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FURTHER CHARACTERIZATION OF THE PUTATIVE GLYCOLIPID RECEPTOR FOR MIF: ROLE OF FUCOSE ASSOCIATED WITH AN ACIDIC GLYCOLIPID *

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SUMMARY: Water soluble glycolipids were extracted from guinea pig macrophages. These glycolipids, when incubated with macrophages, augment the cells' response to migration inhibitory factor. The glycolipids were fractionated by diethylaminoethyl-Sephadex ion exchange chromatography into neutral and acidic fractions. Only the acidic glycolipid fraction was able to enhance the responsiveness of macrophages to migration inhibitory factor. Additional studies indicate that the enhancing activity of these glycolipid preparations can be abrogated by the removal of terminal fucose residues with α-L-fucosidase. The possibility that fucose functions as an essential component of a macrophage glycolipid receptor for migration inhibitory factor is discussed.

INTRODUCTION

The biological responses of cells to a variety of hormones and toxins are increased when their glycolipid receptors are preincubated with these target cells (1-4). It has been previously shown that the response of macrophages to migration inhibitory factor**, a soluble mediator of cellular immunity can be substantially increased by pretreatment with guinea pig macrophage-derived glycolipids (5-8) but not by glycolipids from either guinea pig brain or PMNL, porcine globoside, bovine ceramide, cerebrosides or G_{M2} (5). It was

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**Abbreviations: MIF, migration inhibitory factor; G_{M2},GalNAc-Gal(NANA)-Glc-Cer; GalNAc, N-acetylgalactosaminyl; Gal, galactosyl; NANA, N-acetylneuraminic acid; Glc, glucosyl; Cer, ceramide; DEAE, diethylaminoethyl; HBSS, Hanks' balanced salt solution; MEM, minimum essential medium; C:M, chloroform:methanol; PMNL, polymorphonuclear leukocytes; EDTA, ethylene diaminetetraacetic acid; GSL, glycosphingolipids; GLC, gas-liquid chromatography.

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therefore hypothesized that macrophage glycolipids are part of a MIF receptor associated with macrophages.

Experiments were initiated to further purify and characterize the enhancing macrophage glycolipid, which is found in the aqueous fraction of the Folch partition (5). The ability of macrophage glycolipids to augment the macrophage response to MIF was found to reside in the acidic fraction following DEAE-Sephadex chromatography. Furthermore, the work of Remold (9) demonstrating the importance of fucose in the macrophage response to MIF prompted us to investigate whether L-fucose is a necessary part of these glycolipids. The data presented herein demonstrate that the enhancing activity of glycolipid preparations can be abolished by the removal of terminal fucose, indicating the importance of this carbohydrate for the biological activity of the glycolipid. These results are in agreement with those obtained by treatment of macrophages with glycosidases (9) and further support the hypothesis that glycolipids associated with macrophages are part of a MIF receptor.

MATERIALS AND METHODS

Materials: HBSS, MEM, and medium 199 were purchased from Microbiological Associates, Bethesda, MD. Fluorescamine was obtained from Roche Diagnostics, Nutley, NJ. Egg yolk lecithin, bovine cholesterol and ceramide were obtained from Supelco, Inc., Bellefonte, PA.

Collection of guinea pig cells and tissue: Guinea pig macrophages, polymorphonuclear leukocytes (PMNL) and brain tissue were obtained as previously described (5).

Glycolipid extraction and fractionation: Glycolipid was extracted from guinea pig cells or tissue and partially purified by Folch partition as previously described (5). The biologically active aqueous phase glycolipids were separated into acidic and neutral glycolipids using DEAE-Sephadex (10).

Glycosidase treatment of glycolipids: α-L-fucosidase was purified from rat epididymis using a modification of the Carlsen and Pierce method (11). The enzyme preparation (88 U/mg) was dialyzed briefly against 0.1 M Na citrate buffer pH 6.0. Residual β-N-acetylglucosaminidase activity was destroyed by heating at 70°C for 18 min before use.

Glycolipids were dried in a test tube under N_2 and then resuspended in 0.1 M sodium citrate buffer pH 6.0 in the presence of 0.02% NaN_3 with or without α-L-fucosidase. The ratio of enzyme to substrate was 2.5 units α-L-fucosidase/μg of sphingosine. All tubes were incubated at 37°C for 48 hr. Control incubations consisted of glycolipid exposed to α-L-fucosidase

inhibited in the presence of its monosaccharide product, L-fucose. At the end of the incubation, the samples were quickly frozen and lyophilized. The glycolipid was extracted with C:M:KCl/EDTA 10:10:3 and then brought to dryness. The dried material was resuspended in cold water, followed by extensive dialysis against water at 4°C before lyophilization. The dry residue was resuspended in C:M 2:1, quantified, and used to form liposomes.

Analysis by gas-liquid chromatography: Neutral carbohydrates were identified using gas-liquid chromatography on a Perkin-Elmer model 3920 gas chromatograph equipped with on-column injections and flame ionization detectors. Carbohydrates were modified according to the method of Hakomori and Siddiqui (12) and analyzed as alditol acetates on either a 6' 3% ECNSS-M or 9' 3% Silar 7CP 1/8" diameter stainless steel column. Both liquid phases were on 80-100 mesh Gas Chrome Q supports obtained as packed columns from Applied Science Lab., State College, PA. The ECNSS-M column was held at 170°C for 16 min, programmed at a rate of 1°/min to the final temperature of 210°C and held at this temperature for 16 min. The Silar 7CP column was held at 210°C for 16 min, programmed at a rate of 32°C/min to 245°C and held at this temperature for 16 min. Carrier gas flow rate for the ECNSS-M column was 40 ml/min and 60 ml/min with the Silar 7CP using helium as carrier gas. Both injectors and detectors were held at 250°C. Samples consisting of 1-5 µl in acetone were analyzed. Carbohydrates were quantitated by comparing the peak area of experimental samples obtained by the triangulation method with that of standards similarly modified and analyzed.

Glycolipid analysis: Following hydrolysis and extraction of glycolipid samples, glycolipids were assessed for their sphingosine content using the fluorescamine assay (5).

Liposome production and macrophage preincubation: Liposomes were produced and preincubated with macrophages as previously described (5).

Production and assay of MIF: MIF-rich and control lymphocyte supernatants were obtained from cultures of guinea pig lymph node lymphocytes that were incubated with and without concanavalin A, partially purified over Sephadex G-100 and assessed for activity as previously described (13). MIF activity was expressed as percentage of migration inhibition of macrophages (%I), calculated by the following formula:

$$\%I = \left(1 - \frac{\text{Average migration in MIF containing fractions}}{\text{Average migration in control fractions}} \right) \times 100$$

A %I greater than 20 was considered to be significant MIF activity (13).

Statistical analysis: Changes in %I resulting from pretreatment of macrophages were assessed for statistical significance by a three way analysis of variance test. All raw data in each group of experiments were subjected to logarithmic transformation and analyzed by the statistical analysis system (SAS - 1979 version) general linear models (glm) procedure. This analysis was carried out by Dr. Harriet Peterson, Department of Biostatistics, Harvard School of Public Health.

RESULTS AND DISCUSSION

Fractionation of aqueous phase glycolipids on DEAE-Sephadex: It was previously reported that the enhancing effect of macrophage glycolipids on MIF responsiveness of the macrophages resides in the aqueous phase of the Folch

Table I. Enhancement of the Response of Macrophages to a low dose of MIF by Glycolipids Fractionated on DEAE-Sephadex

		% Inhibition		
		Macrophages preincubated with:		
Exp. #	Medium 199	Acidic	Neutral	Ceramide[a]
1	3	49	10	10
2	-2	33	-10	-17
3	-4	30	-5	10
4	19	35	-1	12
5	14	35	-8	-21

[a]Liposomes made with ceramide instead of macrophage glycolipids. Statistical analysis (see Materials and Methods) of all five experiments indicated the following: macrophages preincubated with acidic glycolipids showed statistically significant enhancement compared to medium 199 ($p < 0.001$), neutral ($p < 0.001$), and ceramide ($p < 0.001$); these latter three groups showed no statistically significant difference between each other.

partition (5). These glycolipids were further separated into acidic and neutral glycolipids using DEAE-Sephadex and tested for their capacity to increase the MIF response of macrophages. Table I shows the results of five experiments. It can be seen that liposomes containing acidic macrophage glycolipids enhanced macrophage responsiveness to MIF. Liposomes containing neutral macrophage glycolipids or ceramide showed no such effect, even at doses high enough to nonspecifically inhibit control migration. Acidic glycolipid liposomes did not affect normal migration in these experiments, and only 0.1-0.4 μg sphingosine equivalents (0.5-2 μg GSL)/3.5 X 10^7 cells were required to increase MIF responsiveness. Preliminary evidence indicates that the activity derived from the DEAE acidic fraction can be correlated with one band in the ganglioside region on thin-layer chromatography (8).

Role of fucose in enhancement of MIF responsiveness by macrophage glycolipids: The experiments of Remold (9) demonstrated that fucose on the macrophage was necessary for the cell's response to MIF, suggesting a role for L-fucose as part of a MIF receptor. Since glycolipids are candidates for a MIF receptor, it was of interest to investigate whether L-fucose was required for the biological activity of the glycolipids. In these experiments, macrophage glycolipids were treated with α-L-fucosidase (Table II). Controls were incubated under the same conditions with either buffer alone (Exp. 1-5)

Table II. α-L-fucosidase prevents glycolipid enhancement of MIF

	% Inhibition		
	Macrophages preincubated with:		
Exp. #	Medium 199	Fucosidase-treated macrophage glycolipid	Control macrophage glycolipid
1	-1	2	23[a]
2	30	15	67[a]
3	12	14	24[a]
4	23	21	35[a]
5	29	30	44[a]
6	25	-17	44[b]
7	25	31	45[b]
8	-26	14	51[b]

[a]Aqueous phase glycolipids pretreated with 0.1 M citrate buffer pH 6.0.

[b]Acidic glycolipids pretreated with α-L-fucosidase in the presence of 0.1 M fucose

Statistical analysis of all eight experiments indicated the following: control glycolipid liposomes showed statistically significant enhancement compared to medium 199 ($p < 0.001$) and fucosidase-treated glycolipid liposomes ($p < 0.001$); there was no statistically significant difference between medium 199 and fucosidase-treated glycolipid liposomes.

or with α-L-fucosidase in the presence of L-fucose (Exp. 6-8). As shown in Table II, treatment of macrophage glycolipids with α-L-fucosidase consistently abrogated their ability to enhance macrophage responsiveness to MIF in eight experiments, whereas control glycolipid preparations enhanced macrophage responsiveness to MIF.

GLC analysis of one of the aqueous phase glycolipid preparations (which was used for experiments 3 and 4, Table II) demonstrated that α-L-fucosidase removed fucose from the glycolipids without loss of glucose or galactose (Table III). Buffer-treated glycolipids retained their normal constituency of carbohydrates, including fucose. Fucose attached to glycolipid per se is not a sufficient criterion for enhancement, since glycolipid from guinea pig PMNL and brain also contain substantial fucose (Table III, footnote c) but do not impart increased MIF responsiveness to macrophages (5), even in amounts five times the effective dose of macrophage glycolipids (Higgins, unpublished observation). Glycolipids extracted from human O type erythrocytes, which contain fucolipids (14, 15), also do not appear to enhance the cell response

Table III. Carbohydrate Content of Guinea Pig Macrophage Glycolipids after α-L-fucosidase Treatment

Macrophage glycolipid[a]	mmoles CHO/mole sphingosine		
	fucose	galactose	glucose
+ buffer[a,b]	147	154	14
+ α-L-fucosidase[a,b]	undetectable	151	17

[a]Preparation of aqueous phase glycolipids after Folch partition.

[b]This glycolipid preparation utilized for experiments 3 and 4 shown in Table II.

[c]Galactose and glucose content were unaffected by α-L-fucosidase treatment.
Guinea pig macrophages contain a substantial quantity of fucose. Guinea pig PMNL and brain also contain lipid-associated fucose but in lower concentrations, 65 and 35 mmoles/mole sphingosine respectively.

to MIF (Bout, Killian, and Liu, unpublished observation). However, a preparation of bovine brain mixed gangliosides, which also contains a fucolipid (16), is able to enhance the MIF response (6), indicating that fucose has to be part of a specific glycolipid structure as a prerequisite for biological activity of the structure.

The results of experiments where macrophage glycolipids were treated with α-L-fucosidase are consistent with those obtained by treatment of the intact macrophage with this enzyme, as the latter rendered the macrophage unresponsive to MIF (9). Taken together, these data suggest that fucose is essential and is being modified by α-L-fucosidase. It has also been shown that sialic acid but not galactose or glucose is needed for the macrophage response to MIF and for the biological activity of macrophage glycolipids (7), further strengthening the concept that lipid-associated fucose and sialic acid act as cell receptors for MIF.

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