Evaluation of synthetic sphingosine, lysosphingolipids and glycosphingolipids as inhibitors of functional responses of human neutrophils

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Human neutrophils, when exposed to soluble stimuli, aggregate, release oxygenated products of arachidonic acid and generate active oxygen species. Sphingolipid-derived products such as sphingosine and lysosphingolipids have been shown to exert selective actions on a variety of cell types, including neutrophils. Therefore, to determine the structural basis for selective inhibition of neutrophil responses by naturally occurring sphingolipids, seven compounds were prepared by total organic synthesis, and their impact on neutrophils in suspension has been studied. The compounds synthesized included sphingosine, psychosine, lactosyl lysosphingolipid, globotriaosyl (Gb₃) lysosphingolipid, galactosyl cerebroside, lactosyl ceramide and Gb₃ ceramide. The neutrophil responses studied were aggregation, leukotriene generation and superoxide anion production. When exposed to non-cytotoxic levels of the synthetic compounds, as monitored by exclusion of Trypan Blue, none of the synthetic sphingolipids inhibited A23187-induced aggregation of neutrophils. Only lactosyl lysosphingolipid, at a concentration of 1 μ M, significantly inhibited aggregation induced by fMetLeuPhe; the other compounds in this series including sphingosine were without effect at equal molar concentrations (1 μ M). Aggregation induced by phorbol 12-myristate 13-acetate (PMA) (0.1 μ M) was significantly blocked by only two of the synthetic sphingolipids (1 μ M). At concentrations below 1 μ M, these inhibitory actions were not evident, nor was it possible to assign a structure–activity relationship for this series of compounds. None of the synthetic sphingolipids effectively inhibited the generation of superoxide anions induced by PMA. In addition, neither synthetic sphingosine nor psychosine affected either the formation or metabolism of leukotriene B₄. Taken together, the results provide further evidence that sphingolipids, when added to intact cells, are not potent selective inhibitors of functional responses of human neutrophils.

INTRODUCTION

Upon activation, human neutrophils aggregate, remodel their membrane lipids and generate mediators of inflammation such as active oxygen species and oxygenated products derived from arachidonic acid [1–5]. These responses can be initiated by the chemotactic peptide fMetLeuPhe, which acts upon specific receptors located on the cell surface [6], or by agents such as the bivalent-cation ionophore A23187 or the tumour promoter phorbol 12-myristate 13-acetate (PMA), both of which bypass early events in stimulus–response coupling. The ionophore circumvents these steps in neutrophil activation by directly mobilizing Ca²⁺, whereas PMA activates protein kinase C [7–9]. These later events are held to play pivotal role(s) in stimulus–response coupling in the neutrophil, as in many other cell types [7–11].

Drawing upon the concept that diacylglycerol, an intermediate in phospholipid biosynthesis, may serve to activate protein kinase C in various cells following receptor–ligand interactions [12], it has been proposed

that sphingosine, an intermediate in the biosynthesis of sphingolipids (including sphingomyelins and glycosphingolipids), may also serve a messenger role in inhibiting protein kinase C [13]. Thus exogenously applied sphingosine and related compounds have been shown to block a wide range of cellular responses (reviewed in [14]). With human neutrophils, for example, sphingosine was reported to inhibit several responses including the generation of superoxide anions as well as the biosynthesis of platelet-activating factor and leukotriene B₄ (LTB₄) [15–17]. Results of studies by Pittet *et al.* [18] indicate that, in addition to inhibiting superoxide anion generation and mobilization of Ca²⁺ in neutrophils, sphinganine (dihydrosphingosine) leads to increased permeabilization at concentrations similar to those which block cellular functions.

To date, the actions of the sphingolipids have been explored with commercial preparations of these substances. However, in the present study, sphingosine and six other naturally occurring sphingolipids were prepared by total organic synthesis and their impact on some of

Abbreviations used: leukotriene B₄ (LTB₄), 5S,12R-dihydroxyeicosa-6,14-*cis*-8,11-*trans*-tetraenoic acid; PMA, phorbol 12-myristate 13-acetate; PBS, (Dulbecco's) phosphate-buffered saline; Me₂SO, dimethyl sulphoxide; Gb₃, globotriaose.

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the functional responses of human neutrophils was evaluated. In the absence of cell lysis, a clear structure-function relationship was not obtained with sphingosine, psychosine, lactosyl lysosphingolipid, globotriaosyl (Gb₃) lysosphingolipid, galactosyl cerebroside, lactosyl ceramide and Gb₃ ceramide.

MATERIALS AND METHODS

Reagents

PMA, fMetLeuPhe, cytochrome *c* (horse heart, type VI), cytochalasin B, ionophore A23187 and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Dimethyl sulphoxide (Me₂SO) was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and h.p.l.c.-grade solvents were from American Scientific Products, Burdick and Jackson (Muskegon, MI, U.S.A.). PMA was stored at -20 °C in Me₂SO. fMetLeuPhe, cytochalasin B and A23187 were each stored in ethanol and diluted in buffer just before use.

Preparation of neutrophil suspensions

For each experiment, peripheral blood (approx. 100–120 ml) was obtained from healthy volunteers. Neutrophils were isolated from heparinized blood directly after venipuncture by dextran sedimentation followed by Ficoll/Hypaque gradient centrifugation [19]. Red cells were removed by hypo-osmotic lysis followed by centrifugation. Isolated cells were suspended in Dulbecco's phosphate-buffered saline (PBS) containing both CaCl₂ (0.6 mM) and MgCl₂ (1.0 mM), pH 7.4, and warmed for 5 min at 37 °C before additions. This buffer was used

throughout. The suspensions contained 98 ± 1 % neutrophils as determined by light microscopy. The integrity of cells in suspension was monitored both before and during incubation by determining their ability to exclude Trypan Blue. These determinations were performed by using light microscopy. The final concentrations of vehicle (either Me₂SO or ethanol) were less than 0.2 % (v/v). In all experiments, vehicle controls were run in parallel.

Preparation of synthetic sphingolipids and related compounds

Each compound depicted in Fig. 1 was prepared by total organic synthesis from intermediates described in [20]. The stereochemistry and complete structure of each compound were confirmed by both i.r. and n.m.r. analyses after isolation and purification by flash column chromatography on silica gel.

1. D-Erythrospingosine. (4*E*,2*S*,3*R*)-2-Amino-4-octadecene-1,3-diol. ¹H n.m.r.; δ (p.p.m.) (250 MHz) (C²HCl₃) 5.73 (1H, dt, *J* 7.4 and 15.1 Hz, C=C-H), 4.98 (1H, dd, *J* 7.2 and 15.1 Hz, C=C-H), 3.83 (1H, dd, *J* 3.2 and 12.3 Hz, CH-OH), 3.41 (1H, dd, *J* 8.4 and 12.3 Hz, CH-OH), 2.37–1.94 (7H, m, C-NH, C-OH, CH-N, CH-O, C=C-CH), 1.45–0.96 (22H, m, CH₂ alkane), 0.82 (3H, t, *J* 6.8 Hz, CH₃).

2. Psychosine. β-Galactopyranosyl(1→1)sphingosine. ¹H n.m.r. δ (p.p.m.) (500 MHz) ([²H₆]Me₂SO/²H₂O, 49:1, v/v) 5.64 (1H, dt, *J* 7.0 and 15.2 Hz, C=C-H), 5.44 (1H, dd, *J* 6.5 and 15.1 Hz, C=C-H), 4.08 (1H, d, *J* 7.4 Hz, H-1), 4.00 (1H, t, *J* 5.4 Hz H₂CHO), 3.68–3.26 (8H, m, CH-O and CHN), 3.21 (s, H²HO),

Fig. 1. Structures of the synthetic sphingolipids and related compounds

The glycosphingolipids used in the present studies were prepared by total organic synthesis as reported previously [20]. The structure of each compound was confirmed by i.r. and n.m.r. analysis (see the Materials and methods section).

2.96 (1H, m, CH-O), 2.00 (2H, q, J 6.8 Hz, C=C-CH), 1.34–1.11 (22H, m, CH₂ alkanes), 0.85 (3H, t, J 6.8 Hz, CH₃).

3. Lactosyl lysosphingolipid. β -Galactopyranosyl(1→4) β -glucopyranosyl(1→1)sphingosine. ¹H n.m.r. δ (p.p.m.) (500 MHz) ([²H₆]Me₂SO/²H₂O, 49:1, v/v) 5.64 (1H, dt, J 7.4 and 15.4 Hz, C=C-H), 5.44 (1H, dd, J 6.6 and 15.4 Hz, C=C-H), 4.22–4.21 (2H, m, H-1, H-1'), 4.03 (1H, t, J 5.6 Hz), 3.74 (1H, dd, J 9.0 Hz, CH-O), 3.76–3.30 (13H, m, CHO), 3.33 (s, ²H₂O), 3.06 (1H, t, J 8.1 Hz, CH-O), 3.01 (1H, m, CHN), 2.00 (2H, q, J 6.5 Hz, C=C-CH), 1.34–1.11 (22H, m, CH₂), 0.85 (3H, t, J 6.7 Hz, CH₃).

4. Gb₃ lysosphingolipid. α -Galactopyranosyl(1→4) β -galactopyranosyl(1→4) β -glucopyranosyl(1→1)sphingosine. ¹H n.m.r. δ (p.p.m.) (500 MHz) ([²H₆]Me₂SO/²H₂O, 49:1, v/v) 5.56 (1H, dt, J 7.6 and 15.5 Hz, C=C-H), 5.43 (1H, dd, J 6.6 and 15.5 Hz, C=C-H), 4.79 (1H, d, J 3.8 Hz, H-1'), 4.26 (1H, d, J 7.6 Hz, H-1'), 4.16 (1H, d, J 7.9 Hz, H-1), 4.05 (1H, t, J 6.5 Hz), 3.82–3.30 (19H, m, CH-O), 3.29 (s, ²H₂O), 3.02 (1H, t, J 8.2 Hz, CH-O), 2.75 (m, 1H, CHN), 1.97 (2H, q, J 6.9 Hz, C=C-CH), 1.32–1.12 (22H, m, CH₂), 0.84 (3H, t, J 6.9 Hz, CH₃).

5. Gb₃ ceramide. R_f = 0.35 (silica, chloroform/methanol/water, 60:25:4, by vol.); [α]_D²⁵ +24.1° (c 0.44 in pyridine); ¹H n.m.r. δ (p.p.m.) (500 MHz) ([²H₆]Me₂SO/²H₂O, 49:1, v/v) 5.53 (1H, dT, J 15.3 and 7.7 Hz, C=C-H), 5.33 (1H, dd, J 15.4 and 7.1 Hz, C=C-H), 4.78 (1H, d, J 3.7 Hz, H-1'), 4.25 (1H, d, J 7.6 Hz, H-1'), 4.15 (1H, d, J 7.8 Hz, H-1), 4.05 (1H, t, J 6.1 Hz, H-5'), 3.93 (1H, dd, J 10.2 and 5.0 Hz, H-1 Cer), 3.88 (1H, t, J 7.5 Hz, H-3 Cer), 3.80–3.28 (18H, m, CH-O and ²H₂O peak), 3.04 (1H, t, J 8.0 Hz, H-2), 2.01 (1H, t, J 7.4 Hz, C=C-CH₂), 1.91 (2H, m, COCH₂), 1.43 (2H, m, COCH₂-CH₂), 1.31–1.11 (50H, m, CH₂), 0.83 (6H, t, J 6.8 Hz, CH₃); ¹³C n.m.r. δ (p.p.m.) (125 MHz) ([²H₆]Me₂SO) 171.7, 131.5, 131.3, 103.8, 103.5, 100.6, 80.7, 77.1, 75.0, 74.8, 74.4, 73.2, 72.8, 71.1, 70.8, 70.7, 69.2, 68.8, 68.6, 60.4, 59.3, 52.9, 39.0, 35.6, 31.8, 29.2, 29.1, 28.8, 28.7, 25.4, 22.2, 13.8.

6. Galactosyl ceramide. R_f = 0.36 (15% methanol in CH₂Cl₂); [α]_D²⁵ –0.20° (c 1.0 in methanol/chloroform, 1:1, v/v); ¹H n.m.r. δ (p.p.m.) (500 MHz) (C²H₅O²/C²HCl₃, 3:2, v/v) 5.70 (1H, dt, J 6.8 and 15.2 Hz, C=C-H), 5.46 (1H, dd, J 7.8 and 15.2 Hz, C=C-H), 4.23 (1H, d, J 7.6 Hz, H-1), 4.19 (dd, J 4.5 and 10.1 Hz, H-a), 4.11 [1H, t, J 7.8 Hz, (HO)CH-C=C], 4.00–3.97 (1H, m, CHO CH-N), 3.86 (1H, d, J 3.3 Hz, H-4), 3.80 (1H, dd, J 6.8 and 11.4 Hz, H-6), 3.74 (1H, dd, J 5.1 and 11.4 Hz, H-6'), 3.60 (1H, dd, J 3.2 and 10.1 Hz, H-a'), 3.56 (1H, dd, J 7.6 and 9.6 Hz, H-2), 3.53–3.48 (2H, m, H-3 and H-5), 2.18 (2H, t, J 7.0 Hz, COCH₂), 2.03 (1H, dt, J 6.8 and 7.2 Hz, C=C-CH₂), 1.28 (52H, m, CH₂), 0.89 (6H, t, J 6.9 Hz, CH₃).

7. Lactosyl ceramide. R_f = 0.65 (silica, CHCl₃/methanol/water, 60:25:4, by vol.); [α]_D²⁵ +0.4° (c 0.4 in Me₂SO); ¹H n.m.r. δ (p.p.m.) (500 MHz) ([²H₆]Me₂SO/²H₂O, 49:1, v/v) 7.46 (1H, d, J 9.1 Hz, N-H), 5.52 (1H, dt, J 7.5 and 15.1 Hz, C=C-H), 5.34 (1H, dd, J 7.0 and 15.1 Hz, C=C-H), 4.19 (1H, d, J 7.0

Hz, H-1'), 4.15 (1H, d, J 7.7 Hz, H-1), 3.96 (m, 1H, CHO), 3.86 (1H, t, J 7.5 Hz, CH-O), 3.77–3.71 (2H, m, H-3', CH-N), 3.60–3.28 (11H, m, CH-O), 3.31 (s, ²H₂O), 3.03 (1H, dd, J 7.9 and 8.0 Hz, H-2), 2.01 [2H, t, J 7.24 Hz, CH₂-C(O)], 1.92 (2H, m, t, J 6.8 Hz C=C-CH₂), 1.42 [2H, m, CH₂-CH₂C(O)], 1.38–1.07 (50H, m, CH₂), 0.84 (6H, t, J 6.7 Hz, 2×CH₃).

The purity of each synthetic sphingolipid was > 98% as determined by n.m.r. Each compound was suspended in Me₂SO/distilled water (50:50, v/v) and stored at –4 °C. They were diluted in PBS just before their incubation with cells.

Neutrophil aggregation

The aggregation response of human neutrophils was studied by monitoring changes in light transmittance utilizing a four channel aggregation profiler (model PAP-4, Biodata Co., Hatboro, PA, U.S.A.). The instrument was used to simultaneously monitor changes in light transmittance with four samples [three exposed to either agonists or synthetic sphingolipids and a fourth group of cells exposed to vehicles (ethanol and/or Me₂SO) alone]. Neutrophils isolated as described above were adjusted to 5 × 10⁶ cells/ml in PBS, pH 7.45, suspended in siliconized cuvettes (BioData Co.) and warmed at 37 °C. Cells were incubated with cytochalasin B (5 µg/ml, final) for 3 min before exposure to agonist (PMA, A23187 or fMet-LeuPhe), as described in [21], or to synthetic sphingolipids (see the Results section for incubation times). Magnetic stirring (approx. 600 rev./min) was employed in each cuvette.

Measurement of superoxide generation

Superoxide anion generation was measured by the reduction of ferricytochrome *c* as in [3,7]. All incubations were performed in duplicate. Aliquots of neutrophils (5 × 10⁶ cells/ml) were placed in plastic tubes (10 mm × 75 mm) with buffer and reagents in a final volume of 1.0 ml. The reaction mixtures containing ferricytochrome *c* (700 µg/ml) were warmed for 5 min at 37 °C followed by addition of the synthetic sphingolipids (at the concentrations indicated), and the incubations were continued for an additional 15 min at 37 °C. Control incubations contained superoxide dismutase (1.0 µg) plus vehicle in addition to ferricytochrome *c*. Agonists (e.g. PMA, 0.1 µM) were added and the incubations were continued for an additional 15 min. Reduction of cytochrome *c* was measured in supernatants obtained by centrifugation (183 g, 5 min, 4 °C) as in [7] using a Cary model 118 spectrophotometer. Superoxide generation is expressed in nmol of cytochrome *c* reduced/15 min per 5 × 10⁶ cells and was calculated from the molar absorption coefficient of reduced cytochrome *c* at 551 nm. Continuous recording of ferricytochrome *c* reduction was monitored as described in [3].

Generation of LTB₄ and related lipoxigenase products

LTB₄, its ω -oxidation product (20-COOH-LTB₄), and the two major non-enzymic hydrolysis products of LTB₄ (6-*trans*-LTB₄ and 12-*epi*-6-*trans*-LTB₄) were extracted from incubations with neutrophils by previously described techniques [22,23]. Briefly, ethanol (1 vol.) was added to incubations, which were placed at 4 °C for 30 min followed by centrifugation. The supernatants were removed, the resulting pellets were resuspended in methanol (1 vol.) and the procedure was repeated twice. The

resulting ethanol- and methanol-containing fractions were pooled and taken to dryness by rotary evaporation.

Materials were resuspended in methanol/water (1:45, v/v) by vortex-mixing in a round-bottomed flask (for 1–2 min), and were then transferred into a glass syringe followed by rapid acidification to pH 3.5. Next, samples from each incubation were rapidly loaded on to cartridges containing ODS silica (C₁₈ Sep-Paks; Waters Associates) and washed with 10 ml of water to give pH 6–7. The materials were eluted with hexane and methyl formate [22]. Materials eluting in the methyl formate fractions were concentrated under argon and injected into reverse-phase-h.p.l.c. system (Waters) equipped with an Altex Ultrasphere-ODS (4.6 mm × 25 cm) column. The column was eluted with methanol/water/acetic acid (70:30:0.01, by vol.) as mobile phase with a flow rate of 1.0 ml/min at approx. 11 730 kPa.

The u.v. detector (model 481; Waters) was set at 270 nm to monitor conjugated triene-containing products. The identity of each of the products was confirmed by coelution with synthetic standards. Retention times in this system were 6.48 ± 0.20 min for 20-COOH-LTB₄, 20.08 ± 0.67 min for 6-*trans*-LTB₄, 22.35 ± 0.70 min for 12-*epi*-6-*trans*-LTB₄, and 25.54 ± 1.21 min for LTB₄. Quantification of these products was achieved by measuring the peak area after h.p.l.c. of known amounts of authentic standards. The percentage recovery of prostaglandin B₂ in these experiments was $77.3 \pm 13.7\%$ (mean $\pm$ s.d. of $n = 3$ experiments with five determinations each).

RESULTS

Actions of sphingolipids on neutrophil aggregation

The seven sphingolipids prepared by total organic synthesis were tested for their ability to either inhibit or stimulate the aggregation of human neutrophils. The compounds studied were sphingosine, psychosine (galactosyl lysosphingolipid), lactosyl lysosphingolipid, Gb₃ lysosphingolipid, galactosyl cerebroside, lactosyl ceramide and Gb₃ ceramide, the structures of which are

depicted in Fig. 1. These compounds were selected because (1) they are naturally occurring products [24], and (2) the series taken together offers a well-defined structure–activity relationship between the products. For example, lactosyl lysosphingolipid and psychosine each share the basic sphingosine structure with the addition of either a lactosyl or galactosyl group to the compound (Fig. 1).

In response to various stimuli, human neutrophils rapidly adhere to one another *in vitro* [21]. In the present study, when exposed to either fMetLeuPhe, ionophore A23187 or phorbol ester, neutrophil aggregation was evident with each stimulus in both a time- and dose-dependent fashion. The aggregation response was monitored utilizing a multi-channel aggregation profiler, which permitted simultaneous recording of the changes in light transmittance. Either stimuli or test compounds were added to three of four separate suspensions of neutrophils. The fourth suspension of cells was exposed in parallel to vehicle. This protocol enabled direct temporal comparisons between cell suspensions incubated in the presence and absence of various agents. A representative series of profiles obtained with cells exposed to increasing concentrations of fMetLeuPhe [(5×10^{-8}) – (5×10^{-6}) M] is shown in Fig. 2(a). In the absence of added stimuli, cell suspensions maintained for up to approx. 20 min at 37 °C did display changes in light transmittance. Dose responses were also obtained with both A23187 and PMA under identical conditions (results not shown, $n = 4$ for each stimulus). The results obtained were similar to those previously reported for A23187, fMetLeuPhe and PMA [21].

When added to neutrophils in suspension at concentrations reported to inhibit O₂^{•−} generation by human neutrophils (i.e. 25 μ M) [15], both lactosyl lysosphingolipid and psychosine, but not sphingosine, provoked rapid changes in light transmittance (Fig. 2b). To investigate a possible basis for this observation, the ability of neutrophils in suspension to exclude Trypan Blue was examined by light microscopy after exposure to each of the seven sphingolipids. The results of these

Fig. 2. Time course of human neutrophil aggregation: evaluation of synthetic sphingolipids

A four channel aggregation profiler (model PAP-4; BioData Co.) was used to study neutrophil aggregation in response to various agonists. This instrument was able to simultaneously monitor changes in light transmittance in four sample cuvettes (three containing test agents and a fourth with cells alone). Neutrophils (5×10^6 cells/ml) were suspended in PBS, pH 7.45, at 37 °C, in siliconized cuvettes. Cytochalasin B (5 μ g/ml) was added approx. 3 min before addition of either stimuli or synthetic sphingolipids. (a) Representative time course and dose–response obtained with neutrophils exposed to fMetLeuPhe (0.05–5.0 μ M). Arrows indicate points of addition. The lower tracing shows the simultaneous monitoring of cells incubated in the absence of fMetLeuPhe. The results are representative of four separate experiments. (b) Representative experiment in which neutrophils were exposed to lactosyl lysosphingolipid (25 μ M), psychosine (25 μ M) or sphingosine (25 μ M) (see the Results section).

Table 1. Neutrophil viability on exposure to various amounts of synthetic sphingolipids

Neutrophils (5×10^6 cells) were suspended in 1 ml of PBS and incubated in the presence or absence of synthetic sphingolipids at the indicated concentrations for 15 min at 37 °C. After incubation, aliquots of cells were removed and assessed for their ability to exclude Trypan Blue by light microscopy. Neutrophil viability is expressed as the percentage of cells excluding Trypan Blue. Results are the means ($\pm$ S.D.) of three separate experiments with cells obtained from different donors ($n = 3$). The levels of significance, P , were determined by a one-tailed paired t test: * $P < 0.05$ when compared with incubations exposed to vehicle alone.

Compound added	Concn. ...	Cell viability (%)		
		50 μ M	10 μ M	1.0 μ M
Vehicle		98.2 $\pm$ 0.7	98.5 $\pm$ 0.3	97.8 $\pm$ 0.4
Sphingosine		97.8 $\pm$ 0.9	97.9 $\pm$ 1.4	97.1 $\pm$ 2.4
Psychosine		0.5 $\pm$ 0.9*	65.9 $\pm$ 16.8*	93.8 $\pm$ 2.1*
Lactosyl lysosphingolipid		3.8 $\pm$ 6.6*	88.3 $\pm$ 3.2*	97.0 $\pm$ 1.5
Gb ₃ lysosphingolipid		87.7 $\pm$ 3.7*	96.3 $\pm$ 2.6	95.7 $\pm$ 2.7
Galactosyl cerebroside		94.6 $\pm$ 3.1	98.2 $\pm$ 1.0	94.6 $\pm$ 3.1
Lactosyl ceramide		97.0 $\pm$ 1.6	95.9 $\pm$ 1.8	95.1 $\pm$ 3.0
Globotriaosyl ceramide (Gb ₃)		95.9 $\pm$ 1.4*	97.7 $\pm$ 0.6	97.1 $\pm$ 1.1

experiments are given in Table 1. When exposed to reported inhibitory concentrations ($> 10 \mu$ M) of these compounds [15–18] for 15 min at 37 °C, neutrophils were unable to exclude Trypan Blue in the presence of psychosine, lactosyl lysosphingolipid or Gb₃ lysosphingolipid (10–50 μ M). These findings suggest that the addition of a galactosyl or lactosyl group to sphingosine renders the compounds cytotoxic at these concentrations.

They are also consistent with the findings of Badwey *et al.* [25] and Pittet *et al.* [18] that both sphinganine and sphingosine provoke the release of cytoplasmic markers. Therefore the following experiments were conducted with final concentrations of synthetic sphingolipids within a concentration range (0.01–1.0 μ M) where $> 95\%$ of the cells exposed to these compounds excluded Trypan Blue.

Fig. 3. Aggregation response of human neutrophils: actions of synthetic sphingolipids at non-cytotoxic levels

Neutrophil aggregation was monitored as described in the legend to Fig. 2 and the Materials and methods section. Cells were incubated with various concentrations of synthetic sphingolipids (▨, 1 μ M; ■, 0.1 μ M; □, 0.01 μ M) for 15 min at 37 °C in PBS, pH 7.45. Next, either fMetLeuPhe (0.5 μ M) (a) or PMA (0.1 μ M) (b) was added to the cells in suspension and changes in light transmittance were monitored for 15 min. In each case, results obtained with cells exposed to synthetic sphingolipids were compared with those obtained simultaneously with cells incubated in their absence followed by addition of either fMetLeuPhe, PMA or A23187. The results are expressed as percentage change in agonist-induced neutrophil aggregation (maximal 100%) after exposure to synthetic sphingolipids. Results represent means $\pm$ S.E.M. of three separate experiments with duplicate determinations within each. Only lactosylsphingolipid gave a significant result with fMetLeuPhe ($P < 0.05$), whereas sphingosine and Gb₃ ceramide gave a significant result with PMA ($P < 0.05$).

Next, neutrophils were exposed to one of three concentrations (1.0, 0.1 or 0.01 μM) of each of the synthetic sphingolipids for 15 min at 37 °C followed by addition of fMetLeuPhe (0.5 μM), PMA (0.1 μM) or the ionophore A23187 (5 μM) (Fig. 3). After addition of fMetLeuPhe, none of sphingosine, galactosyl cerebroside, psychosine, lactosyl ceramide or Gb₃ ceramide showed statistically significant dose-dependent inhibition of neutrophil aggregation. Only lactosylsphingolipid significantly inhibited fMetLeuPhe-induced aggregation at 1 μM (Fig. 3a). In addition, none of the synthetic sphingolipids inhibited A23187-induced aggregation (results not shown).

In the case of PMA-induced aggregation of human neutrophils, only two of the synthetic sphingolipids significantly inhibited aggregation at 1 μM , namely sphingosine and Gb₃ ceramide (Fig. 3b). At lower concentrations, these inhibitory actions were not evident, nor was it possible to assign a clear structure-activity relationship to the inhibition observed at 1 μM for the synthetic sphingolipids studied.

Superoxide anion generation with synthetic sphingolipids

None of the synthetic sphingolipids at 1.0 μM stimulated detectable levels of superoxide anions. In addition, the synthetic sphingolipids did not inhibit PMA-induced generation of superoxide anions. Continuous monitoring of superoxide anion generation was carried out with neutrophils exposed either to synthetic sphingosine or to psychosine. Again, neither of the compounds (10 μM concn.) stimulated or inhibited the generation of superoxide anions (results not shown).

Generation of LTB₄ and related products

Upon its release from membrane sources, unesterified arachidonic acid is converted by human neutrophils to LTB₄, which possesses potent biological actions (reviewed in [5]). The intermediate of the pathway, LTA₄, can also give rise to two non-enzymic hydrolysis products (6-*trans*-LTB₄ and 12-*epi*-6-*trans*-LTB₄) which are less potent than LTB₄ [26,27]. Once generated, LTB₄ is rapidly transformed via sequential ω -oxidation to 20-OH-LTB₄ and 20-COOH-LTB₄ [28]. Two of the synthetic sphingolipids, sphingosine and psychosine, were examined for their impact on LTB₄ generation by human neutrophils. Neither compound altered the levels of either LTB₄ or 20-COOH-LTB₄ generated by A23187-stimulated cells (results not shown). In addition, neither sphingosine (1 μM) nor psychosine (1 μM) affected the generated amounts of the 'all-*trans*'-LTB₄ isomers, suggesting that LTA₄ formation was unaltered in the presence of sphingolipids ($n = 3$). Together, these findings indicate that, under the present conditions, neither sphingosine nor psychosine block the formation of LTB₄ or related compounds generated by human neutrophils.

DISCUSSION

Previous studies have shown that, when added to human neutrophils, sphingosine selectively inhibits the functional responses of these cells, including the generation of superoxide anions and the formation of both platelet-activating factor and LTB₄ [15–18]. Whereas sphinganine does not inhibit phagocytosis, evidence was obtained indicating that sphingosine blocks the release of contents from specific but not azurophilic granules

[15,16]. One site of action of sphingolipids has been shown to be protein kinase C [13], and it recently has been proposed that breakdown products derived from sphingolipids may serve important roles in signal transduction [14]. Since sphingolipids are widely held to play primarily structural roles in membranes, some even serving as antigenic structures [24], the putative involvement of products derived from sphingolipids serving as messengers is particularly interesting. Therefore the present studies were performed to evaluate the actions of several sphingolipids on functional responses of human neutrophils in an effort to obtain evidence for a structure-function relationship for various sphingolipids. To this end, seven sphingolipids were prepared by total organic synthesis, and their structures are shown in Fig. 1. They were sphingosine, psychosine, lactosyl lyso-sphingolipid, Gb₃ lysosphingolipid, galactosyl cerebroside, lactosyl ceramide and Gb₃ ceramide. Each of these compounds is naturally occurring and each has been identified in a wide range of cell types ([24] and refs. cited therein). The identity and stereochemistry of each of the compounds studied here were confirmed by i.r. and n.m.r. (see the Materials and methods section) after total synthesis from intermediates described in [20].

When neutrophils in suspension were exposed to the synthetic sphingolipids at concentrations reported to be inhibitory with sphinganine (i.e. > 25 μM ; cf. [15–18]), rapid changes in light transmittance (T) were observed with each of the lyso compounds except for sphingosine (Fig. 2b). The results of experiments with Trypan Blue exclusion revealed that some of the sphingolipids were highly cytotoxic at concentrations greater than 1.0 μM (Table 1). In particular, psychosine and lactosyl lyso-sphingolipid were the most active of this series in increasing the permeability of neutrophils to Trypan Blue. This observation suggests that the changes in ΔT evoked by these compounds at higher concentrations are the result of cytotoxicity. In fact, studies by Pittet *et al.* [18] have shown that sphinganine, when added to human neutrophils at concentrations similar to those showing inhibitory actions, induces cell permeabilization as assessed by release of quin2 and lactate dehydrogenase as well as by changes in membrane potential. Merrill [29] has also observed that, at concentrations of greater than 1 μM , sphinganine is highly toxic towards cultured Chinese-hamster ovary cells. Although some of the compounds increased the permeability of human neutrophils, synthetic sphingosine did not provoke changes even at concentrations as high as 50 μM (Table 1). Therefore, in order to probe the possible structural basis for differences between these compounds, it was necessary to study them collectively within concentration ranges where > 95 % of the cells exposed to each of the compounds excluded Trypan Blue.

None of the synthetic sphingolipids inhibited A23187-induced aggregation, whereas some showed effects with both fMetLeuPhe- and PMA-induced aggregation (Fig. 3). Although lactosylsphingolipid significantly inhibited aggregation with fMetLeuPhe as stimulus, a dose-response relationship at the concentrations employed was not evident (Fig. 3a). The molecular basis for this level of inhibition remains to be established. Since activation of the neutrophil by fMetLeuPhe involves protein kinase C [10], it may be possible that lactosylsphingolipid can affect this enzyme system. However,

with the present incubation conditions, the basic structure, namely synthetically prepared sphingosine, did not inhibit fMetLeuPhe-induced aggregation (Fig. 3a).

PMA-induced aggregation can involve the direct activation of protein kinase C [12]. In the present study, previous exposure to only two of the synthetic sphingolipids at $1\ \mu\text{M}$ inhibited aggregation (Fig. 3b). These effects, however, were not observed at $< 1\ \mu\text{M}$, nor was it possible to establish a structure-activity relationship among the compounds studied. Taken together, these findings suggest that the inhibitory effects of the synthetic sphingolipids observed with PMA-induced aggregation arise via non-specific interactions. For example, it is conceivable that, when added to cells in suspension, each of the sphingolipids (at the concentrations employed here) may affect the fluidity of the plasma membrane. Such changes in membrane dynamics, which may be equally evoked by the different sphingolipid structures (Fig. 1), may alter the responsiveness of human neutrophils to certain stimuli.

Previous reports have shown that commercial preparations of both sphinganine and sphingosine can inhibit $\text{O}_2^{\cdot-}$ generation [15,18,25]. In contrast with these findings, the present results indicate that the synthetic sphingolipids neither stimulate nor inhibit PMA-induced $\text{O}_2^{\cdot-}$ generation at concentrations which are non-cytotoxic. Even in the presence of toxic levels of psychosine ($10\ \mu\text{M}$), striking inhibition was not observed. Similar experiments with LTB_4 generation did not reveal evidence for an impact of either sphingosine or psychosine on this system. The levels of LTB_4 , its ω -oxidation product and two non-enzymic hydrolysis products of LTA_4 were unchanged following exposure to these compounds.

In summary, the results of the present study indicate that synthetic sphingolipids of unambiguous structure do not inhibit superoxide anion generation, LTB_4 production or aggregation induced by either fMetLeuPhe or A23187 in intact human neutrophils. Two of the synthetic sphingolipids did, at a concentration of $1\ \mu\text{M}$, block PMA-induced aggregation; however, a structure-function relationship was not evident among the compounds in the absence of neutrophil lysis. Although structurally intriguing second messengers may be derived from membrane sphingolipids as proposed recently [14], the present results support those of Pittet *et al.* [18]; namely, that sphingolipids cannot be used as selective inhibitors when added exogenously to intact human neutrophils.

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