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Neoglycolipid Analogues of Ganglioside G_{M1} as Functional Receptors of Cholera Toxin

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ABSTRACT: We synthesized several lipid analogues of ganglioside G_{M1} by attaching its oligosaccharide moiety ($G_{M1}OS$) to aminophospholipids, aliphatic amines, and cholesteryl hemisuccinate. We incubated G_{M1}-deficient rat glioma C6 cells with each of the derivatives as well as native G_{M1} and assayed the cells for their ability to bind and respond to cholera toxin. On the basis of the observed increase in binding of ^{125}I-labeled cholera toxin, it was apparent that the cells took up and initially incorporated most of the derivatives into the plasma membrane. In the case of the aliphatic amine derivatives, the ability to generate new toxin binding sites was dependent on chain length; whereas the C_{10} derivative was ineffective, C_{12} and higher analogues were effective. Increased binding was dependent on both the concentration of the neoglycolipid in the medium and the time of exposure. Cells pretreated with the various derivatives accumulated cyclic AMP in response to cholera toxin, but there were differences in their effectiveness. The cholesterol and long-chain aliphatic amine derivatives were more effective than native G_{M1}, whereas the phospholipid derivatives were less effective. The distance between $G_{M1}OS$ and the phospholipid also appeared to influence its functional activity. The neoglycolipid formed by cross-linking the amine of $G_{M1}OS$ to phosphatidylethanolamine (PE) with disuccinimidyl suberate was less effective than the neoglycolipid formed by directly attaching $G_{M1}OS$ to PE by reductive amination. Furthermore, insertion of a C_8 spacer in the former neoglycolipid rendered it even less effective. Finally, cells treated with neoglycolipids based on phospholipids exhibited a longer lag period before a response to cholera toxin was observed compared to cells treated with G_{M1} or neoglycolipids based on stearylamine or cholesterol. Our results indicate that although $G_{M1}OS$ provides the recognition site for the binding of cholera toxin, the nature of the lipid moiety plays an important role in the action of the toxin.

Cholera toxin (choleragen), an enterotoxin of *Vibrio cholerae*, activates adenylate cyclase in a variety of vertebrate cells [reviewed in Fishman (1990)]. Cholera toxin is an 85-kDa heteroprotomeric protein composed of A (activating) and B (binding) components. The A component (27 kDa) consists of two unequal disulfide-linked peptides: A_1 and A_2. A_1 peptide is an ADP-ribosyltransferase that catalyzes the ADP-ribosylation of the stimulatory G protein of the adenylate cyclase complex, resulting in its persistent activation (Moss & Vaughan, 1988; Gilman, 1987). The B component of the toxin is a 58-kDa pentamer composed of identical subunits (each 11.6 kDa) and binds to specific receptors on the cell surface. The only functional cholera toxin receptor described to date is the ganglioside G_{M1}[1] (Fishman, 1982, 1990). As has been studied in detail, cholera toxin recognizes and binds to the monosialogangliotetraosyl moiety of G_{M1}, each B component binding five $G_{M1}OS$ (Fishman et al., 1978). The structure of the oligosaccharide part of the receptor determines the high specificity of toxin binding. The role of the lipid moiety is less clear. Recently, we developed a method to attach $G_{M1}OS$ to sulfhydryl groups on cell surface proteins of viable

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[1] Abbreviations: G_{M1}, II^3Neu5Ac-GgOse$_4$Cer; $G_{M1}OS$, II^3Neu5Ac-GgOse$_4$; $G_{M1}OSNH2$, 1-amino-1-deoxyglucitol derivative of $G_{M1}OS$; DSS, disuccinimidyl suberate; DMEM/HEPES, Dulbecco's minimal essential medium buffered with 25 mM HEPES; PE, phosphatidylethanolamine; PS, phosphatidylserine. Abbreviations of neoglycolipids and their structures are shown in Figure 1.

cells (Pacuszka & Fishman, 1990). We used G_{M1}-deficient rat glioma C6 cells and found that although the modified cells bind high levels of cholera toxin, its binding did not result in adenylate cyclase activation. On the other hand, modification of the ceramide moiety of G_{M1} by replacing the fatty acid with acetic acid increases its "effectiveness" as measured by higher adenylate cyclase activation (Fishman et al., 1980). As a continuation of these studies, we have coupled $G_{M1}OS$ to lipids of different structure such as aliphatic amines, phospholipids, and cholesteryl hemisuccinate. We have determined whether or not these neoglycolipids can be inserted into the plasma membrane of viable rat glioma C6 cells and act as functional receptors for cholera toxin.

Experimental Procedures

Materials. Cholera toxin was purchased from List Biological Laboratories (Campbell, CA) and (for radioiodination) from ICN Biochemicals (Cleveland, OH). Cholesteryl hemisuccinate, phosphatidyl-L-serine (bovine brain), 5-isobutyl-3-methylxanthine, and stearylamine (octadecylamine) were from sigma (St. Louis, MO). Other aliphatic amines (decyl to hexadecyl), 1,8-diaminooctane, *N*, *N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, and phosphatidyl-L-ethanolamine (dipalmitoyl, synthetic) were from Fluka (Ronkonkoma, NY). Disuccinimidyl suberate was from Pierce Chemical Co. (Rockfort, IL) and sodium cyanoborohydride was from Aldrich (Milwaukee, WI). $NaCNB^3H_3$ (4.7 Ci/mmol) was from Amersham Corp. (Arlington Heights, IL). The preparation of G_{M1} and $G_{M1}OS$ has recently been described (Pacuszka & Fishman, 1990).

Preparation of $G_{M1}OS$ Derivatives of Phospholipids and Aliphatic Amines. $G_{M1}OS$ was coupled to PE, PS, and aliphatic amines via reductive amination in the presence of $NaCNBH_3$. The procedure used was the same as described by Stoll et al. (1988), but the reaction time was extended to 40 h to improve the yields.

Preparation of $G_{M1}OSNH$-X-PE and $G_{M1}OSNHC_8NH$-X-PE. $G_{M1}OS$ was reductively aminated to $G_{M1}OSNH_2$ (Schwarzmann et al., 1983), which was coupled to phosphatidylethanolamine with the cross-linking reagent DSS. Briefly, $G_{M1}OSNH_2$ (5 μmol) and phosphatidylethanolamine (50 μmol) were suspended in 20 mL of anhydrous chloroform/methanol (1:1 v/v) containing 0.1% triethylamine, purged with argon, and sonicated for 10 min in a cleaning bath. Then DSS was slowly added stepwise with stirring (2 μmol in 200 μL of chloroform each time) for 2 h until the molar ratio of $G_{M1}OSNH_2$:phosphatidylethanolamine:DSS of 1:10:5.6 was reached. The reaction was allowed to proceed for an additional 2 h with four more additions of DSS. Finally, 5 μmol of DSS in chloroform/methanol (1:1 v/v) was added, and the reaction mixture was stirred overnight. For each addition, DSS was freshly dissolved.

For the preparation of $G_{M1}OSNHC_8NH$-X-PE, the same procedure was used. $G_{M1}OSNHC_8NH_2$ was obtained by reductive amination of $G_{M1}OS$ with an excess of 1,8-diaminooctane. Briefly, $G_{M1}OS$ (10 μmol) was dissolved in 5 mL of methanol containing 500 μmol of 1,8-diaminooctane, purged with argon, and left for 2 h at 50 °C. Then 7 mg of $NaCNBH_3$ was added and reaction allowed to proceed for 40 h at 50 °C. The reaction mixture was subsequently dried under argon, and excess $NaCNBH_3$ was decomposed with acetic acid. The modified oligosaccharide was desalted on a Bio-Gel P-2 column (1 × 80 cm) and purified on a CM-cellulose column (1 × 40 cm) (Zopf et al., 1978).

Preparation of $G_{M1}OSNH$-X-CHOL. The cholesterol derivative of $G_{M1}OS$ was prepared from $G_{M1}OSNH_2$ and cholesteryl hemisuccinate by two-step procedure. First, the carboxylic group of cholesteryl hemisuccinate was reacted with *N*-hydroxysuccinimide in the presence of dicyclohexylcarbodiimide to form an amino ester; second, the latter was coupled via an amide bond with the amino group of $G_{M1}OSNH_2$. Briefly, cholesteryl hemisuccinate (200 μmol), *N*-hydroxysuccinimide (190 μmol), and dicyclohexylcarbodiimide (200 μmol) were stirred in 1 mL of dry dimethylformamide at room temperature. Then 1 mL of dry chloroform was added and the reaction mixture clarified by filtration. The filtered material was concentrated under argon to semidryness, redissolved in benzene, and lyophilized. The resulting white powder was stored under vacuum over phosphorus pentoxide. In the second step, $G_{M1}OSNH_2$ (5 μmol) was stirred overnight at room temperature with 150 μmol of the above amino ester of cholesteryl hemisuccinate in 8 mL of dry dimethylformamide containing 50 μmol of triethylamine. Then excess triethylamine was neutralized with acetic acid and the reaction mixture dried under argon.

Purification of Neoglycolipids. A similar procedure was used for the purification of all neoglycolipids. The dried reaction product was dissolved in a small volume of chloroform/methanol (2:1 v/v). Chloroform was added to increase the ratio to 9:1, and the sample was applied to a small silica gel column packed in chloroform. The weight ratio of neoglycolipid to silica gel was about 1:1000. The column was washed with 10 column volumes of chloroform/methanol (9:1 v/v) followed by 5 column volumes of chloroform. Neoglycolipids were eluted with solvent A, chloroform/methanol/aqueous 0.25% $CaCl_2$ (60:35:8 v/v/v), and their purity was checked by thin-layer chromatography with the same solvent (Fishman et al., 1979). Appropriate fractions were combined and repurified on Sephadex G-25 Superfine columns packed in chloroform/methanol/water (60:30:4.5 v/v/v) (Fishman et al., 1979). The weight ratio of neoglycolipid to Sephadex was about 1:500. $G_{M1}OSNHC_{12}$ was purified further by preparative thin-layer chromatography with solvent system B, chloroform/methanol/0.25% $CaCl_2$ (5:4:1 v/v/v).

Partial Characterization of Neoglycolipids. Neoglycolipids were analyzed on precoated silica gel plates (E. M. Merck) in solvent system A or B. For the detection of separated neoglycolipids, the plates were exposed to iodine vapors or sprayed with water. The following sprays were used to detect specific components: orcinol (hexoses), resorcinol (sialic acid), molybdenum blue (phospholipid), phosphotungstic acid (cholesterol), and acetic anhydride/sulfuric acid (Liebermann–Burchard assay for cholesterol). Carbohydrate compositional analyses were performed by anion-exchange chromatography with pulsed amperometric detection (Hardy, 1989) as outlined in the Dionex Workbook and Manual (Dionex Corp., Sunnyville, CA). To release monosaccharides, samples (10 nmol) were hydrolyzed in 2 M HCl at 100 °C for 2 h. For characterization of sialic acid, mild acid hydrolysis was used, i.e., 2% acetic acid at 100 °C for 2 h.

Cells and Cell Culture. Rat glioma C6 cells were cultured as described (Zaremba & Fishman, 1984) except that the medium was supplemented with 5% Nu-Serum IV (Collaborative Research, Waltham, MA) instead of fetal bovine serum. For most of the in situ toxin binding experiments, cells were grown in 24- × 16-mm wells of cluster dishes. For other experiments 12- × 22-mm and 6- × 35-mm clusters as well as flasks were used.

Treatment of Cells with Glycolipids. Stock solutions of neoglycolipids and G_{M1} in organic solvents were stored at −80 °C. Portions were dried under argon, traces of solvents re-

moved under vacuum, and the residues redissolved in water. Neoglycolipids were added to serum-free DMEM/HEPES as described for G_{M1} and other gangliosides (Fishman et al., 1980). After the cells were incubated with the glycolipids at 37 °C for 1 h unless otherwise indicated, the cells were washed three times with ice-cold Dulbecco's phosphate-buffered saline. Cells were used promptly for ^{125}I cholera toxin binding, cAMP accumulation, and studies on formation of toxin A_1 peptide. For ^{125}I toxin binding to membranes and adenylate cyclase assays, cells were lysed in 1 mM Tris-HCl/2 mM EDTA, pH 7.4 (lysing buffer) at 4 °C, for 5–10 min.

Binding of ^{125}I Cholera Toxin. Binding to cells in clusters was done as described by Spiegel (1985). In some experiments, binding was done at 4 °C for 1 h in Eagle's minimal essential medium buffered with 25 mM HEPES. Binding to cell membranes in suspension was performed on crude membrane preparations according to the method of Miller-Podraza et al. (1982). Briefly, cell lysates were centrifuged at 39000*g* for 30 min. Pellets were suspended in a small volume of the lysing buffer; after removal of "nucleoproteins" (Zaremba & Fishman, 1984), the crude membranes were used immediately or frozen at −80 °C. Portions of the membrane preparations were incubated with ^{125}I cholera toxin and unlabeled toxin, as indicated, in 200 μL of assay buffer containing 0.1% bovine serum albumin in a 96-well GV Millititer plate (Millipore Corp.) for 1 h at room temperature, vacuum-filtered, and washed three times with about 200 μL of ice-cold wash buffer (Pacuszka & Fishman, 1990). The filters were cut out and the bound radioactivity was determined in a γ counter.

Determination of cAMP and Adenylate Cyclase Activity. The treated and washed cells were incubated at 37 °C in DMEM/HEPES containing 1 mM isobutylmethylxanthine and 0.01% bovine serum albumin with or without 10 nM cholera toxin. After the indicated times, the medium was rapidly aspirated, and cAMP concentration was determined by a radioimmune assay (Zaremba & Fishman, 1984). For the determination of adenylate cyclase activity, cell lysates were centrifuged at 150*g* at 4 °C for 2 min. Portions of the supernatants (10 μg of protein) were assayed as described previously (Zaremba & Fishman, 1984).

Generation of A_1 Peptide of Cholera Toxin. The method of Kassis et al. (1982) was followed. Briefly, cells were incubated with glycolipids in 6- × 35-mm well clusters and washed as described above. One milliliter of ice-cold Eagle's minimal essential medium buffered with 25 mM HEPES and containing ^{125}I cholera toxin (5 nM, 6×10^6 cpm per well) and 0.1% BSA was added. Binding was allowed to proceed for 45 min at 4 °C. Then the cells were washed as before and incubated in fresh DMEM/HEPES containing 0.01% BSA at 37 °C for 60 min. The cells were extracted as described (Kassis et al., 1982), and the amount of A_1 peptide generated was determined after SDS–polyacrylamide gel electrophoresis by counting the appropriate gel slices in a γ counter.

Other Methods. Sialic acid content was determined by its reaction with resorcinol (Svennerholm & Fredman, 1980) and protein concentration by the method of Lowry et al. (1951).

Results

Partial Characterization of Neoglycolipids. The neoglycolipids that we synthesized are shown in Figure 1. For all of the neoglycolipids analyzed, the ratio of sialic acid to galactose to galactosamine was close to 1:2:1. Glucitol was not detected, while glucose was found only in small or trace amounts. Sialic acid released during mild acid hydrolysis was identified as *N*-acetylneuraminic acid; other *N*-acyl-substituted or deacylated derivatives were not detected. Neoglycolipids

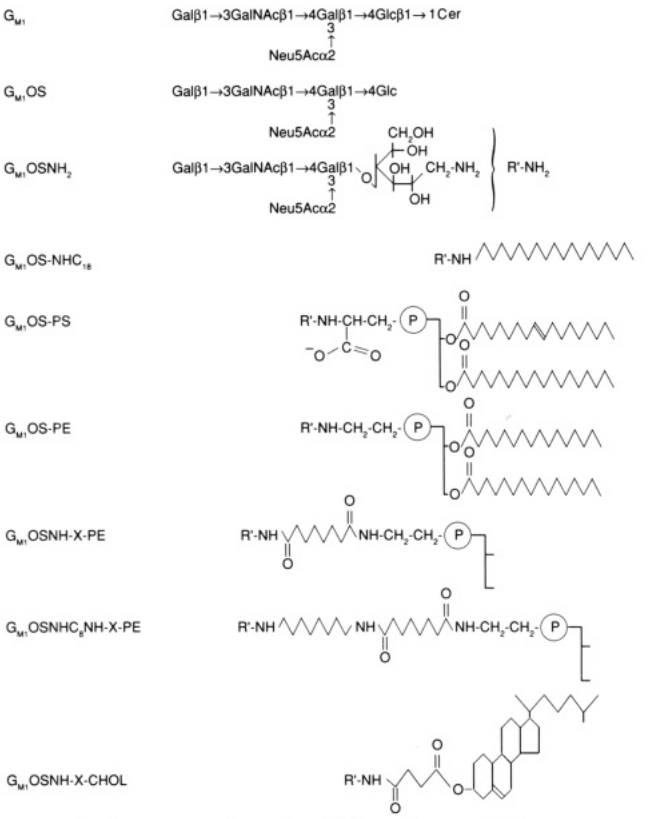

FIGURE 1: Structures of neoglycolipid analogues of G_{M1}.

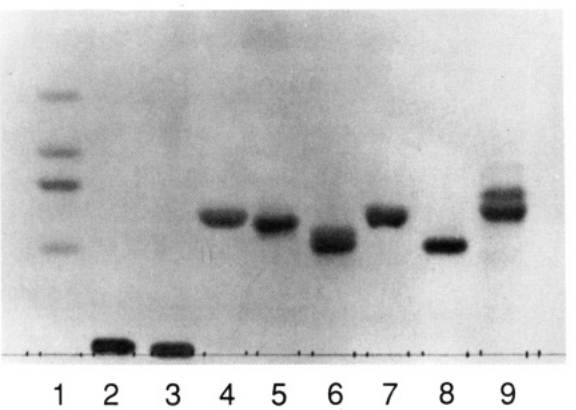

FIGURE 2: Analysis of neoglycolipids by thin-layer chromatography. Portions (5 nmol) of G_{M1}OS (lane 2), $G_{M1}OSNH_2$ (lane 3), G_{M1}OSNH-X-CHOL (lane 4), $G_{M1}OSNHC_{18}$ (lane 5), G_{M1}OS-PS (lane 6), G_{M1}-PE (lane 7), G_{M1}OSNH-X-PE (lane 8), and $G_{M1}OSNHC_8$NH-X-PE (lane 9) were chromatographed on thin-layer silica gel in solvent system A and detected with orcinol spray as described under Experimental Procedures. Lane 1 shows ganglioside standards from top to bottom: G_{M3}, G_{M2}, G_{M1}, and G_{D1a} (1 nmol each of sialic acid).

had much higher mobilities on thin-layer silica gel in solvent system A than G_{M1}OS and $G_{M1}OSNH_2$ and migrated between the G_{M1} and G_{D1a} standards (Figure 2). Under the same conditions, their unmodified lipid moieties migrated close to the solvent front (not shown). All neoglycolipids were stained on the thin-layer chromatograms with orcinol (hexoses) and resorcinol (sialic acid), while only phospholipid-based neoglycolipids gave a blue color with molybdenum blue reagent at room temperature. The cholesterol derivative stained pink-grey with phosphotungstic acid and greenish blue with Liebermann–Burchard reagent. When the thin-layer chromatograms were first exposed to iodine vapors or sprayed with water, all of the spots detected also were stained with orcinol or resorcinol. The yields of the various neoglycolipids were

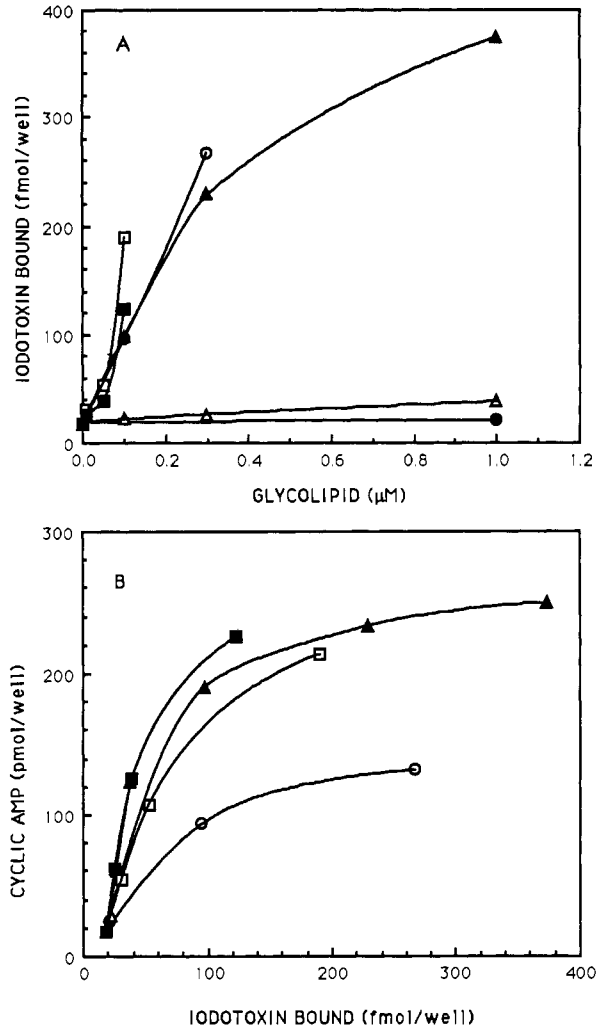

FIGURE 3: Cholera toxin binding and cAMP accumulation in rat glioma C6 cells treated with G_{M1} and aliphatic amine derivatives of G_{M1}OS. Cells were incubated with the indicated concentrations of (○) G_{M1}, (●) $G_{M1}OSNHC_{10}$, (△) $G_{M1}OSNHC_{12}$, (▲) $G_{M1}OSNHC_{14}$, (□) $G_{M1}OSNHC_{16}$, and (■) $G_{M1}OSNHC_{18}$ for 1 h at 37 °C, washed, and assayed for specific ^{125}I cholera toxin binding (A, B) and cholera toxin stimulated cAMP accumulation (B) as described under Experimental Procedures.

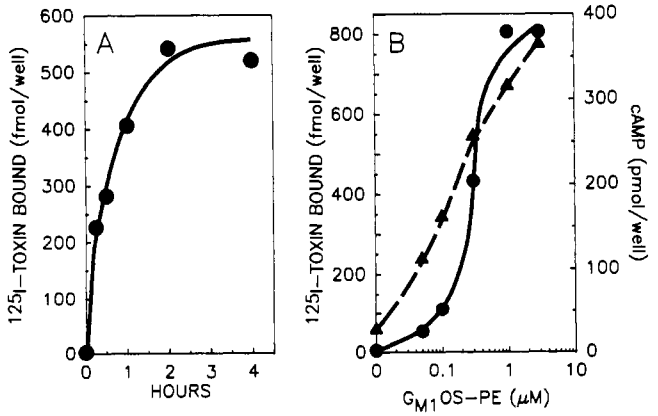

FIGURE 4: Effect of time and concentration on uptake of G_{M1}OS-PE by rat glioma C6 cells. Cells were incubated (A) with 1 μM G_{M1}OS-PE for the indicated times or (B) for 1 h with the indicated concentrations of G_{M1}OS-PE at 37 °C. The cells then were washed and assayed for (●) specific ^{125}I cholera toxin binding or (▲) cholera toxin activated cAMP accumulation as described under Experimental Procedures.

over 80% and, after final purification, about 50–60% on the basis of sialic acid recovery.

Table I: Effect of Neoglycolipid Treatment on Rat Glioma C6 Cells As Measured by Cholera Toxin Binding and Stimulation of cAMP Accumulation[a]

treatment	concn (μM)	^{125}I toxin bound (fmol/well)	cAMP content (pmol/well)
control		7.1	35.5
G_{M1}	0.1	108	172
	0.3	443	229
G_{M1}OS-PE	0.1	327	210
	0.3	1204	267
G_{M1}OS-PS	0.1	252	169
	0.3	1380	255
G_{M1}OSNH-X-PE	0.1	752	112
	0.3	2021	136
$G_{M1}OSNHC_8NH$-X-PE	0.1	233	52.4
	0.3	408	86.0
$G_{M1}OS$-NHC_{18}	0.1	403	333
G_{M1}OSNH-X-CHOL	0.05	183	274
	0.1	373	312

[a] The cells were treated with G_{M1} and neoglycolipids at the indicated concentrations for 1 h at 37 °C, washed, and assayed for specific ^{125}I cholera toxin binding and cholera toxin stimulated cAMP accumulation as described under Experimental Procedures. Each 16-mm well of cells usually contained 100 μg of protein; values were corrected for variations in protein concentration and are expressed as the means of triplicate determinations, which varied less than 10%. Each experiment was repeated at least twice with similar results.

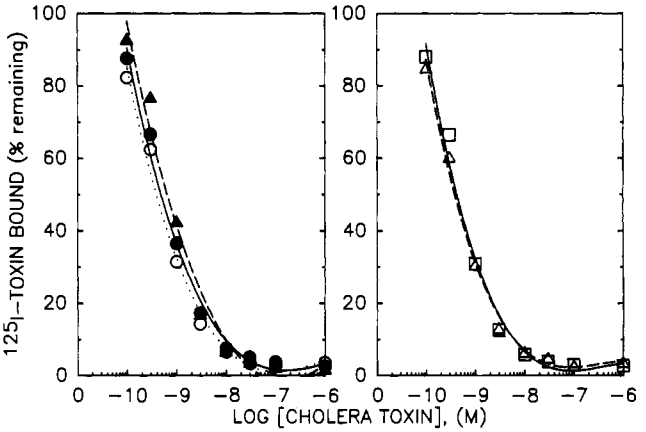

FIGURE 5: Competition binding of cholera toxin to membranes from rat glioma C6 cells treated with G_{M1} or neoglycolipids. Membranes were prepared from cells treated with (●) G_{M1}, (○) G_{M1}OS-PE, (□) G_{M1}OSNH-X-PE, (△) $G_{M1}OSNHC_8NH$-X-PE, and (▲) G_{M1}OSNH-X-CHOL, and portions were incubated with 0.13 nM ^{125}I cholera toxin in the presence of increasing concentrations of unlabeled toxin as described under Experimental Procedures.

Incorporation of Neoglycolipids into C6 Cells. In most of the experiments, incorporation of glycolipids into cells was detected by measuring the increase in ^{125}I cholera toxin binding after incubating the cells in serum-free medium containing the glycolipids. By this criterion, most of the neoglycolipids were taken up by the cells and incorporated into cell surface membranes (Table I and Figure 3A). There appeared to be differences in the amounts taken up. When present in the medium at the same concentration, most of the neoglycolipids were incorporated more extensively than G_{M1}. Exceptions were $G_{M1}OSNHC_{10}$, which was not incorporated at all, and $G_{M1}OSNHC_{12}$, which was taken up poorly (Figure 3A). As studied in more detail for G_{M1}OS-PE, incorporation was time dependent for at least 2 h (Figure 4A) and concentration dependent up to approximately 1 μM (Figure 4B).

Characterization of ^{125}I Cholera Toxin Binding to Neoglycolipids. The affinity of cholera toxin for the various neoglycolipids incorporated into C6 cell surface membranes was determined by competition binding (Figure 5). The

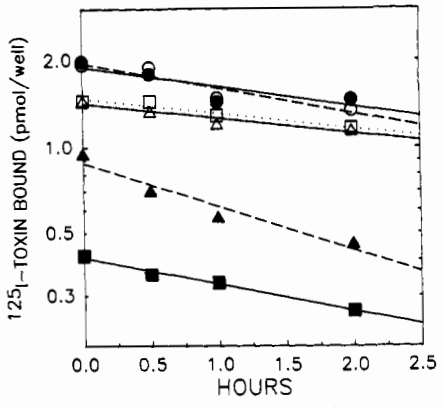

FIGURE 6: Disappearance of toxin binding sites from rat glioma C6 cells treated with neoglycolipids. Cells were incubated at 37 °C for 1.5 h with (●) 1 μM G_{M1}, (○) 1 μM G_{M1}OS-PE, (□) 0.3 μM G_{M1}OSNH-X-PE, (△) 1 μM G_{M1}OSNHC$_8$NH-X-PE, (▲) 0.1 μM G_{M1}OSNH-X-CHOL, or (■) 0.1 μM G_{M1}OSNHC$_{18}$. The cells were washed and incubated in fresh medium at 37 °C for the indicated times. Then the cells were assayed for specific ^{125}I cholera toxin binding at 4 °C as described under Experimental Procedures.

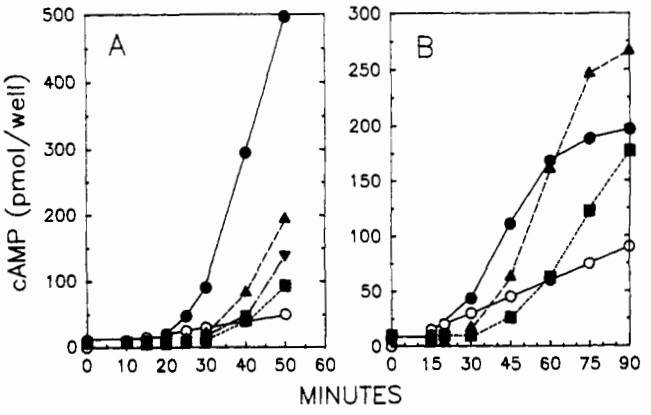

FIGURE 7: Time course for stimulation of cAMP accumulation by cholera toxin in control and neoglycolipid-treated rat glioma C6 cells. (A) Cells were incubated at 37 °C for 1 h with (○) no addition or with 0.1 μM (●) G_{M1}, (▲) G_{M1}OS-PE, (▼) G_{M1}OS-PS, or (■) G_{M1}OSNH-X-PE. The cells then were washed and incubated in medium containing 1 mM IBMX and 10 nM cholera toxin and assayed for cAMP content at the indicated times. (B) Cells were incubated at 37 °C for 1 h with (○) no addition or with 0.1 μM (●) G_{M1}, (▲) G_{M1}OS-PE, or (■) G_{M1}OSNH-X-PE. The cells were washed and incubated in medium containing 10 nM cholera toxin at 4 °C for 30 min. After the cells were washed once with ice-cold phosphate-buffered saline, they were incubated in medium containing 1 mM IBMX at 37 °C for the indicated times and assayed for cAMP content.

displacement curves obtained for ^{125}I cholera toxin binding to membranes from cells treated with G_{M1} and with its neoglycolipid analogues were essentially superimposable, with half-maximal inhibition occurring between 0.5 and 0.7 nM unlabeled cholera toxin.

The incorporation of the neoglycolipids also was studied directly with ^{3}H-labeled G_{M1}OS-PE, G_{M1}OS-NHC$_{18}$, and G_{M1}OSNH-X-CHOL and was found to be similarly time dependent (data not shown). Initially, the molar ratio of neoglycolipid incorporated into cells to cholera toxin bound was between 5:1 and 6.5:1, which is characteristic for multivalent binding as is the case for G_{M1} taken up by C6 cells (Fishman et al., 1983). With longer times of exposure, the molar ratio increased, indicating that some of the inserted neoglycolipids may be undergoing internalization.

To follow the disappearance of the cell surface neoglycolipids, cells were incubated at 37 °C for 90 min with G_{M1} or neoglycolipids, washed, and incubated in fresh medium at 37 °C. At different times, the cells were then assayed for ^{125}I cholera toxin binding (Figure 6). The cholesterol and octadecylamine derivatives of G_{M1} seemed to disappear more rapidly than G_{M1}, whereas the derivatives based on PE seemed to disappear at a rate similar to G_{M1}. On the basis of our previous studies with G_{M1}, this disappearance represents internalization (Fishman et al., 1982).[2]

Cholera Toxin Stimulated cAMP Accumulation in Neoglycolipid-Treated C6 Cells. Incubation of C6 cells with G_{M1} and neoglycolipids resulted in not only increased toxin binding but also an enhanced response to cholera toxin as measured by cAMP accumulation (Table I). In most cases, the magnitude of the cAMP response increased with increased ability to bind cholera toxin until a maximal response was obtained, as shown for cells treated with G_{M1} and G_{M1}OS-NHC$_{14}$ (Figure 3B) and G_{M1}OS-PE (Figure 4B). The magnitude of the response, however, was not the same for various neoglycolipids relative to the increase in toxin binding. The "effectiveness" of glycolipid receptors seemed to be related to the structure of their lipid moiety as the phospholipid derivatives were less effective than G_{M1} and the hexadecylamine and cholesterol derivatives were more effective. Furthermore, the insertion of a spacer between the phospholipid and oligosaccharide moieties apparently diminished its effectiveness. G_{M1}OS-PE was more effective than G_{M1}OSNH-X-PE, and G_{M1}OSNHC$_8$NH-X-PE was the least effective. Thus, in a separate series of experiments, cell treated with G_{M1} or the latter three neoglycolipids bound similar amounts of toxin (374–393 fmol/well), but the fold stimulation of cAMP accumulation by the toxin compared to control cells was 10.3, 5.9, 3.6, and 2.2, respectively.

Although the chain length of the aliphatic amine had a pronounced effect on the ability of the resulting neoglycolipid to be incorporated into the cells (Figure 3A), it had less of an effect on the ability of the inserted derivative to act as a functional receptor for cholera toxin (Figure 3B). The decylamine derivative was not taken up and thus had no effect on the cAMP response (24.3 pmol/well compared to 17.4 for control cells). The dodecylamine derivative, which was taken up poorly, did confer an enhanced response to cholera toxin. All of the aliphatic amine derivatives that were incorporated by the cells were much more "efficient" receptors than G_{M1} because cells binding less toxin produced the same or greater amounts of cAMP (Figure 3B).

Characteristics of the Lag Phase. Cells exposed to cholera toxin exhibit a characteristic lag before an increase in either cAMP levels or adenylate cyclase activity is observed (Fishman, 1980). When C6 cells pretreated with G_{M1} or the neoglycolipids derived from octadecylamine and cholesterol were incubated at 37 °C in the presence of cholera toxin, there was no increase in cAMP levels until 20 min, after which time levels increased with similar kinetics (data not shown). In contrast, cells pretreated with neoglycolipids derived from the phospholipids exhibited a longer lag period (Figure 7A). In order to preclude the possibility that these differences might be due to variations in the rates of toxin binding, binding was allowed to occur at 4 °C. The temperature of the medium was then shifted to 37 °C, and the time course of cAMP accumulation was determined (Figure 7B). Again, the cells treated with neoglycolipids based on PE had a longer lag period than those treated with G_{M1}; however, by 1 h cAMP levels in the cells treated with G_{M1}OS-PE and with G_{M1} were the

[2] Preliminary experiments indicate that some of the G_{M1}OS-PE, G_{M1}OSNH-X-CHOL, and G_{M1}OS-NHC$_{18}$ taken up by C6 cells underwent metabolism.

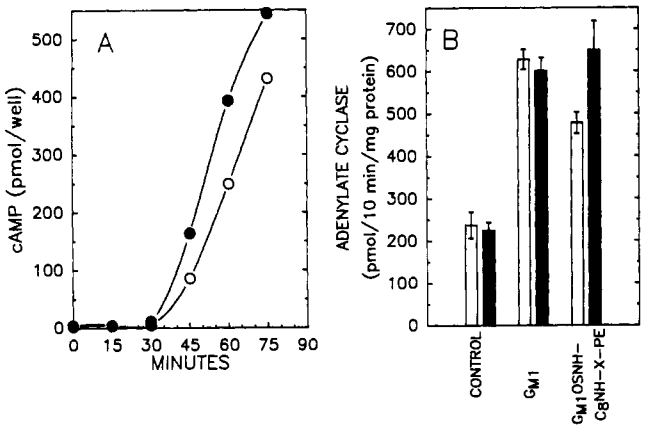

FIGURE 8: Effect of chloroquine on response of cells treated with $G_{M1}OSNHC_8NH$-X-PE to cholera toxin. (A) Cells were incubated with 1 μM $G_{M1}OSNHC_8NH$-X-PE in the (○) absence and (●) presence of 0.1 mM chloroquine for 1 h at 37 °C. The cells then were washed and incubated in medium containing 1 mM IBMX and 10 nM cholera toxin with and without chloroquine. At the indicated times, the cells were assayed for cAMP content. Chloroquine had no effect on the initial uptake of neoglycolipid, as control cells and chloroquine-treated cells bound 14.6 and 14.5 pmol of ^{125}I toxin/mg of protein, respectively. (B) Cells were incubated without or with 0.3 μM G_{M1} or $G_{M1}OSNHC_8NH$-X-PE for 1 h in the absence (open bars) or presence (closed bars) of chloroquine, washed, incubated with cholera toxin for 50 min, and assayed for adenylate cyclase activity as described under Experimental Procedures.

same, and the levels in the former began to exceed those in the latter. A similar situation was reached by 90 min in the cells treated with $G_{M1}OSNH$-X-PE.

Effects of Chloroquine and Monensin on Effectiveness of Neoglycolipids as Receptors. The differences in lag times described above raised the possibility that cholera toxin bound to cell surface neoglycolipids derived from phospholipids may be internalized and processed by a pathway different from that of toxin bound to G_{M1}. Janicot et al. (1988) reported that, in isolated rat heptocytes, chloroquine and monensin inhibited activation of adenylate cyclase by cholera toxin. Chloroquine (0.1 mM) had its most pronounced effect on C6 cells treated with $G_{M1}OSNHC_8NH$-X-PE and caused a significant increase in their response to cholera toxin (Figure 8A). This enhancement was also observed when adenylate cyclase activity was directly measured (Figure 8B). In some but not all experiments, chloroquine also potentiated to a small extent cholera toxin stimulated cAMP production in cells treated with the other phospholipid derivatives. In contrast, chloroquine had a no effect (Figure 8B) or sometimes a small inhibitory effect on cells treated with G_{M1} or the cholesterol derivative (data not shown). Monensin had no significant effect on the responsiveness of cells treated with G_{M1} to cholera toxin but enhanced the responsiveness of cells treated with $G_{M1}OSNHC_8NH$-X-PE (Table II).

Generation of A_1 Peptide of Cholera Toxin. C6 cells treated with G_{M1} or neoglycolipids were incubated with ^{125}I cholera toxin at 4 °C, washed, and incubated for 1 h at 37 °C in fresh medium. Then the amount of A_1 peptide formed was determined. As previously reported (Kassis et al., 1982), this represented between 1% and 2% of the total bound toxin, and no substantial differences were observed among the different neoglycolipids. In cells exposed to chloroquine, a 2.5–4-fold increase in the amount of A_1 peptide generated was observed. In contrast, Janicot et al. (1988) had reported that chloroquine reduced the generation of A_1 peptide in rat hepatocytes by 2–4-fold. The effect of chloroquine was not unique to C6 cells as we observed a similar increase in the generation of A_1 peptide in mouse neuroblastoma cells (data not shown).

Table II: Effect of Monensin on Cholera Toxin Mediated cAMP Accumulation by Rat Glioma C6 Cells Treated with Different Glycolipids[a]

glycolipid treatment	cyclic AMP accumulation (pmol/well)	
	−monensin	+monensin
none	14.6 ± 1.8	16.7 ± 2.9
G_{M1}	169 ± 17.8	155 ± 2.7
$G_{M1}OS$-PE	130 ± 15.9	147 ± 18.0
$G_{M1}OSNHC_8NH$-X-PE	36.5 ± 1.7	76.5 ± 6.7

[a] The cells were treated with 0.3 μM glycolipid for 30 min at 37 °C, and 10 μM monensin was added as indicated. After an additional 30 min, the cells were washed and incubated in fresh medium containing 1 mM IBMX plus or minus monensin at 37 °C. After 5 min, cholera toxin was added, and the cells were incubated for an additional 50 min and assayed for cAMP as described under Experimental Procedures. The values represent the means ± SD of triplicate determinations from a representative experiment.

Discussion

In the present study, we utilized relatively simple chemical procedures to covalently attach the oligosaccharide moiety of G_{M1} to defined lipids. Either $G_{M1}OS$ was directly attached via reductive amination or $G_{M1}OS$ was reductively aminated and coupled to the lipid. With these methods, the neoglycolipid analogues of G_{M1} were prepared in good yield. Carbohydrate compositional analyses indicated that the oligosaccharide moiety remained intact except for the expected inability to detect glucose due ot its reductive amination. Most of the neoglycolipids migrated on thin-layer chromatography as homogeneous spots. $G_{M1}OS$-PS was more diffuse due to its fatty acid composition represented by stearic (41%) and oleic (27%). $G_{M1}OSNHC_8NH$-X-PE migrated as two spots; the reason for this heterogeneity remains unknown. Because both spots reacted with cholera toxin when the chromatogram was overlaid with ^{125}I toxin (not shown) and, after incubation with C6 cells, only one class of binding sites was formed, we did not attempt to separate the two components.

All of the neoglycolipids that we synthesized were readily taken up and incorporated into the surface membrane of rat glioma C6 cells. This was determined on the basis of the observed increase in ^{125}I cholera toxin binding. These cells are deficient in G_{M1} and bind very little (Fishman, 1980). The only neoglycolipid that failed to be incorporated was $G_{M1}OSNHC_{10}$. Possibly the hydrocarbon moiety is too short or insufficiently hydrophobic to be inserted into the lipid bilayer of the plasma membrane. All of the other neoglycolipids except $G_{M1}OSNHC_{12}$ appeared to be taken up more effectively than G_{M1} when present in the medium at the same low concentrations. We had obtained similar results with a G_{M1} derivative in which the fatty acid had been replaced with an acetyl group (Fishman et al., 1980). Even though incorporation of the neoglycolipids was more effective than G_{M1}, the affinity of cholera toxin for these different receptors was essentially the same. This is consistent with there being only one class of high-affinity receptors present on the cell membrane irrespective of the nature of the lipid moiety.

We examined the uptake of $G_{M1}OS$ derivatives of PE, octadecylamine, and cholesterol in more detail by using ^3H-labeled neoglycolipids. Uptake was time dependent as had been observed for $[^3H]G_{M1}$ (Fishman et al., 1980). In addition, binding of cholera toxin to the cellular neoglycolipids appeared to be multivalent, as the ratio of bound toxin to incorporated neoglycolipids was similar to that obtained for C6 cells treated with G_{M1} (Fishman et al., 1983). As each toxin can bind to five $G_{M1}OS$, most of the neoglycolipids initially taken up by the cell are on the surface. With time, however, the neo-

glycolipids appeared to be internalized, as is G_{M1} (Fishman et al., 1983).

The neoglycolipids differed most in their effectiveness as functional receptors when the effectiveness was measured by cAMP accumulation in cells that had bound comparable amounts of cholera toxin. The most efficient receptors had a medium- to long-chain aliphatic amine or cholesterol as their lipid moiety. In this regard, G_{M1} in which the fatty acid had been replaced with an acetyl group is much more efficient than G_{M1} itself (Fishman et al., 1980). The length of the characteristic lag period before a rise in cAMP levels is observed was the same for these neoglycolipids and for G_{M1}. Thus, the enhanced response was not due to a more rapid activation of adenylate cyclase. The less efficient receptors were those derived from phospholipids, especially those in which a spacer had been inserted between the oligosaccharide and the phospholipid. Thus, the order of effectiveness was G_{M1} > G_{M1}OS-PE > G_{M1}OSNH-X-PE > G_{M1}OSNHC$_8$NH-X-PE. With these derivatives, we observed a longer lag period before cholera toxin dependent cAMP levels began to rise. One simple explanation is that the oligosaccharide is displaced farther away from the membrane by the spacers. It is still controversial as to whether the oligosaccharide of G_{M1} lies on the same axis as the ceramide and extends straight out from the membrane or is bent toward it (Tettamanti, 1988). In addition, the hydrocarbon spacers may increase the tendency of the oligosaccharide to bend toward the cell surface. Finally, the cholesterol derivative also has a spacer between G_{M1}OS and the lipid (see Figure 1), yet it is a very efficient receptor.

It is believed that, during the lag period, the A component of cholera toxin penetrates into the lipid bilayer of the membrane and is reduced to generate the active A_1 peptide, which then catalyzes the ADP-ribosylation of the stimulatory G protein of adenylate cyclase at the cytoplasmic side of the membrane (Fishman, 1990). The length of the lag period is dependent on a number of factors including the density of toxin receptors, the amount of toxin bound, and the temperature (Fishman, 1980). Although the role that the lipid moiety of G_{M1} plays in promoting the penetration of the A subunit across the membrane is unknown, cholera toxin induces a perturbation in model membranes containing G_{M1} (Moss et al., 1977). It has been suggested the perturbation arises from the toxin-induced multivalent clustering of the gangliosides into a microdomain of the membrane (Fishman, 1990). Thus, the phospholipid derivatives may be less effective in promoting this process, as reflected in the longer lag period.

On the other hand, the longer lag period may be due to the bound cholera toxin entering the cell through another pathway. On the basis of the inhibitory effects of chloroquine and monensin, it has been proposed that, in hepatocytes, endocytosis of cholera toxin is required for its activation of adenylate cyclase (Houslay & Elliott, 1981; Janicot et al., 1988). In contrast, we found that chloroquine had no or a slight inhibitory effect on toxin action in G_{M1}-treated rat glioma C6 cells[3] and actually potentiated the response in cells treated with the phospholipid derivatives without altering the lag period. Monensin appeared to have effects similar to those of chloroquine; it did not alter the responsiveness of G_{M1}-treated cells and enhanced that of cells treated with G_{M1}OSNHC$_8$NH-X-PE. These results are consistent with the possibility that cholera toxin bound to neoglycolipids derived from phospholipids is undergoing endocytosis instead of directly penetrating the plasma membrane. The A component or its active A_1 peptide must still penetrate the endosomal or lysosomal membrane in order to gain access to its cellular target G protein. In this regard, we found that chloroquine caused an increase in the amount of A_1 peptide generated by the cells irrespective of the nature of the toxin receptor. In hepatocytes, however, chloroquine was reported to cause a decrease in the formation of A_1 peptide (Janicot et al., 1988).

These differences may reflect differences in cell type as well as differences in the intracellular compartments in which cholera toxin is processed. In hepatocytes, cholera toxin bound to cell surface G_{M1} may undergo endocytosis and be processed in the mildly acidic milieu of endocytic vesicles, thus explaining the inhibitory effects of both chloroquine and monensin (Janicot et al., 1988). In contrast, toxin bound to cell surface G_{M1} of rat glioma C6 cells as well as other types of cells[3] may be processed at the plasma membrane (Fishman, 1990). Finally, toxin bound to phospholipid derivatives of G_{M1}OS on the surface of C6 cells may undergo endocytosis and processing in the more acidic lysosomal compartment. This may explain the positive effect of both chloroquine and monensin. Although the effects of monensin are usually associated with disruption of membrane vesicular transport through the Golgi apparatus (Tartakoff, 1983), the ionophore catalyzes the exchange of sodium ions with protons across membranes and thus should cause alkalization of lysosomes (Pressman, 1976).

In summary, our results indicate that the nature of the moiety to which G_{M1}OS is conjugated is not important for the binding of cholera toxin as long as the conjugate is able to insert into the cell membrane. It is important, however, for the subsequent ability of cholera toxin to activate adenylate cyclase. In this regard, they complement our recent studies on attaching G_{M1}OS to cell surface proteins (Pacuszka & Fishman, 1990). There we demonstrated that although cholera toxin binds to such neoganglioproteins, it is unable to activate adenylate cyclase in rat glioma C6 cells. We believe that the neoglycolipid analogues of G_{M1} may be useful for exploring the function of gangliosides in other systems. G_{M1} has neuritogenic and neuronotrophic effects both in vitro and in vivo (Ledeen, 1984) and has been implicated as a biphasic modulator of cell growth (Spiegel & Fishman, 1987).

[3] The lack of a consistent and substantial inhibitory effect of chloroquine or other lysosomotrophic compounds such ammonium chloride and methylamine on cholera toxin action also was observed in murine neuroblastoma NB41A cells, human fibroblasts, and Friend erythroleukemic cells. All of these agents were very effective in inhibiting the metabolism of bound ^{125}I cholera toxin to trichloracetic acid soluble material.

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Transcriptional Activation of the Lipoprotein Lipase Gene in Macrophages by Dexamethasone†

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ABSTRACT: The effect of dexamethasone on lipoprotein lipase (LPL) gene expression during macrophage differentiation was investigated by using the human monocytic leukemia cell line THP-1 and human monocyte-derived macrophages. Addition of dexamethasone to THP-1 cells increased steady-state levels of LPL mRNA and LPL mass accumulation in the medium during PMA-induced differentiation by 4-fold. Studies with human monocyte-derived macrophages showed a similar effect of dexamethasone on LPL expression. Peak LPL mRNA levels were achieved 24-h post-dexamethasone addition to THP-1 cells. Optimal stimulation of LPL mRNA occurred when dexamethasone was added 24 h after induction with PMA. Thereafter, there was rapid decline in responsiveness to dexamethasone. Induction of LPL mRNA in THP-1 cells was completely blocked by actinomycin D, suggesting that induction was transcription dependent. The stability of LPL mRNA was not influenced by dexamethasone. Treatment of THP-1 cells with PMA led to a 2-fold increase in specific binding of dexamethasone and a 4-fold increase in glucocorticoid receptor mRNA within 12 h. Thus, dexamethasone stimulates LPL gene expression during differentiation of human macrophages, a process that involves induction of glucocorticoid receptor synthesis and activation.

Lipoprotein lipase (LPL)[1] is an extracellular triglyceride hydrolase important in lipoprotein and energy metabolism (Eckel, 1989). It is synthesized by many tissues, but most abundantly in muscle and adipose tissue where its regulation has been extensively studied. In these tissues, LPL is regulated by hormones that control energy storage and utilization, such as insulin, catecholamines, glucocorticoids, thyroxin, and growth hormone (Semenkovich et al., 1989; Ong et al., 1988; Friedman et al., 1978; Ball et al., 1986; Robinson & Speake, 1989; Marikawa et al., 1982; Miller et al., 1989; Ailhaud et al., 1986). LPL also is synthesized and secreted by macrophages (Khoo et al., 1981; Chait et al., 1982; Wang-Inverson et al., 1982; Mahoney et al., 1982). In contrast to the many studies concerning regulation of LPL in adipocytes, little is known about the hormonal regulation of LPL in macrophages. In adipose tissue, LPL activity can be stimulated by insulin (Ong et al., 1988; Semenkovich et al., 1989) and depressed by catecholamines (Friedman et al., 1978; Ball et al., 1986). In contrast, insulin does not appear to regulate LPL in macrophages, while catecholamines result in a slight inhibition of LPL activity (Kawakami et al., 1986; Behr & Kraemer, 1986). The human LPL gene has been sequenced (Deeb & Peng, 1989; Kirchgessner et al., 1989), and two sequence motifs homologous to known glucocorticoid regulatory elements have been found in the 5′ upstream region of the human LPL gene, suggesting that glucocorticoids may regulate LPL gene expression. Both stimulatory (de Gasquet et al., 1975;

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[1] Abbreviation: LPL, lipoprotein lipase.

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