We have previously established that preincubation of guinea pig macrophages with crude preparations of macrophage glycolipids enhances their response to a mediator of cellular immunity, migration inhibitory factor. This biologically active moiety found in the extract was attributed to a single glycolipid component. This component was purified to homogeneity by phase partition, ion exchange, adsorption, and thin layer chromatography. It migrates in the gangliotetraose region on thin layer plates, contains sialic acid and sphingosine. There is no apparent identity with any of the common gangliosides possessing the gangliotetraose structure. Homogeneity was demonstrated by rechromatography in four additional solvent systems. These results are discussed in the context of their implications for a deeper understanding of the putative receptor function of this glycolipid.

The interaction between the macrophage and immune lymphocytes plays a pivotal role in cellular immunity with important implications for the host's defense against microbes, and for tumors and transplantation rejection. This interaction and subsequent changes in macrophage function are mediated by secreted products of lymphocyte activation. One of these mediators, migration inhibitory factor (MIF), inhibits the migration of macrophages out of capillary tubes (1, 2). The mechanisms of MIF influence on the macrophage are not well understood at this time.

Previous studies from this laboratory have focussed on the role of glycolipids as putative receptors on the macrophage for MIF. It was demonstrated that the response of macrophages to MIF is enhanced when the cells are pretreated with macrophage glycolipids (3, 4). Fucose and sialic acid were shown to be essential for the enhancing activity of glycolipids (5, 6). Furthermore, recent studies indicated that only enhancing glycolipids are able to bind specifically MIF, lending more support for the hypothesis that macrophage glycolipids serve as membrane receptors for guinea pig MIF (7). There is an increasing amount of data to suggest that certain glycolipids are able to function as part of membrane receptors for toxins and viruses (8, 9), hormones (10), and interferon (11).

In this study, it was our objective to purify the glycolipid, which enhances the MIF response of macrophages, to homogeneity so that we may be able to define its chemical nature. This will eventually allow an investigation into the mechanisms of MIF action on the macrophage and the function of glycolipids in this interaction.

EXPERIMENTAL PROCEDURES

Materials

Hanks' balanced salt solution, minimal essential medium, Medium 199, penicillin G, streptomycin, and HEPES buffer were purchased from Microbiological Associates, Bethesda, MD. Fluoresceamine (Fluram) was obtained from Roche Diagnostics, Nutley, NJ. Sili- acid (Unisil) was purchased from Clarkson Chemical Company, Inc., Williamsport, PA. Precast silica G plates, egg yolk lecithin, bovine cholesterol, GT, GM1, GM2, and bovine brain mixed gangliosides were obtained from Supelco Inc., Bellefonte, PA. Silica 60 plates were from EM Laboratories, Inc., Elmsford, NY; primuline from Eastman; and resorcinol from Sigma.

Methods

Production of Migration Inhibitory Factor—Production of MIF-enriched and control lymphocyte supernatants proceeded as previously described (12). Sensitized lymph node lymphocytes (2.4 X 10⁹/μl) from guinea pigs were incubated with and without concanavalin A (10 μg/ml) for 24 h at 37°C. The cultures were harvested and centrifuged to remove cells. At this point concanavalin A was added to the control cultures. These supernatants were partially purified by gel filtration on Sephadex G-100. Fractions eluting in the 25,000- to 55,000-dalton range were pooled, concentrated to 100-fold of the original volume of supernatant, and stored at −70°C.

Assay for Migration Inhibitory Factor—Aliquots of the concentrated MIF supernatant were diluted in minimal essential medium containing 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% normal guinea pig serum. A capillary tube migration assay (13) was used to assess the response of normal guinea pig peritoneal exudate cells (more than 80% macrophages), which had been previously preincubated with glycolipid liposomes or with Medium 199 (see below), to MIF. Migration inhibitory factor activity was expressed as the percentage of migration inhibition of these cells (%I).

%I = 1 - \frac{\text{average migration in MIF-containing fractions}}{\text{average migration in control fractions}} \times 100

At least 20% inhibition was considered as significant activity. Suboptimal amounts of MIF were used such that MIF, by itself, caused little or no inhibition of macrophage migration. This allowed the detection of enhancing effects.

Liposome Production—Liposomes were prepared according to the method of Kinsky (14) with some modifications. Lecithin, cholesterol,
and test glycolipid were dried together in vacuo in a molar ratio of 2:2:2:6:1 (lecithin/cholesterol/sphingosine content). For example, a typical liposome preparation of 1.5 ml contained 57.5 μg of lecithin, 34 μg of cholesterol, and 10 μg of sphingosine equivalents. Liposomes were formed under nitrogen, 25 mM HEPES-buffered saline by agitation with a few glass beads on a Vortex mixer. They were sonicated at 4 °C for 30 min in a Mettler Electronic 4.6 ultrasonic cleaning bath and centrifuged at 27,000 × g for 30 min at 4 °C. Liposomes remaining in suspension were characterized as small in diameter (1000 Å) and were used in these experiments for up to 2 weeks while being stored under nitrogen at 4 °C.

Test for Enhancement of MIF Response of Guinea Pig Peritoneal Exudate Cells—Peritoneal exudate cells (3.5 × 10⁶/ml) were preincubated in Medium 199 containing 25 mM HEPES with and without liposomes for 1 h at 37 °C with gentle agitation to resuspend the cells. Liposomes containing test glycolipids were at a concentration of 0.1 to 0.6 μg of sphingosine equivalents/ml. Cells were then washed by centrifugation with cold Hanks’ balanced salt solution and assessed for their response to a low dose of MIF in the capillary tube migration assay.

Collection of Guinea Pig Peritoneal Exudate Cells—Peritoneal exudate cells were obtained from guinea pigs as previously described (13). Briefly, animals were injected with sterile mineral oil (25 ml) into their peritoneum. Three days later, the guinea pigs were bled by heart puncture and the peritoneal cavity lavaged with Hanks’ balanced salt solution. These cells were then immediately used for the MIF assay or stored at −20 °C until needed for lipid extraction.

Lipid Extraction—Peritoneal exudate cells were extracted as previously described (3). Briefly, 10 ml of packed cells were homogenized on ice with a Potter-Elvehjem apparatus in an equal volume of 0.1 M KCl, 0.01 M EDTA (KCl/EDTA) for 5 min. A second homogenization used 15 volumes (based on original volume of material) of chloroform/methanol (2:1). This was stirred under nitrogen for 45 min at 4 °C. The homogenate was filtered through a scinttered glass funnel and the filtrate saved. Particulate matter was homogenized with 15 volumes of chloroform/methanol (1:1) and then with 15 volumes of chloroform/methanol (1:2) in the exact same manner as the previous homogenizations. The filtrates were combined and concentrated in vacuo to near dryness with gentle warming (35 °C). The concentrated filtrate was partitioned by adding 30 ml of chloroform/methanol (2:1) and 6 ml of KCl/EDTA and allowing the phases to separate overnight at 4 °C. Each phase was then washed three times with the opposite theoretical phase except for the last wash of the organic lower phase which was done with water to allow complete recovery of all gangliosides. Washings were pooled with its appropriate parent phase. The combined aqueous upper phases were concentrated in vacuo to dryness, solubilized in KCl/EDTA, dialyzed against cold saline and then water, lyophilized, extracted with chloroform/methanol (2:1), and filtered. Approximately 1500 μg of sphingosine was obtained from 10 ml of packed cells.

Glycolipid Fractionation—Aqueous-phase glycolipids were first treated with mild alkaline to remove phospholipids. Acidic and neutral glycolipids were separated on either DEAE-cellulose (15) or activated silicic acid columns (16).

When using DEAE-cellulose, aqueous-phase glycolipids (800 to 1200 μg of sphingosine equivalents) were percolated onto a glass column (2 × 10 cm) equilibrated with chloroform/methanol (2:1). All nonacidic lipids were eluted sequentially with chloroform/methanol (2:1) and methanol at a rate of 60 ml/h or less. This was followed by chloroform/methanol/ammonia/potassium acetate (4:1:2:50 mM) and methanol to recover the acidic lipids. Both fractions were reduced to dryness under vacuum, dissolved in KCl/EDTA, dialyzed against 2000 times volume of precooled distilled water, lyophilized, and then redissolved in chloroform/methanol (2:1).

Silicic acid (100–200 mesh) was packed in a small Pasteur pipette on top of glass wool and sand. A 1-cm long bed was sufficient to accommodate the amount of glycolipid from one preparation of aqueous glycolipids, which was layered on the column in 0.5 ml. Neutral glycolipids were eluted with 2 ml of chloroform/methanol (2:1) for maximum yield of gangliosides. This was followed by 2 washes of 1 ml of chloroform/methanol/water (10:10:3) to elute gangliosides. The silicic acid was stirred vigorously during the second rinse and gentle pressure was applied to the column.

Thin Layer Chromatography—Silica G plates (500 μm) were developed in Solvent 2, chloroform/methanol/water (60:45:10). Silica 60 plates (250 μm) were developed either in Solvent 2, chloroform/methanol/2.5 M NH₄OH (60:35:5); Solvent 3, 1-propanol/water/NH₄OH (6:2:1); Solvent 4, 1-butanol/pyridine/water (9:6:4 with 0.1% KCl); or Solvent 5, chloroform/methanol/water (50:45:10 with 0.02% CaCl₂, 2H₂O). Bands were detected by spraying plates with the non-destructive fluorescent primuline (17) or with resorcinol (18). Each Tlc band was extracted from the silica gel with chloroform/methanol/KCl/EDTA (10:10:1), filtered, and tested for enhancing activity.

Chemical Analysis—Glycosphingolipids were analyzed for their...
sphingosine content by measuring the hydrolyzed and extracted sphingosine (19) with fluorescamine (20).

Statistical Analysis—Changes in the percentage of migration inhibition resulting from pretreatment of macrophages were analyzed for statistical significance by the Students' t test.

RESULTS

The procedure for the purification of the enhancing glycolipid is depicted in Fig. 1. The initial stages of purification were described in two previous reports (3, 5). It was found that the upper aqueous-phase lipids and not the lower organic-phase lipids of the Folch partition contained the ability to enhance the macrophage response to MIF (3). Fractionation of the aqueous lipids by DEAE-Sephadex chromatography into neutral and acidic glycolipids revealed the enhancing activity to reside in the acidic fraction (5).

Essentially similar results were obtained when we used DEAE-cellulose or silicic acid columns to separate neutral and acidic glycolipids. Thus, previous results could be confirmed by using a technique based on similar principles (ion exchange) or dissimilar principles (Unisil adsorption). Silicic acid chromatography offered the advantages of being simpler and faster to use with better yields (50–70%) than with DEAE-cellulose (30–50%). A TLC band pattern developed by resorcinol staining to show sialic acid-containing species is shown in Fig. 2. There appear to be at least five detectable bands migrating in the ganglioside region with possibly two of them not corresponding to the common ganglioside species, GT1, GD1a, and GM1. Under preparative TLC conditions (300 μg of sphingosine equivalents spotted on a 500-μm Silica Gel G plate) five other bands migrating above GMI of RF values ranging from 0.5 to 0.9 could be detected with primuline (data not shown). The majority of sphingolipid migrated in the ganglioside region with the total yield averaging 75%. Each of the resorcinol and primuline-positive bands were extracted to test for its enhancing activity. The results are shown in Fig. 3. Band 3 was the only species possessing enhancing activity. All others were inactive even when tested with two to five times the amount used for band 3. In each experiment, a preparation of the unpurified aqueous lipids was included to serve as a positive active control. Due to their low sphingosine content, bands 7–10 were combined and then tested for activity, which proved to be negative.

Although the active material migrated as one band in Solvent System 1, we asked the question whether co-migrating contaminations could be separated in a more polar solvent system, such as Solvent System 2. The band pattern is depicted in Fig. 4. Band 3 from several TLC preparations (Solvent System 1) were combined before proceeding to Solvent System 2. Approximately 100 μg of band 3 was applied to Silica 60 plates with yields steadily reaching 70–80%. Band 3 was resolved into three species, a singlet and a doublet. The singlet and the doublet were then extracted from the plate.
and run separately in Solvent System 2 to give the picture shown in Fig. 4. The singlet was positive for sialic acid and migrated just ahead of GM1. The doublet also stained for sialic acid and migrated ahead of and with GM2. Approximately 50 μg of sphingosine equivalents of each species was obtained. They were then tested for enhancing activity (Fig. 5). In seven experiments the singlet but not the doublet was able to enhance the macrophage response to migration inhibitory factor. The singlet band was found to be homogeneous since it migrated as one band on Silica Gel 60 plates run in Solvent Systems 3, 4, and 5.

**DISCUSSION**

The data presented in this report demonstrate that the ability of lipid extracts from guinea pig peritoneal macrophages to enhance the macrophage response to MIF or to bind MIF (3, 4). If the enhancing glycolipid is a ganglioside, then this would not be inconsistent with the known essential role of sialic acid in the macrophage response to MIF and in the enhancing ability of macrophage glycolipids (6). Although only one glycolipid component, the singlet, exhibited enhancing activity, there still exists the possibility that the other nonactive components may act synergistically with the singlet component to elicit an even more pronounced enhancement of the macrophage response to MIF. In the context of our knowledge of the essential role of fucose in the macrophage response to MIF (21) and in the enhancing ability of macrophage glycolipids (5) we may speculate that a fucolipid could serve as the synergistic component to the singlet ganglioside. It is interesting to note that a fucolipid was isolated and characterized from rat macrophages and was postulated to have a receptor function for MIF (22). The other alternative is that the singlet component is a fucoganglioside. Such structures have been found in bovine liver, thyroid, and brain (23-26); pig adipose tissue (27); boar testes (28); rat hepatoma (29); human brain, kidney, and erythrocytes (30-32); and rabbit brain (33). We are presently investigating the chemical composition and structure of enhancing glycolipid to help resolve these problems.

Now that the enhancing glycolipid has been purified to homogeneity we can begin to clearly define the relationship of its enhancing activity to a putative receptor function for MIF. The singlet is being tested for its ability to bind MIF and subsequent recovery of active MIF. It has been shown that there is a correlation between the ability to enhance the macrophage response to MIF and its ability to bind MIF (4, 7). Further studies will be required to unequivocally demonstrate a receptor function for the singlet glycolipid. To help achieve this goal, studies are in progress to generate a monoclonal antibody to the enhancing glycolipid.

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