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Igal Gery, ... , Roscoe O. Brady, John A. Barranger

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Research Article

Although the enzymatic lesion in Gaucher's disease is well established, little is known concerning the pathogenic mechanisms involved in the clinical manifestations of the disease. In order to obtain insight into this unexplored aspect of Gaucher's disease, we examined the effects of glucocerebroside (GL₁) at the cellular level in monolayers of cultured murine macrophages. The addition of GL₁ to these cultures stimulated the macrophages to release increased amounts of lymphocyte-activating factor (LAF) and lysosomal enzymes into the medium. These responses were proportional to the amount of GL₁ added to the culture. At higher levels of GL₁ (≥ 20 $\mu\text{g/ml}$), lactic dehydrogenase, a cytoplasmic enzyme was also released indicating cellular damage at these doses. Intracellular LAF also increased in macrophages incubated with the high doses of GL₁, demonstrating an increase in total LAF production by these cells. Lipopolysaccharide acted synergistically with GL₁ and stimulated the release of exceedingly high levels of LAF which had a molecular weight profile similar to that of LAF released by exposure to lipopolysaccharide alone. Unlike GL₁, galactocerebroside, sphingomyelin, and ceramidetrihexoside, exerted little or no effect on the release of macrophage products. The effect of GL₁ was selective for macrophages since addition of this material to mouse lens epithelial cells had no detectable cytotoxic effect and it was only slightly toxic to lymphocytes or P815 cells in concentrations [...]

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IGAL GERY, J. SAMUEL ZIGLER, JR., ROSCOE O. BRADY, and

JOHN A. BARRANGER, *Laboratory of Vision Research, National Eye Institute and Development and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205*

ABSTRACT Although the enzymatic lesion in Gaucher's disease is well established, little is known concerning the pathogenic mechanisms involved in the clinical manifestations of the disease. In order to obtain insight into this unexplored aspect of Gaucher's disease, we examined the effects of glucocerebroside (GL₁) at the cellular level in monolayers of cultured murine macrophages. The addition of GL₁ to these cultures stimulated the macrophages to release increased amounts of lymphocyte-activating factor (LAF) and lysosomal enzymes into the medium. These responses were proportional to the amount of GL₁ added to the culture. At higher levels of GL₁ (≥ 20 $\mu\text{g/ml}$), lactic dehydrogenase, a cytoplasmic enzyme was also released indicating cellular damage at these doses. Intracellular LAF also increased in macrophages incubated with the high doses of GL₁, demonstrating an increase in total LAF production by these cells. Lipopolysaccharide acted synergistically with GL₁ and stimulated the release of exceedingly high levels of LAF which had a molecular weight profile similar to that of LAF released by exposure to lipopolysaccharide alone. Unlike GL₁, galactocerebroside, sphingomyelin, and ceramidetrihexoside, exerted little or no effect on the release of macrophage products. The effect of GL₁ was selective for macrophages since addition of this material to mouse lens epithelial cells had no detectable cytotoxic effect and it was only slightly toxic to lymphocytes or P815 cells in concentrations at which macrophages were clearly affected. A direct relationship was observed between the cytotoxicity of the sphingolipids and their accumulation in various cells. Macrophages accumu-

lated large amounts of GL₁ but not sphingomyelin, whereas the other cells examined in this investigation did not accumulate either of these lipids. Human monocytes, like murine macrophages, also release increased amounts of LAF when incubated with GL₁. The effect of GL₁ was dose-responsive and synergy was found with lipopolysaccharide. The relevance of these findings to the pathogenesis of Gaucher's disease is considered.

INTRODUCTION

Gaucher's disease, an autosomal recessive lipid storage disorder, is characterized by the deficiency of glucocerebrosidase and the accumulation of its substrate, glucocerebroside (GL₁)¹ in phagocytic cells ("Gaucher cells") throughout the reticuloendothelial system. The disease is multifaceted and its pathogenesis is not completely understood (1, 2).

Very little is known about the effects of GL₁ on macrophages (M ϕ) in culture and to the best of our knowledge, no study on this topic has been reported previously. The present study was aimed at examining the effects of GL₁ on M ϕ cultures. The parameters tested included the release of lysosomal and cytoplasmic enzymes, as well as of a monokine, lymphocyte-activating factor (LAF (3-6), also designated Interleukin 1 (7)). The specificity of the responses of M ϕ to GL₁ was established by examining the effects of other clinically relevant sphingolipids, namely,

¹Abbreviations used in this paper: CTH, ceramide trihexoside; FCS, fetal calf serum; Gal-cer, galactocerebroside; GL₁, glucocerebroside; LAF, lymphocyte-activating factor; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; M ϕ , macrophages; NIH, National Institutes of Health; PBS, Dulbecco's phosphate-buffered saline; ROS, rod outer segments; SPM, sphingomyelin.

galactocerebroside (Gal-cer), sphingomyelin (SPM) and ceramidetrihexoside (CTH) which accumulate in Krabbe disease, Niemann-Pick disease, and Fabry disease, respectively.

METHODS

Reagents. GL₁, SPM, and CTH, were purchased from Supelco, Inc. Bellefonte, Pa. Gal-cer, purified from sheep brain, was a gift from Dr. A. Gal (National Institutes of Health, Bethesda, Md.). The sphingolipids were suspended in water by sonication and sterilized by irradiation with 10,000 rad. GL₁ was examined for purity by thin-layer chromatography; the preparation used here gave a single spot on silica gel G plates in a solvent system of chloroform:methanol:water (60:35:4). In addition, high-performance liquid chromatography of the perbenzoylated glycolipid by the method of Ullman and McCluer (8) gave a single peak on a Ultrasphere-Si column (Altex Scientific, Inc., Berkeley, Calif.). Soybean intralipid (SB-IL) was supplied by Cutter Medical (Berkeley, Calif.) in a concentration of 10% and was further diluted in the tested medium. Quartz silica particles, <5 μ m, a gift from Dr. P. Davies (Merck Institute for Therapeutic Research, Rahway, N. J.), were suspended in Dulbecco's phosphate-buffered saline (PBS, NIH Media Unit); lipopolysaccharide W from *Salmonella typhimurium* (LPS, Difco Laboratories, Detroit, Mich.), phytohemagglutinin P (Difco Laboratories), concanavalin A and lysophosphatidyl choline (LPC, or lysolecithin, from egg, Sigma Chemical Co., St. Louis, Mo.) were dissolved in PBS.

Preparations of cell cultures. Cultures were incubated in a humidified atmosphere at 37°C with 5% CO₂ in air. All media contained 100 U penicillin and 100 μ g streptomycin per ml. (a) Peritoneal M ϕ were collected by lavage from Swiss outbred mice (6–8 wk of age, NIH breeding facilities), washed once and resuspended in M-199 medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (FCS, Gibco Laboratories). Cultures were set up with 3–4 \times 10⁶ cells/ml, in two types of dishes, according to the experimental design (see below). After an incubation for 2 h, nonadherent cells were removed by thorough washing and attached M ϕ were used for testing. (b) Human monocytes were isolated from the mononuclear fraction of peripheral leukocytes of normal donors. Mononuclear cells, fractionated on Isolymp gradients (Gallard-Schlesinger Chemidex, Inc., Carle Place, N. Y.) were cultured at 5–7 \times 10⁶ cells/ml in RPMI 1640 medium (Gibco Laboratories), with 10% FCS. Following an incubation for ~1 h, the nonadherent cells were removed by thorough washing and the remaining monocytes were tested as described below. (c) Murine lens epithelial cells were kindly provided by Dr. Paul Russell (National Eye Institute). These cells were originally isolated (9) and used in this study after being periodically subcultured for ~4 yr. (d) Normal human skin fibroblasts were supplied by Human Genetic Mutant Cell Repository (Camden, N. J.) and subcultured 3–5 times before being used in this study. (e) Mouse thymus or spleen lymphocytes were obtained from CBA/N mice (6–9 and 8–12 wk of age, respectively, NIH breeding facilities) by gentle teasing and used after washing once. (f) Cells of the P815 cell line (murine mastocytoma) were kindly provided by Dr. G. M. Shearer (National Cancer Institute) and subcultured in suspension once a week until being used.

Content and release of cell products. Monolayer cell cultures were set up in multiwell plates, 17 \times 24 mm (Linbro

Chemical Company, Hamden, Conn.) and used immediately after removal of the nonadherent cells. Agents to be tested were added in 1 ml of M-199 medium (for M ϕ) or RPMI 1640 medium (for human monocytes), with 5% FCS, and the cultures were further incubated for 20 h. After incubation, the supernates were collected and centrifuged at 900 g for 15 min. Monolayers assayed for intracellular LAF activity were washed three times with Hanks' balanced salt solution (NIH media unit), covered with 1 ml of M-199 or RPMI 1640 medium (for M ϕ or monocytes, respectively) with 5% FCS, and frozen at –20°C. After thawing, the cells were scraped off by rubber policeman, sonicated and filtered (0.45 μ m). Monolayers tested for intracellular enzyme activities were washed three times with PBS, lysed with 1.0 ml of 0.1% Triton X-100 and removed by scraping.

Chromatography on sephadex columns. Supernates from M ϕ cultures were concentrated (\times 8) by Amicon UM-10 membranes (Amicon Corp. Scientific Sys. Div.) and fractionated on G-75 superfine Sephadex columns (2.6 \times 35 cm), equilibrated with RPMI 1640 medium with HEPES (Gibco Laboratories). Elution, with the same medium, was carried out by gravity and the fractions, 3 ml each, were supplemented with FCS (5%) before being filtered (0.45 μ m). Bovine β ₁- and γ -lens crystallins were used as molecular weight markers.

LAF assay. The original method (3) was modified for microcultures as follows. Samples were diluted in RPMI 1640 medium (Gibco Laboratories) with 5% FCS and added in triplicate to flat bottomed microplates (Linbro, 8 \times 6 mm), in aliquots of 0.1 ml. Thymocytes were added in aliquots of 1.5 \times 10⁶ cells in 0.1 ml shortly after being mixed with PHA at a final dilution of the commercial product of 0.5 μ l/ml. After an incubation for 66 h the cultures were pulsed for 6 h with [³H]thymidine (New England Nuclear, Boston, Mass., 2 Ci/mM, 0.5 μ Ci/well) and harvested by a MASH II (Microbiological Associates, Walkersville, Md.). The radioactivity in the collecting filter discs was measured by an LKB 1216 Rackbeta scintillation counter. The levels of LAF activity in the tested M ϕ preparations are expressed in two ways: (a) the mean counts per minute values \pm standard error of the mean of [³H]thymidine uptake in thymocyte cultures stimulated by one dilution of the tested preparation and (b) "LAF units". 1 unit was defined as the LAF activity found in supernate of the control M ϕ culture to which no additions were made; the unit values in tested preparations were calculated by the dilution needed to bring the LAF activity to the 1 unit level. The use of LAF units is introduced here in order to simplify the data presentation and to accurately illustrate the exceptionally high LAF activities in certain preparations obtained in this study; the dose-response curves of LAF activity of these preparations gave a high plateau at a wide range of dilutions and all dilutions (1:10–1:640) were taken into account when LAF units were calculated. Control cultures, incubated with the medium alone, incorporated 150–400 cpm. The sphingolipids tested here had no apparent effect on the thymocyte cultures.

Enzyme assays. β -N-Acetylglucosaminidase (hexosaminidase) was determined by the method of Pugh et al. (10). The assays were carried out in duplicate, with the means differing from the actual values by <15%. Lactate dehydrogenase (LDH) was assayed by the method of Amador et al. (11). The levels of enzymatic activities in the supernates are presented as percent of the total activity in the culture (supernate + cell lysate).

Inhibition of nucleic acid synthesis. This assay was carried out in flat bottomed microplates (6 \times 8 mm, Linbro), with the cultures set up in triplicate, in a final volume of 0.2 ml of RPMI 1640 medium with 5% FCS. The cultures

consisted of (a) 4×10^4 P815 cells, (b) murine lens epithelial cells, cultured 20 h before being used, at 2.5×10^4 cells/well, or (c) 3×10^5 mouse spleen cells, collected shortly before use and stimulated with concanavalin A ($2 \mu\text{g/ml}$). After addition of the tested compounds, the cultures were incubated for 24 or 48 h and pulsed with [^3H]uridine (New England Nuclear, 8 Ci/mmol) or [^3H]thymidine, respectively, $0.5 \mu\text{Ci/well}$, for the last 6 h of incubation. All cultures were harvested by MASH II, with the lens epithelial cells being treated with trypsin beforehand (12).

Accumulation of labeled particles.² Monolayer cultures were set up in duplicate in plastic petri dishes, 35 mm in diam (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) and consisted of freshly prepared peritoneal M ϕ and confluent monolayers of lens epithelial cells and skin fibroblasts cultured for 48 h in advance. After removal of the original culture media, the labeled particles were added, suspended in 2-ml aliquots of RPMI 1640 medium with HEPES, supplemented with 5% FCS. The labeled particles included [^3H]GL₁ (New England Nuclear, 8 Ci/mmol), [^{14}C]SPM (New England Nuclear, 45 mCi/mmol) or ^3H -labeled bovine retinal rod outer segments (ROS). [^3H]GL₁ was prepared from the same unlabeled GL₁ used in this study and co-chromatographed with it on thin layer silica gel G. Labeled ROS were prepared as described (13) and kindly provided by Dr. Paul O'Brien (National Eye Institute). Unlabeled sphingolipids, at $30 \mu\text{g/ml}$, were added to the suspensions of the labeled GL₁ and SPM. After incubation for various intervals, at 37°C or 4°C , the monolayers were washed thoroughly with PBS and lysed in 0.5 ml NaOH, 0.2 N. The radioactivity was measured by scintillation counting in aliquots of 0.1 ml, treated with 0.5 ml of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill) and mixed with 10 ml of Betafluor (National Diagnostics Inc., Advanced Applications Institute Inc., Parsippany, N. J.) The protein content of the monolayer lysates was determined by the method of Lowry et al. (14). The results are presented as both the specific uptake, (mean disintegrations per minute per milligram protein) and as the percent of the total added isotope. The levels of specific uptake in the individual dishes differed by $<10\%$ from the mean values.

RESULTS

Effects of GL₁ on the release of LAF and enzymes by M ϕ . The results obtained in two experiments in which the release of various cell products was measured after incubation of M ϕ monolayers with various concentrations of GL₁ are illustrated in Fig. 1. The addition of GL₁ caused an increase in the release of the monokine, LAF, the lysosomal enzyme, hexosaminidase, and the cytoplasmic enzyme, LDH, in a dose-dependent fashion. The increase in release of LDH indicates cell damage (15, 16). It is noteworthy that the release of LAF and hexosaminidase was particularly elevated at toxic doses of GL₁, although an apparent increase in LAF release was observed in cultures with no detectable elevation of LDH release (with GL₁ at $\sim 20 \mu\text{g/ml}$ or less). A known M ϕ stimulant, LPS,

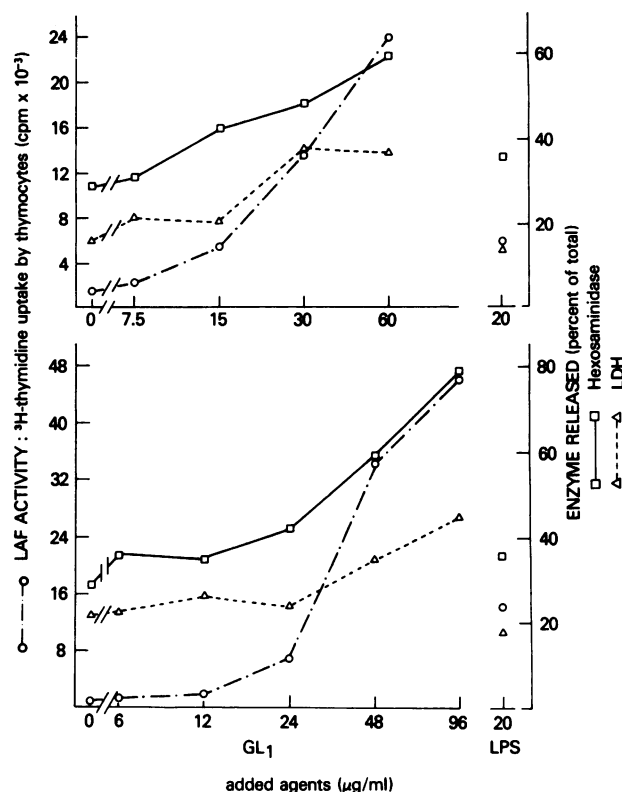


FIGURE 1 Effects of GL₁ at different concentrations on release of LAF, hexosaminidase and LDH by murine peritoneal M ϕ cultures. The levels of LAF activity presented here were obtained with the supernates diluted to 1:40.

moderately increased the release of LAF and hexosaminidase, but had no effect on LDH release. GL₁ also increased the release of two other lysosomal enzymes, β -galactosidase and glucuronidase (data not shown), but the changes were less consistent than those observed with hexosaminidase. Silica particles, a known cytotoxic agent for M ϕ (17), were used as control in other experiments to examine the effect of damage on the M ϕ product release. The effects of silica resembled those of GL₁, namely, at subtoxic concentrations ($<25 \mu\text{g/ml}$), only LAF release was increased, whereas at toxic higher doses, larger amounts of LAF were released, along with an increased release of LDH and hexosaminidase.

Synergy between GL₁ and LPS. M ϕ monolayers incubated with combinations of GL₁ and LPS released LAF in amounts greatly exceeding those secreted by cultures stimulated by the individual agents (Table I). It is noteworthy that the release of substantial amounts of LAF in the presence of LPS and GL₁ at $10 \mu\text{g/ml}$ was not accompanied by an increase in the release of LDH; the combination of LPS and GL₁ at $30 \mu\text{g/ml}$ produced very high levels of LAF, along with elevated LDH release. Hexosaminidase secretion in these experi-

² The term "accumulation" was used in the present study to describe the process in which labeled particles become associated with cell monolayers. This process may include both binding and actual phagocytosis or pinocytosis.

TABLE I
Synergy between GL₁ and LPS*

Agent added to Mφ cultures μg/ml	Mφ products in supernates			
	LAF activity		Enzymes¶	
	[³ H]Thymidine‡ uptake	LAF§ units	Hexosaminidase	LDH
None	394±81	1	25.3	20.1
GL ₁ , 10	425±106	1	29.7	22.4
GL ₁ , 30	426±37	2	37.5	36.5
LPS, 20	2,670±338	5	20.0	18.2
LPS + GL ₁ , 10	5,171±447	10	26.2	18.4
LPS + GL ₁ , 30	33,827±1,837	60	47.6	46.7

* The data recorded here are of one experiment, the findings were reproduced in three other experiments performed on different dates.

‡ Data represent counts per minute±standard error.

§ LAF activity is presented as mean cpm of [³H]thymidine incorporated by thymocyte cultures, in triplicate, incubated with the tested supernates at a final dilution of 1:160. The definition of "LAF units" is detailed in Methods.

¶ Levels of enzymatic activities in supernates, as percent of total activities in culture (supernate + cell lysate).

ments paralleled the release of LDH and reached particularly high levels in cultures with LPS and GL₁ at 30 μg/ml.

Chromatographic analysis of LAF released by GL₁ + LPS. The possibility that the high levels of LAF activity released by the combination of GL₁ plus LPS are due to a mediator other than LAF was examined by testing the chromatographic profiles of the mediator activity released by these cultures; the chromatographic properties of LAF have been well defined in previous studies (5, 18). Fig. 2 shows the chromatographic comparison between the activities released by Mφ incubated with LPS alone or with the combination of LPS plus GL₁. The results demonstrate a close similarity between the elution profiles of the LAF activity from the two types of Mφ cultures, despite the substantial difference in their levels of activity. It seems, therefore, that the synergistic effect of GL₁ plus LPS brings about the release of extremely large amounts of the same mediator secreted by cultures stimulated with LPS alone.

Effects of GL₁ on intracellular LAF. In view of the cytotoxic effect of high doses of GL₁ on Mφ, intracellular LAF activity was also measured, to test the possibility that the increased release of LAF by GL₁ was due only to leakage of the intracellular pool of the mediator. The data of a typical experiment are presented in Table II; similar results were repeatedly obtained in other experiments. GL₁ at 80 μg/ml (a toxic dose, see Fig. 1) substantially increased the release of

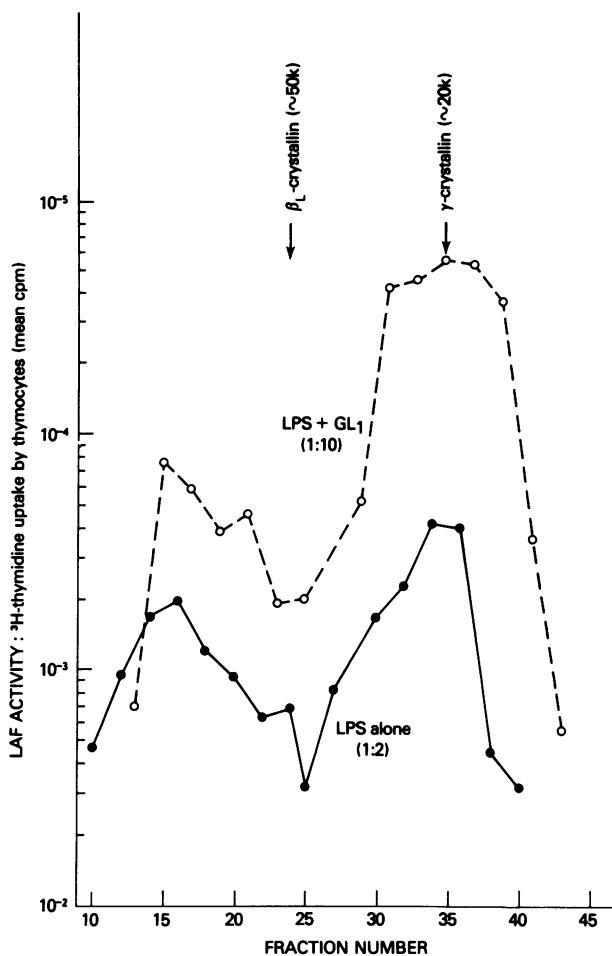


FIGURE 2 Chromatographic profiles of LAF activities released by murine Mφ cultured with LPS alone (at 20 μg/ml) or with LPS + GL₁ (at 20 and 60 μg/ml, respectively). The fractionation was carried out on Sephadex G-75 superfine columns and the fractions were tested at the recorded dilutions.

LAF and moderately elevated the level of intracellular LAF activity. LPS differed from GL₁ by remarkably increasing the intracellular LAF activity, while having only a small effect on LAF release. The combination of GL₁ and LPS again showed synergy in LAF release, along with the stimulation of high intracellular activity (which surpassed the elevated activity found in cultures with LPS alone). It is apparent, therefore, that the increase in LAF release by Mφ cultured with GL₁ is not simply the result of depletion of the intracellular LAF pool. Rather, incubation with GL₁ brings about an increase of both intracellular and extracellular activities.

Effects of other sphingolipids on Mφ. Three other sphingolipids involved in storage lipid diseases were tested, CTH, SPM and Gal-cer. As shown in Table III, these lipids caused minimal or no increase in LAF

TABLE II
Effects of GL₁ on Intracellular and Extracellular Levels
of LAF Activity of Mφ*

Agent added to Mφ†	Supernates		Cell lysates	
	[³ H]Thymidine uptake‡	LAF units	[³ H]Thymidine uptake‡	LAF units
None	381±94	1	1,245±204	3
GL ₁	7,001±950	16	5,703±665	10
LPS	1,662±355	3	30,169±525	60
GL ₁ + LPS	40,574±548	100	51,467±5,679	100

* The data recorded here are of one experiment; the findings were reproduced in three other experiments.

† GL₁ was added to the concentration of 80 μg/ml and LPS, to 20 μg/ml.

‡ The values of [³H]thymidine uptake recorded here were obtained with the supernates and lysates added at a final dilution of 1:40, and are expressed as mean counts per minute±standard error.

secretion and, unlike GL₁, were not synergistic with LPS. At the doses tested, none of the lipids other than GL₁ had a detectable effect on the release of hexosaminidase or LDH (data not shown).

TABLE III
Release of LAF by Mφ Cultured with Different Sphingolipids

Tested lipid	Concentra- tion	LPS added, 20 μg/ml	LAF activity	
			[³ H]Thymidine uptake*	LAF units
None	—	No	383±103	1
		Yes	4,525±528	5
GL ₁	40	No	5,654±553	5
	120	No	17,332±524	20
	40	Yes	29,739±986	100
CTH	40	No	724±76	1
	120	No	1,487±318	2
	40	Yes	4,068±134	6
SPM	40	No	651±144	1
	120	No	698±218	1
	40	Yes	3,673±505	5
Gal-cer‡	60	No	467±50	1
	120	No	515±98	1
	60	Yes	2,346±524	4

* The values of [³H]thymidine uptake recorded here were obtained with the supernates added at a final dilution of 1:40, and expressed as mean counts per minute±standard error.

‡ Gal-cer was tested in a separate experiment. Supernate of LPS alone (at 1:40 dilution) stimulated in this experiment an uptake of 1,760±230, or 3 LAF units.

Cytotoxicity of GL₁ to cells other than Mφ. The possibility that GL₁ affects Mφ simply as a nonspecific cytotoxic agent was examined by testing its effects on cells other than Mφ. The cytotoxic effects of GL₁ and other lipids were measured by their capacity to inhibit nucleic acid synthesis; this effect has been shown to be directly related to cytotoxicity (19). The data of two experiments are shown in Fig. 3. Lens epithelial cells were found refractory to GL₁, whereas spleen and P815 cells were moderately inhibited by high concentrations of this sphingolipid. SPM and CTH affected cells other than Mφ similarly to GL₁ (data with CTH are not shown). All tested cells were highly inhibited by the surface active agent, lysophosphatidyl choline (LPC) and moderately affected by soybean intralipid (SB-IL).

Accumulation of GL₁ by Mφ. The relationship between the effect of the lipids and their interaction with the cells was further analysed by measuring the accumulation of [³H]GL₁, [¹⁴C]SPM and ³H-labeled ROS by monolayers of Mφ and two other cell types, human skin fibroblasts and murine lens epithelium. The sphingolipids were added to the cultures in the presence of 30 μg/ml of the nonradioactive lipid. The data recorded in Table IV show a striking difference between the Mφ and the other tested cells in their capacity to accumulate [³H]GL₁. Mφ cultures accumulated ~10% of the added tracer during an incubation of 20 h, while the accumulation of radioactivity by the other cells was <1%. On the other hand, Mφ, like the other cells, did not accumulate a significant amount of [¹⁴C]SPM. The selective lack of

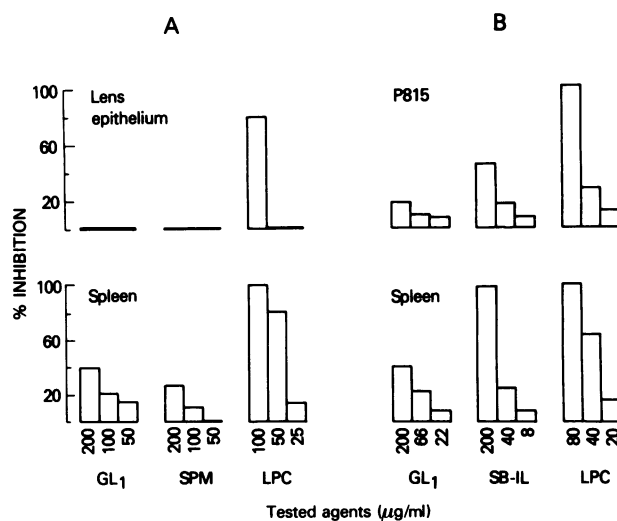


FIGURE 3 Inhibitory activity of GL₁ and other lipids on the nucleic acid metabolism of various cell cultures. Incorporation of [³H]thymidine was used in experiment A and of [³H]uridine in experiment B. % inhibition = 100 - mean cpm in cultures with lipid/mean cpm in control cultures × 100.

TABLE IV
Uptake of [³H]GL₁ and Other Particles by Mφ, Fibroblasts, and Epithelial Cells

Exp.	Particles tested	Incubation time	Temp.	Cell monolayers		
				Mφ	Skin fibroblasts	Lens epithelium
		<i>h</i>	<i>°C</i>			
I.	³ H-GL ₁	20	37	700,402 (9.3)*	9,742 (0.1)	4,569 (0.15)
			4	5,246 (0.1)	6,462 (0.03)	26,987 (0.8)
	[¹⁴ C]SPM	20	37	21,673 (0.4)	6,539 (0.1)	5,194 (0.3)
			4	4,796 (0.1)	8,449 (0.05)	2,678 (0.1)
	³ H]ROS	20	37	30,580 (7.6)	12,149 (1.4)	24,353 (9.2)
II.	³ H]GL ₁	3	37	201,196 (4.3)	2,861 (0.02)	4,472 (0.1)
			4	3,947 (0.1)	1,046 (0.01)	7,255 (0.2)
		20	37	569,390 (10.4)	7,360 (0.1)	5,054 (0.1)
			4	4,402 (0.1)	7,266 (0.04)	19,714 (0.3)
	³ H]ROS	3	37	26,478 (5.8)	8,677 (0.7)	33,675 (4.2)
			4	2,542 (0.6)	307 (0.02)	2,216 (0.2)

* Mean disintegrations per minute of labeled particles per milligram protein of cell lysate; in parentheses, the uptake calculated as percent of total activity added per dish. The labeled particles were added in aliquots containing (per dish): [³H]GL₁, Exp. I, 7.5×10^4 dpm and Exp. II, 8.6×10^5 ; [¹⁴C]SPM, 5×10^5 dpm; [³H]ROS, Exp. I, 6.6×10^4 dpm and Exp. II, 8.2×10^4 dpm.

accumulation of [³H]GL₁ by the cells other than Mφ contrasted sharply with their capacity to phagocytose ³H-labeled ROS, which consist of discs of cell membranes (13, 20). Lens epithelial cells phagocytosed ³H-labeled ROS similarly to Mφ. Skin fibroblasts, although inferior to the other cells in their capacity to phagocytose ROS, nonetheless took up considerably more ROS than the sphingolipids.

The molecular form of the label accumulating in Mφ incubated with [³H]GL₁ was examined and found to be similar to that of the original [³H]GL₁ added to the cultures. Chloroform/methanol extracts of the cell lysates resulted in >90% recovery of activity. Further, >90% of the radioactivity in these extracts cochromatographed with GL₁ on thin layer silica gel G plates. In addition, no change in molecular form was found in the label present in the media of cultures of Mφ or other tested cells. These data indicate, therefore, that no significant degradation of GL₁ took place under the conditions employed.

The accumulation of the particles by the cell monolayers was reduced in most systems when carried out at 4°C, thus indicating that active phagocytosis was involved. An exception to this pattern was found in cultures of lens epithelium incubated with [³H]GL₁; in all repeated experiments the accumulation of this lipid was markedly higher at 4°C than at 37°C.

Effects of GL₁ on human monocyte cultures. Monolayers of human blood monocytes were affected

by GL₁ similarly to the murine Mφ. Although some donors did not yield GL₁-responsive monocytes, those who did, retained their responsiveness in repeated tests for >2 yr. Results obtained with cells from a typical responsive donor are shown in Table V. GL₁ increased the release of LAF by the monocyte cultures, with an apparent dose responsiveness, and synergized with LPS to induce the release of very high levels of the mediator. SPM had no detectable effect on the release of LAF by these cultures.

DISCUSSION

Mφ cultures were found in this study to be useful in studying pathophysiologic activities of GL₁ at the cellular level. Mφ monolayers incubated with GL₁ released increased amounts of a specific mediator, LAF, as well as the lysosomal enzyme, hexosaminidase. In addition, GL₁ increased the release of a cytoplasmic enzyme, LDH, which is indicative of cell damage (15, 16). It is assumed, therefore, that the increased secretion of LAF and lysosomal enzymes is due at least in part to cellular injury. It should be pointed out, however, that increased LAF secretion was observed in Mφ cultures incubated with GL₁ at doses that had no detectable effect on LDH release. Moreover, substantial amounts of LAF were released by the combination of low doses of GL₁ and LPS, with no elevation of LDH (Table I). It is not known at this

TABLE V
Release of LAF by Human Monocytes Cultured
with GL₁ and Other Agents*

Agent added μg/ml	LAF Activity in Supernate	
	[³ H]Thymidine uptake†	LAF units
None	791±178	1
GL ₁ , 13	2,554±545	4
GL ₁ , 40	5,056±533	10
GL ₁ , 120	5,838±520	10
LPS‡	1,876±206	3
LPS + GL ₁ , 13	11,950±1,855	20
LPS + GL ₁ , 40	20,604±1,958	40
SPM, 120	494±183	<1

* The data recorded here were obtained with monocytes of one donor; similar results were obtained with cultures from six other donors.

† The [³H]thymidine values recorded here were obtained with the supernates at a final dilution of 1:160, and expressed as mean counts per minute±standard error.

‡ LPS was added to the concentration of 25 μg/ml.

time whether the toxicity of GL₁ in culture has any bearing on the pathogenesis of Gaucher's disease in vivo. Plasma of Gaucher's patients contains ~10–15 μg GL₁/ml. This is in the same range that produces toxicity in our cultures. Moreover, storage Mφ (Gaucher cells) may contain several hundred micrograms of GL₁ per 10⁶ cells. Biopsies of liver or bone marrow from these patients often show evidence of cell damage (21). Whether this results from toxic products released from Mφ (22) remains to be tested. Our data and the observation that Gaucher tissues contain excess lysosomal enzymes (23) support this possibility.

The relationship between Mφ damage and production of LAF was further examined by measuring the levels of intracellular LAF activity in the GL₁-affected cultures. Lysates of these Mφ contained more LAF activity than lysates of untreated controls (Table II). It seems, therefore, that the increased release of LAF by toxic doses of GL₁ is not due merely to a leakage of the cellular content of this mediator. Rather, the results suggest that injured Mφ increase their production of LAF and indeed, similar observations were made in experiments not included here, in which Mφ cultures were incubated with silica, a selective cytotoxic agent for Mφ (17). It is also noteworthy that Mφ "activation" is often accompanied by cell damage and increased LDH release, as has been shown in cultures incubated with high concentrations of antigen-antibody complexes or zymosan (16).

Of a particular interest is the finding that GL₁ synergizes with LPS to induce the secretion of ex-

cessive amounts of LAF by murine or human Mφ. The combined stimulation provides, therefore, a new method for obtaining high levels of LAF, a mediator of various biological reactions (5, 6, 24, 25).

The selectivity of the effect of GL₁ on Mφ was underlined by the finding that three other sphingolipids, Gal-cer, SPM and CTH had very little effect on these cells in terms of LAF release (Table III). This selectivity was further demonstrated by studying the cytotoxic activities of GL₁ on a variety of cells. Lens epithelial cells were unaffected by GL₁ at all tested doses, whereas lymphocytes or P815 cells, which are more susceptible to cytotoxic agents, were minimally affected by GL₁ at concentrations that increased markedly the release of LDH from Mφ, i.e., 20–100 μg/ml. (The release of LDH is considered a reliable method for determining Mφ injury (15, 16)). The selectivity of GL₁ for Mφ may derive from the unique capacity of these cells to bind or take up GL₁. A direct relationship was found between the levels of accumulation and of cytotoxic activity of the sphingolipids as shown by the following results: (a) lens epithelial cells accumulated negligible amounts of GL₁ and were refractory to the damaging effects of this lipid, whereas Mφ accumulated large quantities of GL₁ and showed high susceptibility to its effects. (b) SPM was not accumulated by Mφ and had minimal or no effect on these cells. The relationship between accumulation and damaging capacity of GL₁ was further confirmed in experiments in which untreated ("resident") Mφ were compared to Mφ activated in vivo by BCG bacteria. The activated Mφ accumulated 3–10 times less [³H]GL₁ than the resident Mφ and were refractory to its damaging effects.³

The selectivity of GL₁ for Mφ in vitro (Table IV) may be related to the exclusive accumulation of this sphingolipid in the reticuloendothelial system of patients with Gaucher's disease (1, 2). The mechanism that regulates the selective uptake of GL₁ by Mφ is not known. The possibility that cells other than Mφ did not accumulate GL₁ because of high levels of the specific enzyme, glucocerebrosidase (GL₁ase) is not consistent with the finding that the levels of this enzyme were similar in Mφ and lens epithelial cells (114.7 and 128.7 U/mg protein, respectively). It is known that glucose moieties have some affinity for the mannose receptor of Mφ (26). The possibility that this or another receptor is involved in binding and uptake of glycolipids is currently under investigation.

The present study thus shows that Mφ cultures may be useful for investigating the interactions between GL₁ and the reticuloendothelial system. Although the

³ Gery, I., R. Prendergast, and J. A. Barranger. Production and release of lymphocyte-activating factor by macrophages. Effects of macrophage activation in vivo. In preparation.

relationship between the phenomena observed in vitro and those occurring in vivo is not clear, it is tempting to speculate that the findings reported here are related to certain clinical features of Gaucher's disease. It is conceivable that an increased release of products by GL₁-affected M ϕ could participate in bringing about damage to liver and bone marrow (21), increased levels of lysosomal enzymes (23), or the abnormally high frequency of gammopathies (27) in Gaucher's disease.

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