Stimulation of erythroblast maturation in vitro by sphingolipids

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Abstract A lipid factor previously isolated from leukocytes and found to stimulate basophilic erythroblast formation in an in vitro system of incubated rabbit bone marrow cells has been analyzed by thin-layer chromatography, gas–liquid chromatography, and gas–liquid chromatography–mass spectrometry. The biologically active components are sphingosine ceramides of tetracosanoic and dehydrotetracosanoic acids. Tests of a series of related ceramides show a high degree of structural specificity for the C_{24}-*N*-acyl compounds with significant but markedly lower activity of the C_{22} analog. Commercially available sphingomyelin shows activity comparable to that of the tetracosanoic acid ceramide. Sphingosine and tetracosanic acid supplied in equimolar amounts have negligible activity. The results, in the context of other findings, suggest a possible supportive role of plasma ceramides and sphingomyelins in red cell maturation.

Supplementary key words erythrocyte maturation in vitro · basophilic erythroblasts · tetracosanoic (lignoceric) acid ceramides · sphingomyelin · erythropoiesis

Erythropoietic activity has been demonstrated for a number of lipid-soluble substances, notably for several steroids (1–4) and for at least one nonsteroidal lipid, i.e., batyl alcohol (glycerol 1-octadecyl ether) (5, 6). The physiological significance of these effects remains uncertain, however. Borsook, Jiggins, and Wilson (7) recently reported that a lipid-soluble material extractable from rabbit leukocytes had significant erythropoietic activity in an in vitro assay system (8), but the chemical identity of the material responsible for this activity was not known.

In this paper we present the results of further studies of the erythropoietically active lipid from rabbit leukocytes and show that its erythropoietic activity resides in sphingosine ceramides of tetracosanoic acid and tetracosenoic acid. These are major components of the normally occurring ceramides of human plasma (9). Synthetic ceramides of tetracosanoic (lignoceric) acid and *cis*-15-tetracosenoic (nervonic) acid showed activity comparable to that of the material isolated from leukocytes, while the ceramide of *cis*-13-docosenoic acid showed lower but still appreciable activity. Sphingomyelin also was active in the same system. To our knowledge this is the first reported evidence for erythropoietic activity of sphingolipids.

EXPERIMENTAL

Assay for erythropoietic activity

The assays were carried out by a slight modification of the method previously described in detail by Borsook, Jiggins, and Wilson (8). In outline, the method involved the incubation of a suspension (1 ml) of rabbit bone marrow cells in homologous serum in the presence of added erythropoietin and with the addition of the test substance in 95% ethanol (10 μl). Control incubations received the same volume of 95% ethanol. The erythropoietic effect was assessed in terms of the increase in the number of basophilic erythroblasts in the presence of the test substance as compared with that which occurred in the controls. In order to provide an internal standard against which the basophil count could be measured, a fixed number of chicken erythrocytes was added to each flask at the end of the incubation. In order to ensure that this procedure was effective, it was necessary to remove antibodies, which occasionally caused clumping of the chicken erythrocytes and thus interfered with the final cell count. This was accomplished by modifying the original method (8) to include a step in which the serum to be used as the incubation medium (after preheating at 56°C for 30 min, cool-

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry.

[1] Part of this work was carried out during this author's stay as a guest scientist in the laboratory of Dr. Jan Sjövall in 1972.

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ing, and centrifugation) was "absorbed" by exposure to chicken erythrocytes, which were then removed by centrifugation.

Test and control incubations were always carried out in duplicate. For all except the results shown in Table 3, at least two, and in some cases four or six, replications of the tests were made with different marrow cell suspensions.

Chromatographic methods

Column chromatography was performed with silica gel (E. Merck, column chromatography sorbent, 60–200 mesh), which was washed twice with spectrograde methanol, dried in air, and then activated by heating at 120°C for 30 min. The gel was then partially deactivated by addition of 4% of its weight of water before use.

TLC was carried out on 0.25-mm layers of silica gel G on plates 20 × 20 or 5 × 20 cm, activated for 30 min at 110°C before use. Material was visualized on the plates after separation by exposure to iodine vapor. For the localization of the separated bands, when the material was to be recovered for biological testing, two clean glass plates were clamped against the adsorbent surface by means of spring clips to leave a gap of a few millimeters running the length of the plate. This exposed strip was stained with iodine where it intersected with the bands of separated materials. The stained portion was discarded when the bands were scraped and eluted with 10% methanol in methylene chloride.

GLC–MS was carried out by means of an LKB 9000 instrument. Trimethylsilyl ethers were injected onto columns (3 m × 3.5 mm) of 1.5% SE-30 on 80–100 mesh Chromosorb WHP. Column and separator temperatures were 380 and 290°C, respectively. The ion source temperature was 310°C and electron energy was 22.5 eV. Retention times were measured relative to that of trimyristin (about 37 min) and were: *N*-palmitoyl sphingosine 0.24 and *N*-tetracosanoyl sphingosine 1.01.[5] Mass spectra were recorded repetitively after an initial period of 2 min with an initial interspectra interval of 9.9 sec, increasing at the rate of 0.6 sec/min. Data were recorded and stored by means of an off-line computer system as described by Reimendal and Sjövall (10).

Solvents were of spectrograde quality. Ceramides of known structure were obtained through the courtesy of Dr. K. A. Karlsson and Dr. B. Samuelsson. *N*-Tetracosanoyl sphingosine was synthesized from tetracosanoic acid (Fluka A.G.) and sphingosine by the method of Hammarström (11). Sphingosine (sulfate salt) mixed ceramides and sphingomyelin were purchased from Calbiochem. Reference steroids were synthesized in our laboratories by previously published methods.

[5] Relative retention times of sphingosine ceramides of other fatty acids were: 17:0, 0.29; 18:0, 0.34; 19:0, 0.42; 20:0, 0.49; 21:0, 0.59; 22:0, 0.71; 23:0, 0.85; 24:1, 0.97.

Isolation and analysis of erythropoietically active lipid

The preparation of an ethanol extract of rabbit leukocytes and its in vitro assay for erythropoietic activity have been described elsewhere (7). Preliminary experiments carried out on a small scale indicated that the biologically active material migrated on TLC in the solvent system chloroform–methanol 19:1 with an R_F of 0.4. This TLC system was therefore used to monitor the column effluent in larger-scale preparations such as the following.

The ethanol-extracted material from leukocytes derived from 6 l of rabbit blood was extracted with hexane (7 ml) in several small quantities, and the total hexane extract was applied to 60 g of silica gel packed in hexane in a column 2 × 50 cm. Bioassay of the residue remaining after hexane extraction showed it to be without measurable erythropoietic activity.

The column was eluted with 25-ml fractions consisting of solvent mixtures that comprised a discontinuous gradient with the concentration of the more polar component of each fraction approximately double that of the preceding one. Fractions 1–8 were benzene in hexane, 1–100%; fractions 9–20 were ethyl acetate in benzene, 0.06–100%; fractions 21–36 were methanol in ethyl acetate, 0.01–100%; and fractions 37–42 were water in methanol, 0.06–4%.

Material with the mobility on TLC of the biologically active substance was found in fractions 27–34, inclusive (0.32–32% methanol in ethyl acetate). These fractions (which included a tailing portion of the cholesterol peak, found mainly in fractions 21–24) were combined and chromatographed on a preparative thin-layer plate. Visualization of a 5-mm strip with iodine vapor revealed eight bands. Assay of the basophil-stimulating activity of the materials eluted from the individual bands showed that the major part of the biological activity migrated with R_F 0.4. Inasmuch as the principal emphasis of the work was to identify the biologically active material, estimates of recovery of the original activity through the various purification steps were only approximate but suggested that at least 60–80% of the activity was recoverable in this TLC fraction. Closely similar results were obtained in the analysis of a second large batch of rabbit leukocytes using essentially the same procedures.

Analysis of erythropoietically active lipid by GLC–MS and its further fractionation by TLC

Further information concerning the composition of the biologically active fraction came from analysis by GLC–MS. The unknown material was subjected to procedures for conversion of hydroxyl groups to trimethylsilyl ether derivatives and of ketonic groups to methoximes (12). A series of mass spectra obtained from the product of these reactions suggested that the original material consisted of

a complex mixture of 7- and 25-hydroxycholesterols with a variety of ceramides.

This information guided further attempts to purify the biologically active fraction by TLC. Various solvent systems were tried for the separation of authentic 7α-hydroxycholesterol and 25-hydroxycholesterol from ceramides of known structure. On the basis of these tests, the material isolated by TLC with R_F 0.4 was applied to a second TLC plate (5 × 20 cm) and developed three times in the solvent system hexane–ether–acetic acid 50:50:1 with the plate dried in air at room temperature between runs. Iodine staining revealed four bands, one of which remained at the origin while the others ran with R_F values of 0.1, 0.17, and 0.23, corresponding approximately to the R_F values of ceramides, 7α-hydroxycholesterol, and 25-hydroxycholesterol, respectively. These bands and the intervening regions were scraped from the plate and their contents were eluted and tested for biological activity. The single most active fraction (about 50% of the applied activity) was recovered from the band with R_F 0.1. Approximately 12 and 14% of the biological activity was recovered from the "blank" areas of greater and lesser polarity (R_F 0.05 and 0.14, respectively). About 20% remained at the origin and about 6% migrated with R_F 0.17. The less polar areas, including the band with R_F 0.23, showed negligible or small negative activities.

TABLE 1. Percentage composition of ceramides (*N*-acyl sphingosines analyzed as di-*O*-trimethylsilyl ethers) isolated from rabbit leukocytes

GLC Peak No.	Fatty Acid	% (from GLC)[a]	% (from [M − a] ion)[b]
1	16:0	13.5	16.1
2	17:0	0.1	0.6
3	18:0	1.9	2.8
4	19:0	<0.1	0.1
5	20:0	1.4	1.8[c]
6	21:0	0.8	0.3
7 (a)	22:0	17.8	15.1
7 (b)	22:1		1.7
8	23:0	5.5	4.8
9 (a)	24:0	59.0	25.0
9 (b)	24:1		26.7
9 (c)	24:2		5.0

[a] From a GLC analysis where a flame ionization detector was used. This does not distinguish between the saturated and unsaturated fatty acid derivatives (a) and (b) in peak 7 and (a), (b), and (c) in peak 9.

[b] [M − a] ion: the fragment ion remaining after cleavage of the sphingosine chain between C-2 and C-3, resulting in the loss of a trimethylsilyloxy-C_{16}-alkenyl fragment (fragment "a" according to Samuelsson and Samuelsson [13]). This calculation is based on the assumption that the same percentage of total ionization resides in M − a for all molecular species and that all species are ionized to the same extent.

[c] The value for the [M − a] ion in this case is inaccurate due to coincidence with a fragment of equal mass (*m/e* 426) formed by loss of the fatty acyl amido chain (+1H).

Definitive characterization by GLC–MS

The fraction R_F 0.1, suspected to be a mixture of ceramides, was now converted to the trimethylsilyl ether derivative and analyzed by GLC–MS. The data obtained bore a general similarity to those reported by Samuelsson (9) for ceramide mixtures from human blood, and the observed fragmentation patterns were in good agreement with those reported and discussed in detail by Samuelsson and Samuelsson (13) for synthetic ceramides. Molecular ions were not identified, but peaks of M − 15 (loss of methyl from one trimethylsilyl ether grouping) were identified for sphingosine ceramides of saturated fatty acids ranging from palmitic (C_{16}, M − 15 = *m/e* 666) to tetracosanoic (C_{24}, M − 15 = *m/e* 776). In the latter case the analogous ions (*m/e* 774 and *m/e* 772) corresponding to monounsaturated and diunsaturated derivatives were noted. Loss of the fatty acyl amido chain (+1H), by cleavage between N and C-2 of sphingosine, gave the characteristic peak *m/e* 426, corresponding to the bis-trimethylsilyloxy derivative of the sphingosine hydrocarbon chain for each ceramide. Similarly, the characteristic fragment *m/e* 311, formed by cleavage between C-2 and C-3 of sphingosine, was found in all cases. Cleavage at the same site but with retention of the charge by the amido fragment gave a series of peaks characteristic of the various fatty acid moieties. The fragment ion current chromatograms (10) corresponding to these fragments together with a GLC analysis using a flame ionization detector provided the data recorded in **Table 1** for the composition of the total mixture of ceramides. No evidence for the presence of steroids in this material was obtained. Dihydrosphingosine derivatives, if present, could have constituted no more than 3.0% of the material and were not represented in significant amounts in any one fraction.

Tests of synthetic ceramides for erythropoietic activity in vitro

The above findings prompted a series of in vitro tests of erythropoietic activity with synthetic ceramides, including the ceramides of tetracosanoic acid and of *cis*-15-tetracosenoic acid. Sphingomyelin and mixed commercially available ceramides were also tested. The results of these tests are shown in **Table 2.** It can be seen that the sphingosine ceramides of tetracosanoic acid and its *cis*-15-dehydro derivative had significant and approximately equal biological activities. Sphingomyelin showed comparable activity. The log dose-response curves comparing the activities in the same bone marrow cell preparation of the synthetic *N*-tetracosanoyl sphingosine and the naturally occurring ceramide mixture isolated in our experiments (**Fig. 1**) show the latter material to be less active than the synthetic material, as might be expected, because about one-half of the natural mixture comprises biologically inactive components.

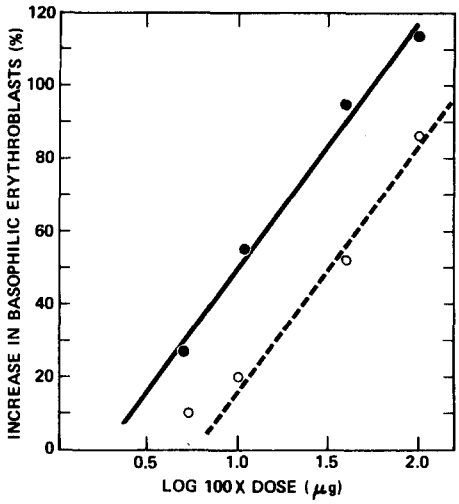

Fig. 1. Dose-dependent response of bone marrow cell suspension to incubation with synthetic *N*-tetracosanoyl sphingosine (●) or ceramide mixture extracted from leukocytes (○). Ordinate shows mean increase in basophilic erythroblasts in test incubations in excess of mean increase in controls, expressed as percentage of increase in controls.

On the basis of the foregoing results, the erythropoietic activity of the C_{24}-acyl sphingosines and of sphingomyelin could have reflected the breakdown of these compounds followed by reutilization of the C_{24}-acyl and/or sphingosine fragments for incorporation into some different compound(s). These possibilities are rendered unlikely by the results of the tests shown in **Table 3,** in which the basophil response to *N*-tetracosanoyl sphingosine is compared with responses to combinations of sphingosine and tetracosanoic acid at three different concentrations and to sphingosine or tetracosanoic acid alone. The acid and base, when present together in amounts (5 μg each) that could have resulted in an approximately tenfold higher concentration of ceramide than in the incubation with the C_{24}-acyl ceramide itself, gave a response that was only about half of that given by the ceramide. Neither acid nor base, when present alone, gave any increase in basophilia.

TABLE 2. Effects of various substances on basophilic erythroblast counts in incubated suspensions of rabbit bone marrow cells[a]

Test Substance	Number of Tests	Response[b]
N-Tetracosanoyl sphingosine	6	109 ± 4.5
Ceramides of leukocytes[c]	2	82 ± 9
Mixed ceramides (commercial)	4	67 ± 5
Sphingomyelin (commercial)	4	79 ± 20
N-cis-15-Tetracosenoyl sphingosine	2	99 ± 17
N-Tetracosanoyl 1,3,4-trihydroxy-sphinganine	2	93 ± 23
N-cis-13-Docosenoyl sphingosine	2	34 ± 12
N-cis-11-Eicosenoyl sphingosine	2	15 ± 3
N-trans-11-Eicosenoyl sphingosine	2	−3 ± 14
N-Octadecanoyl sphingosine	2	−13 ± 8
N-cis-9-Octadecenoyl sphingosine	2	−3.5 ± 3.5
N-Octadecanoyl 1,3,4-trihydroxy-sphinganine	2	18 ± 20
N-2-Hydroxyoctadecanoyl sphingosine	2	10.5 ± 0.5
N-Hexadecanoyl sphingosine	2	8 ± 4.5

[a] Assay conditions as described by Borsook et al. (8) with modifications described in the text. Each test consisted of duplicate incubations for tests and controls. Test substance added in a concentration of 1 μg in 10 μl of 95% ethanol to each 1-ml incubation.

[b] (mean increase of basophils per 100 added chicken erythrocytes − mean increase in controls) × 100/mean increase in controls. Values given ± SEM when n > 2 or ± range when n = 2.

[c] Purified by TLC; composition given in Table 1.

DISCUSSION

When the composition of the ceramide mixture isolated in these studies is compared with that described for whole plasma by Samuelsson (9), the most striking similarity is that the ceramides of tetracosanoic acid and of its monounsaturated derivative are the most abundant species. Samuelsson (9) found a combined total of 3.22 ± 0.92 μg of 24:0 and 24:1 ceramides of sphingosine, 1.35 ± 0.48 μg of 22:0 and 22:1 ceramides, and about 1.0 μg of ceramides of other fatty acids per milliliter of healthy human plasma. It was therefore important to determine whether the amounts of ceramide added to our test incubations were significant in comparison with the amounts that, on the basis of Samuelsson's results, might be present as endogenous constituents of the serum that was used as the incubation medium. Serum (50 ml) that had been treated as for the standard test incubations was therefore extracted with ethanol, and the extract was worked up as described to yield a TLC fraction corresponding to the ceramides. This fraction, examined by GLC as in the case of the leukocye extract, was found to contain very small amounts of material with the gas–liquid chromatographic retention time of *N*-tetracosanoyl and *N-cis*-15-tetracosenoyl sphingosines (≲0.04 μg/ml) together with similar trace amounts of other ceramides. Thus, the amounts of ceram-

TABLE 3. Effects of tetracosanoic acid and sphingosine, separately and in combination, on basophilic erythroblast counts in incubated rabbit bone marrow cells

Test Substances[a]	Response[b]
Tetracosanoyl ceramide (1 μg)	90
Sphingosine (0.5 μg), tetracosanoic acid (0.5 μg)	6
Sphingosine (1.0 μg), tetracosanoic acid (1.0 μg)	19
Sphingosine (5.0 μg), tetracosanoic acid (5.0 μg)	52
Sphingosine (5.0 μg)	10
Tetracosanoic acid (5.0 μg)	−2

[a] Added in amounts shown, otherwise conditions as indicated in Table 2.

[b] Increase in basophils (per 100 added chicken erythrocytes) in excess of increase in controls, expressed as percentage of increase in controls. Values are averages of duplicate incubations using aliquots of a single marrow cell preparation.

ides added in the test system were about 25-fold greater than the endogenous amounts.

Evidently, the various procedures to which the plasma is subjected in the preparation of serum as used in the test system result in essentially complete loss of ceramides. The mechanism(s) responsible for this loss is unknown and may merit further investigation.

Although relatively few ceramide analogs have so far been compared, the structural specificity of the ceramides of the C_{24}-carboxylic acids as stimulators of basophil formation is striking. Even the C_{22}-acyl analog appears to be less active, and essentially no activity is shown by shorter-chain *N*-acyl sphingosines. The substitution of 1,3,4-trihydroxysphinganine for sphingosine in the C_{24}-acyl ceramides appears not to affect activity significantly. It remains to be determined whether 2-hydroxylation of the C_{24}-acyl chain affects the activity of the ceramide. Further experiments with other closely related structural analogs are suggested by the present results.

The physiological significance of these findings can, of course, be determined only by further experiments, and work directed to this end is in progress. A reasonable (though speculative) interpretation of our results is that the maturing red cell precursor is dependent in the in vitro test system upon an external supply of tetracosanoyl sphingosine or *N-cis*-15-tetracosenoyl sphingosine for the synthesis of cell membrane components. The utilization of the intact ceramide moiety in vitro is strongly suggested by the results in Table 3, and one possible metabolic role for ceramides is suggested by the similar biological activity of sphingomyelin, a product of ceramide metabolism (14, 15). Conversely, sphingomyelin may be active because it undergoes partial degradation to ceramide which is then utilized in the synthesis of some different material, e.g., glycosphingolipids (16, 17).

We wish to emphasize that we do not infer from these results that leukocytes, from which the active lipid has been isolated, necessarily play a role in erythropoiesis as a source of ceramides or of sphingomyelin. Rather, we are intrigued by the fact that, in addition to their abundance in the free form in plasma, the C_{24}-*N*-acyl sphingosines are the most abundant ceramide moieties of plasma sphingomyelins (Ref. 18 and other references therein). This fact is consistent with the possibility that both the C_{24} ceramides and sphingomyelins of plasma play closely related roles in supporting erythrocyte maturation in vivo. Several possible metabolic roles for the plasma ceramides, any of which may be reflected in our results, are discussed by Samuelsson (9). Moreover, there remains the possibility that other classes of plasma lipids play some role in erythropoiesis.

It is known that among the many enzymes that are lost in the course of erythrocyte maturation are those that are involved in lipid biosynthesis (19). One or more enzymes involved in sphingolipid synthesis may be lost at a particularly early stage. On the basis of the present results, a C_{24}-acyl CoA:sphingosine acyltransferase is a reasonable candidate for deletion,[6] though in the absence of more specific data this remains a matter for speculation.

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[6] The results shown in Table 3, reflecting some stimulatory effect of sphingosine and tetracosanoic acid present together in high concentration, suggest that deletion is incomplete in the cell preparations used in these studies.

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