in approximately 40 cells of CPS-Ts and CPS-LNC with computerized color change using a Digital Image Processing System. All the monitored cells had virtually the same color change on the display. The data are expressed as the mean of four cells. *Error bars*, SD.

the observed G_1 arrest. It is known that retardation of the G_1 to S phase progression augments IL-2 production in Th1 cells, because the late G_1 phase is the most productive period for IL-2 (Dröge, 1986; Farrar *et al*, 1980; Stadler *et al*, 1981). Given that the late G_1 phase is associated with IL-4 production in Th2 cells, the ability of sphingosine to retard the cell cycle from G_1 to S phase may result in increased production of IL-4 and decreased DNA synthesis in Th2 cells. It is therefore likely that both sphingosine-induced elevation of $[Ca^{++}]_i$ and retardation of G_1 to S phase progression result in augmented IL-4 production.

C2 did not affect the proliferation of any of the types of cells examined, except for only marginal augmentation in nonstimulated lymph node cells and spleen cells. When monitored with cytokine production, C2 inhibited CPS-LNC but not CPS-Th2 cells. Ceramides have pleiotropic functions in cell proliferation and differentiation, depending on the cell type. C2 inhibits proliferation and induces the differentiation of a leukemic cell line HL-60 (Okazaki *et al*, 1989) and of transformed keratinocytes (Wakita *et al*, 1994), whereas cultured fibroblasts proliferate in response to C2 (Olivera *et al*, 1992). The present study suggests that the involvement of ceramides in Th2-cell kinetics was minimal, but endogenously synthesized ceramides might exert different effects on T cells. In fact, our preliminary studies have shown that DNA synthesis in 24-2 and 28-4 cells is increased when natural ceramides are used instead of synthetic ceramides (Tokura *et al*, unpublished observation).

Because a large amount of free sphingosine exists in the stratum corneum (Wertz and Downing, 1990; Wakita *et al*, 1992), it is plausible that sphingosine diffuses backwards and affects infiltrating T cells in some skin disorders. On the other hand, because of their complicated structure (Wertz and Donald, 1991), naturally occurring ceramides in the stratum corneum are less cell permeating than synthetic ceramides and thus are unlikely to penetrate from the stratum corneum to the viable epidermal layer. Because the keratinocyte-derived cytokines IL-1α and tumor necrosis factor–α are induced after epidermal injury (Kupper, 1990) and activate Th2, but not Th1 cells (Solari *et al*, 1990; Tokura *et al*, 1994b), the injured epidermis may permit Th2 propagation. Such an expanded Th2 cell population may be further stimulated to produce cytokines, with concomitant G_1 arrest when exposed to sphingosine that diffuses from the destroyed stratum corneum. As a result, Th2 cells may suppress delayed-type hypersensitivity effector Th1 cells by release of IL-10 (Li *et al*, 1994; Yagi *et al*, 1996), resulting in downregulation of exaggerated cutaneous hypersensitivity reactions. On the other hand, sphingosine in the epidermis might play a provocative role in Th2-mediated skin disorders, such as atopic dermatitis (Van der Heijden *et al*, 1991) and cutaneous T-cell lymphoma (Vowels *et al*, 1992). In addition, the most biologically potent species of sphingosine, dihydroxysphingenine, is increased in psoriatic lesions (Gaver and Sweeley, 1966). Whether such increased amounts of sphingosine are involved in the pathogenesis of psoriasis is a question to be elucidated.

Table III. Sphingosine (Sph)-Induced Retardation of the G_1 to S Phase Progression in CPS-Ts

Materials Added[a]	Percentage of Cells (± SD)		G_2M/G_1 Ratio (± SD)
	G_0G_1	$S+G_2M$	
BSA	85 ± 3	15 ± 2	2.08 ± 0.05
Sph	94 ± 3[b]	6 ± 1[b]	1.87 ± 0.04[b]
C2	81 ± 2[c]	19 ± 2[c]	1.95 ± 0.06[c]

[a] CPS-Ts were cultured for 96 h in the presence of sphingosine, C2, or BSA (control) at 5 μM. The data were expressed as the mean ± SD of three independent experiments.
[b] $p < 0.05$ compared with corresponding BSA group.
[c] Statistically not significant compared with corresponding BSA group.

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