Enzymatic and Chemical Oxidation of Gangliosides in Cultured Cells: Effects of Choleragen†

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ABSTRACT: Cell surface glycolipids of normal human fibroblasts and NCTC 2071 cells (transformed mouse fibroblasts) were labeled by incubating the intact cells with either galactose oxidase or sodium periodate, followed by reduction of the oxidized sugar residues with NaB^3H_4. In intact human fibroblasts, incorporation of ^3H was increased with increasing time of exposure to galactose oxidase prior to treatment with NaB^3H_4. Following limited exposure to galactose oxidase, more label was incorporated into the larger glycolipids. Although labeling of the monosialoganglioside G_{M1} was maximal by 16 h, not all of the G_{M1} in the intact cells appeared to be accessible to galactose oxidase, since 10 to 12 times more G_{M1} was labeled when cells were disrupted before incubation with the enzyme. The human fibroblasts contained approximately 8×10^6 molecules of G_{M1} per cell. Maximal binding of choleragen (5×10^5 molecules of [^{125}I]choleragen per cell) completely prevented oxidation of G_{M1} in intact fibroblasts by galactose oxidase but only partially protected the sialic acid moiety of G_{M1} from oxidation by periodate. Choleragen had little effect on the enzymatic or chemical oxidation of other glycolipids. NCTC 2071 cells do not contain endogenous G_{M1} but incorporate exogenous G_{M1} from the culture medium. When bound to NCTC 2071 cells, exogenous G_{M1} was protected by choleragen from oxidation by galactose oxidase or periodate. Thus, G_{M1} molecules in the fibroblast membrane, whether endogenous or taken up from the incubation medium, are, after interaction with choleragen, less accessible to oxidation by periodate or galactose oxidase.

The initial step in choleragen action appears to be binding of the B subunits of the toxin to the monosialoganglioside G_{M1}[1] on the cell surface (Cuatrecasas, 1973a,b; Holmgren et al., 1973, van Heyningen, S., 1974; van Heyningen, W. E., 1974). The affinity of the ganglioside for choleragen has been shown to be dependent on the structure of the oligosaccharide side chain (Holmgren et al., 1973; Cuatrecasas, 1973a,b; Fishman et al., 1976). Normal human fibroblasts, which respond to choleragen, are capable of synthesizing G_{M1} and contain 8×10^6 molecules of G_{M1} per cell (Fishman et al., 1977). In contrast, NCTC 2071 cells, transformed mouse fibroblasts, do not respond to choleragen, and are unable to synthesize G_{M1} from precursor sugars (Fishman et al., 1976; Moss et al., 1976). These fibroblasts bind exogenous G_{M1} and thereafter are responsive to choleragen (Moss et al., 1976). The availability of these two types of cells allowed us to study the interaction of choleragen with both exogenous and endogenous G_{M1} using enzymatic and chemical oxidation of specific portions of the G_{M1} molecule to probe their role in choleragen binding.

Experimental Procedure

Oxidation of Gangliosides of Intact Cells. The growth conditions, ganglioside content, and choleragen responsiveness of NCTC 2071 cells (American Type Culture Collection, Rockville, Md.) and normal human fibroblasts (Manganiello and Breslow, 1974) have been described (Moss et al., 1976; Fishman et al., 1977). Human fibroblasts, grown to confluency in Eagle's medium containing 10% fetal calf serum, were washed twice with 5 mL of Hank's medium and then incubated with or without 100 μg of choleragen in 10 mL of Hank's medium for 2 h at 37 °C. NCTC 2071 cells grown to confluency in NCTC 135 medium were incubated in 10 mL of fresh NCTC medium with or without 100 μg of choleragen for 2 h at 37 °C. All cells were then washed twice with Dulbecco's phosphate-buffered saline containing calcium and magnesium.

For experiments with galactose oxidase, the cells were incubated with 20 units of the enzyme in 10 mL of the same medium for 3 h at 37 °C or as indicated for specific experiments. The cells were washed four times with phosphate-buffered saline and 2 mL of the same buffer was added to each dish followed by 1 mCi of NaB^3H_4 in 0.1 mL of *N,N'*-dimethylformamide. After incubation at room temperature for 10 min, cells were washed five times with 2 mL of phosphate-buffered saline and harvested for quantification of ^3H in gangliosides.

As the NCTC cells did not adhere to the dishes following incubation with galactose oxidase, they were collected by centrifugation. The sedimented cells were washed three times with 3 mL of phosphate-buffered saline, suspended in 0.5 mL of the same medium, incubated with 2 mCi of NaB^3H_4 at room temperature for 10 min, and collected by centrifugation after washing four times with phosphate-buffered saline. ^3H in gangliosides was determined as described below.

Incubations with 5 mM sodium periodate in 2 mL of phosphate-buffered saline were carried out at 37 °C for 20 min. After adding 0.2 mL of 0.1 M glucose to each dish, the cells were washed four times with phosphate-buffered saline, scraped from the dishes, suspended in 0.5 mL of phosphate-buffered saline, and further incubated with 2 mCi of NaB^3H_4 as described above.

Isolation, Quantification, and Analysis of Labeled Gan-

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[1] Abbreviations used are: G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; G_{M3}, *N*-acetylneuraminylgalactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; GL-2, galactosylglucosylceramide; GL-3, galactosylgalactosylglucosylceramide; GL-4, *N*-acetylgalactosaminylgalactosylgalactosylglucosylceramide; NANA-7, 7-carbon analogue of *N*-acetylneuraminic acid or 5-acetamido-3,5-dideoxyl-L-arabino-2-heptulosonic acid.

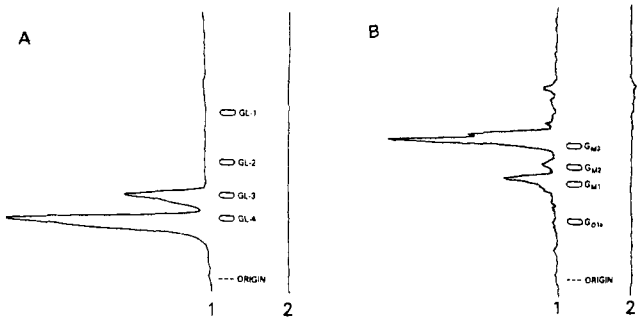

FIGURE 1: Labeling of glycolipids of intact human fibroblasts with galactose oxidase and NaB^3H_4. Human fibroblasts were incubated for 3 h with or without galactose oxidase (20 units/10 mL), and then washed and treated with 1 mCi of NaB^3H_4 per dish for 10 min. Glycolipids were isolated and chromatographed as described under Methods. (A) Radioscans of neutral glycolipids from cells (9 mg of protein) incubated with (scan 1) or without (scan 2) galactose oxidase; (B) radioscans of gangliosides from same cells incubated with (scan 1) or without (scan 2) galactose oxidase. The positions of glycolipid standards are indicated.

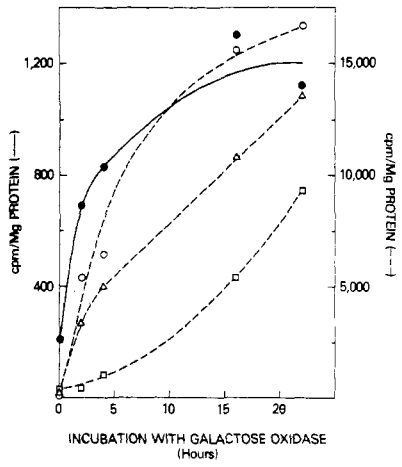

FIGURE 2: Effect of time of exposure to galactose oxidase on labeling of glycolipids of intact human fibroblasts. Human fibroblasts were incubated with galactose oxidase (20 units/10 mL) as indicated, washed, and treated with 1 mCi of NaB^3H_4 before isolation of glycolipids. Isolated glycolipids were separated into neutral and ganglioside fractions and chromatographed as described under Methods. Areas of the chromatograms corresponding to the individual glycolipids were scraped and counted for ^3H as described under Methods; GL-4 (○ - - - ○), GL-3 (△ - - - △), GL-2 (□ - - □), and G_{M1} (● — ●).

gliosides. Gangliosides were extracted and separated from neutral glycolipids and chromatographed as previously described (Fishman et al., 1977). Neutral glycolipids were purified by saponification and separated by chromatography with chloroform–methanol–water (65:25:4, v/v). The chromatograms were scanned with a Varian radioscanner. The ganglioside chromatograms were then stained with resorcinol and those with neutral glycolipids were charred (Simmons et al., 1975) to permit visualization of glycolipids. Radioactivity was quantified by calculation of peak areas from the scan or by liquid scintillation counting after scraping silica gel from the corresponding areas of the chromatograms into counting vials and adding 1 mL of water and 10 mL of Aquasol. When calibrated with $[^3H]G_{M1}$ of known radioactivity, both procedures gave a linear response over a 100-fold range.

Radioactive gangliosides were recovered from the thin-layer chromatograms by scraping the silica gel from the plate and eluting the radioactive material with chloroform–methanol–water (10:10:3, v/v). To determine the distribution of radioactivity in individual sugar residues, samples of eluates were heated at 100 °C for 2 h in 3 N HCl. The hydrolysates were taken to dryness under vacuum and the sugars were separated by descending paper chromatography with ethyl acetate–pyridine–water (72:20:23, upper phase) as the developing solvent (Colombo et al., 1960). In this system, sugars usually found in gangliosides are well resolved. The chromatograms were scanned for radioactivity and areas corresponding to the standards were cut from the paper for radioassay as described above. Other samples of eluates were heated at 80 °C for 1 h in 0.1 N HCl to release sialic acid. The hydrolysates were diluted tenfold with water and applied to small columns of Dowex-1-X8 formate (0.7 mequiv of 200–400 mesh in a Pasteur pipette). Columns were eluted with 2 mL of water and 4 mL of 0.5 N formic acid (modified from Suttajit and Winzler, 1971). Recoveries of authentic $[^3H]$NAN-7 and $[^3H]$NAN-7 from $[^3H]G_{M3}$ prepared from G_{M3} by periodate oxidation and NaB^3H_4 reduction were 78 and 70%, respectively.

Materials. NaB^3H_4 (7.5–10.5 Ci/mmol, Amersham/Searle), 10 mCi/mL, in *N,N'*-dimethylformamide was stored at −80 °C. Galactose oxidase (65 units/mg of protein) was purchased from Worthington Biochemical Corp., choleragen from Schwarz/Mann, sodium periodate from Fisher Scientific Co., and carrier-free $Na^{125}I$ (~17 Ci/mg) and Aquasol from New England Nuclear. $[^{125}I]$Choleragen was prepared by a modification of the method of McFarlane (1958); G_{M3} and G_{M1} were obtained as previously described (Fishman et al., 1972).

Results

Reduction of Glycolipids in Galactose Oxidase Treated Human Fibroblasts with NaB^3H_4. After incubation of human fibroblasts with galactose oxidase for 3 h, exposure to NaB^3H_4 resulted in labeling of several glycolipids, whereas incorporation of ^3H into cells that had not been treated with the enzyme was minimal (Figure 1). ^3H was found in GL-4 and GL-3 (Figure 1A) as well as in gangliosides G_{M1} and G_{M2} (Figure 1B). The largest fraction of the label was associated with an unidentified glycolipid which migrated slightly ahead of G_{M3} on chromatography of the ganglioside fractions (Figure 1B). This material became labeled in cells grown with $[^{14}C]$galactose but not in those grown with *N*-acetyl$[^3H]$mannosamine (Fishman et al., 1977).

Increasing the time of incubation with galactose oxidase increased the amount of ^3H incorporated into cell glycolipids during a subsequent exposure to NaB^3H_4 (Figure 2). Labeling of G_{M1} and GL-4 appeared to be maximal after 16 to 22 h with galactose oxidase, whereas the extent of labeling of GL-2 and GL-3 was still increasing at that time (Figure 2). Even after exposure of cells to galactose oxidase for 22 h, however, most of the G_{M1} did not become labeled with NaB^3H_4, as estimated by comparison of the specific radioactivity of the recovered G_{M1} to that of NaB^3H_4. Chromatograms of glycolipids from cells incubated for 22 h with galactose oxidase exhibited faint new bands corresponding to the oxidized products slightly ahead of G_{M1}, GL-4, GL-3, and GL-2 (Suzuki and Suzuki, 1972). The oxidized products represented approximately 10% of each of these glycolipids.

Labeling of G_{M1} with NaB^3H_4 was much greater when an isolated particulate fraction was incubated with galactose oxidase than it was after enzymatic treatment of the intact cells (Table I). When the presumed G_{M1} was eluted and chromatographed in a second solvent system, most of the radioactivity from intact cells and particulate fractions again migrated as

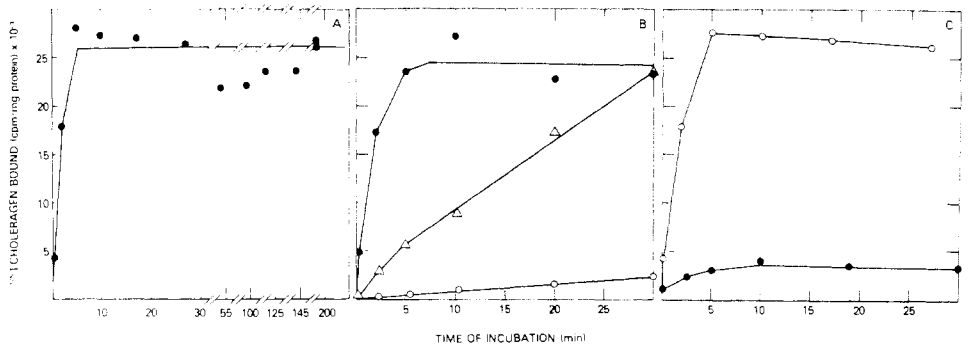

FIGURE 3: (A) Time course of [^{125}I]choleragen binding to fibroblasts. Human fibroblasts were grown to confluency. 12.8 μg of [^{125}I]choleragen (2.1 $\times$ 10^6 cpm) was added to each dish in 10 mL of Hanks' medium. At the indicated time, the cells were washed twice with 5 mL of phosphate-buffered saline. Two milliliters of trichloroacetic acid was added, the cells were harvested, and the precipitate was suspended in 1 N NaOH. The total protein and radioactivity were then determined. (B) Effect of [^{125}I]choleragen concentration on binding to fibroblasts. Cells were incubated with 11.5 μg (●), 1.15 μg (△), and 0.115 μg (○) of [^{125}I]choleragen in 10 mL of Hank's medium. At the indicated times, bound radioactivity and protein were determined. (C) Effect of anticholeragen antibodies on binding of [^{125}I]choleragen. 900 units of anticholeragen antibodies in 10 mL of Hank's medium were added to fibroblasts (●), followed by 11.5 μg of [^{125}I]choleragen (○,●). At the indicated times, protein and radioactivity were determined as described above. Anticholeragen antibodies were obtained from the U.S.-Japan Cooperative Medical Science Program of the National Institutes of Health through Dr. Carl E. Miller.

TABLE I: Labeling of G_{M1} in Intact Cells and in Particulate Fractions by Galactose Oxidase and NaB^3H_4.[a]

Preparation	Protein (mg)	^{3}H in G_{M1} (dpm) I	II[b]
Intact cells	4.3	54 600	54 000
Particulate fraction[c]	1.5	710 000	540 000

[a] Intact human fibroblasts and a particulate fraction from the same number of cells were incubated with galactose oxidase and NaB^3H_4. Gangliosides were separated by thin-layer chromatography on silica gel with chloroform–methanol–0.25% aqueous $CaCl_2$ (60:35:8, v/v) and ^{3}H in G_{M1} was quantified by radioscanning. [b] After radioscanning of the original chromatogram, the areas corresponding to G_{M1} were scraped from the plate and eluted with chloroform–methanol–water (10:10:3). Samples were chromatographed again using chloroform–methanol–2.5 N NH_4OH (60:35:8, v/v) containing 20 mg of $CaCl_2 \cdot 2H_2O$ (van Den Eijnden, 1971). [c] The particulate fraction was prepared by homogenization of the fibroblasts in 0.25 M sucrose, 20 mM Tris (Cl^-), pH 7.5, and centrifuging at 100 000g for 40 min. The precipitate was resuspended in phosphate-buffered saline.

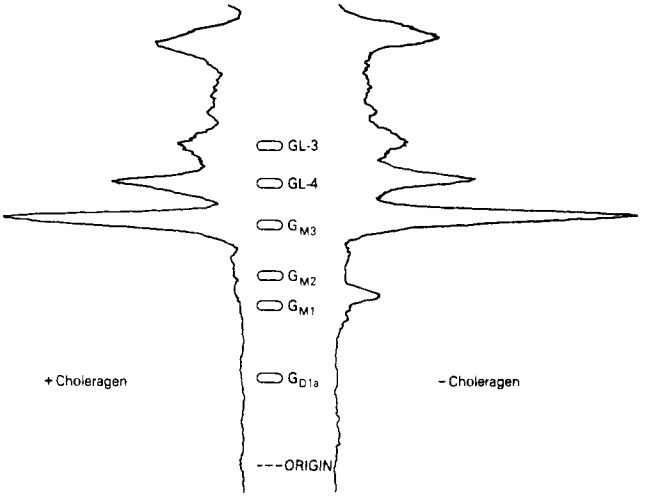

FIGURE 4: Effect of choleragen on labeling of G_{M1} of human fibroblasts by galactose oxidase and NaB^3H_4. Cells were incubated for 2 h with or without 10 μg/mL of choleragen. The cells were washed, incubated with galactose oxidase for 2 h, and treated with 1 mCi per dish of NaB^3H_4 as described in the legend to Figure 1. Glycolipids were extracted (Fishman et al., 1977) and chromatographed in chloroform–methanol–0.25% $CaCl_2$ (60:35:8, v/v). Radioscans of chromatograms of glycolipids from cells incubated with or without choleragen are shown.

G_{M1}. Thus, it is unlikely that the greater incorporation of ^{3}H in disrupted cells was due to a contaminant.

After acid hydrolysis of G_{M1} from the intact fibroblasts incubated with galactose oxidase and NaB^3H_4, 87% of the ^{3}H was recovered with galactose and 9% with galactosamine (the hydrolysis product of *N*-acetylgalactosamine).[2] After hydrolysis of [^{3}H]G_{M1} prepared in free solution as previously described (Moss et al., 1976), 98% of the ^{3}H was recovered with galactose and 2% with galactosamine.

Effect of Choleragen on Enzymatic and Chemical Modification of G_{M1} in Human Fibroblasts. When fibroblasts were incubated with [^{125}I]choleragen (1 μg/mL) maximal binding occurred in ~30 min (Figure 3A). With lower concentrations of choleragen maximal binding required considerably longer periods of incubation (Figure 3B). Anticholeragen antibody prevented the subsequent binding of choleragen (Figure 3C). The maximal amount of [^{125}I]choleragen bound to the intact fibroblasts was approximately 5 $\times$ 10^5 molecules per cell similar to that reported by Bennett et al. (1975). The G_{M1} content was approximately 8 $\times$ 10^6 molecules per cell (Fishman et al., 1977). Incubation of human fibroblasts with choleragen under conditions that yielded maximal binding (Figure 3) before treatment with galactose oxidase markedly reduced the subsequent incorporation of ^{3}H into G_{M1} but had little effect on labeling of other glycolipids (Figure 4 and Table II). If anything, exposure of the cells to choleragen increased the labeling of GL-3 and GL-4 slightly. The G_{M1} from control and treated cells was eluted from chromatograms and acid hydrolyzed; the hydrolysates were chromatographed on paper, and ^{3}H in galactose was quantified. There was a 91.5% reduction of [^{3}H]galactose in the G_{M1} from cells treated with choleragen. This value is identical to the reduction of ^{3}H in G_{M1} caused by exposure of the cells to choleragen (Table III).

After incubation of human fibroblasts with $NaIO_4$ and

[2] The major labeled sugars recovered from the other glycolipids after acid hydrolysis and chromatography were as follows: for GL-3, galactose (92%) and, for GL-4, galactosamine (96%).

TABLE II: Effect of Choleragen on Labeling of Human Fibroblast Glycolipids by Galactose Oxidase and NaB^3H_4.[a]

Glycolipid	[^{3}H] Incorp (dpm/mg of protein)10^{-3}	
	−choleragen	+choleragen
G_{M1}	266	23
G_{M2}	32	31
GL-4	3910	4890
GL-3	3050	3890

[a] Cells were incubated for 2 h with and without 10 μg/mL of choleragen. The cells were washed and incubated for 2 h with galactose oxidase (20 units/2 mL), washed, and treated with 6 mCi of NaB^3H_4. Glycolipids were extracted, separated into neutral and ganglioside fractions, and further separated by thin-layer chromatography as described under Methods. Incorporation of ^{3}H was determined by radioscanning the chromatograms.

TABLE III: Effect of Choleragen on Oxidation of Human Fibroblast Gangliosides by $NaIO_4$.[a]

Choleragen Concn (μg/mL)	^{3}H in Gangliosides[b] (cpm/mg of protein)10^{-3}	
	G_{M3}	G_{M1}
0	246	19
1	203	12
10	214	7
100	236	7

[a] Human fibroblasts were incubated for 2 h with choleragen as indicated, washed, and treated with $NaIO_4$ and NaB^3H_4 as described under Methods. Gangliosides were scraped from plates for radioassay. [b] G_{M3} and G_{M1} isolated from cells treated with NaB^3H_4 without prior exposure to $NaIO_4$ or choleragen contained, respectively, 13 800 and 2320 cpm/mg of protein.

exposure to NaB^3H_4, some ^{3}H was found in G_{M1} and G_{D1a} but most of the radioactivity in the ganglioside fraction was recovered in G_{M3} which is the major ganglioside component of these cells (Fishman et al., 1977) (Figure 5). The mobilities of the modified gangliosides were slightly faster than those of their native counterparts. A small peak of radioactivity, which migrated slightly ahead of the G_{M3} standard, was resorcinol positive and may represent a modified G_{M3}. Most of the radioactivity in G_{M1} isolated from cells treated with $NaIO_4$ and NaB^3H_4 (82 and 100% in two experiments)[3] was recovered in the sialic acid fraction after mild acid hydrolysis of the ganglioside. Similarly, in the G_{M3} and G_{D1a} from cells labeled by this procedure, most of the ^{3}H was in the sialic acid fraction. In cells that had bound choleragen, G_{M1} was partially protected from periodate oxidation, whereas G_{M3} was not (Table III). The degree of protection of G_{M1} was a function of choleragen concentration. Choleragen did not, however, decrease the extent of G_{M1} oxidation when more drastic conditions of periodate oxidation were employed (data not shown).

Oxidation of Exogenous G_{M1} Bound to NCTC 2071 Cells. When NCTC 2071 cells, which contain no G_{M1} detectable by the usual methods, were incubated with galactose oxidase and then with NaB^3H_4, ^{3}H was incorporated into GL-3 and GL-2. ^{3}H was also recovered in an unidentified glycolipid which

[3] These values have been corrected for the percentage recovery of [^{3}H]NANA-7 carried through the same procedure. Some destruction of NANA-7 occurs during the hydrolysis (Suttajit and Winzler, 1971).

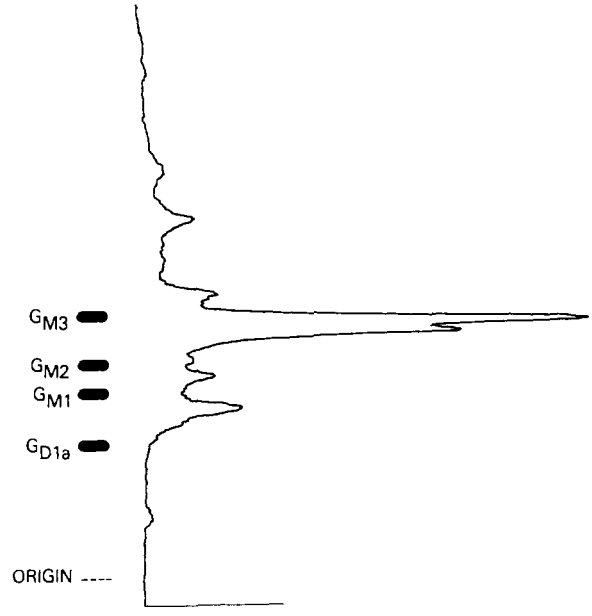

FIGURE 5: Labeling of gangliosides of human fibroblasts with $NaIO_4$ and NaB^3H_4. Human fibroblasts were incubated with 5 mM $NaIO_4$ for 20 min, and then with 10 mM D-glucose before washing and exposure to 1 mCi of NaB^3H_4 per dish for 10 min. Gangliosides were isolated and chromatographed as described under Methods. Radioscan of the ganglioside chromatogram is shown. The radioactive peaks corresponding to periodate-modified G_{D1a} and G_{M1} migrate ahead of the G_{D1a} and G_{M1} standards.

migrated faster than G_{M3} on the ganglioside chromatograms and which became labeled in cells grown with [^{14}C]galactose (Moss et al., 1976). Only a trace amount of radioactivity was associated with a ganglioside that corresponded chromatographically to G_{M2} (Fishman et al., 1976).

The NCTC 2071 cells do not respond to choleragen (Moss et al., 1976) and presumably do not bind the toxin.[4] Incubation of these cells with choleragen did not influence the pattern of labeling of glycolipids found after treatment of the cells with galactose oxidase and NaB^3H_4 (data not shown). As shown in Figure 6, in NCTC 2071 cells that had bound exogenous G_{M1}, the ganglioside was labeled by treatment with galactose oxidase and NaB^3H_4. Incubation of the cells with choleragen before exposure to galactose oxidase markedly decreased the incorporation of ^{3}H into G_{M1} (Figure 6 and Table IV). Exogenous G_{M1} bound to the NCTC 2071 cells was also modified by periodate and this was partially prevented by incubation of cells with choleragen before exposure to periodate (Table IV). Choleragen was much more effective in protecting the exogenous G_{M1} bound to NCTC 2071 cells from oxidation by galactose oxidase than by sodium periodate.

Discussion

Previous workers have provided evidence that the monosialoganglioside G_{M1} is the membrane receptor for choleragen. G_{M1} was found to be the most effective glycolipid for precipitating and inactivating choleragen (Holmgren et al., 1973), for inhibiting the binding of choleragen to membranes and intact cells (Cuatrecasas, 1973a,b), and for enhancing chol-

[4] [^{125}I]choleragen did not bind significantly to NCTC 2071 cells containing no exogenous gangliosides or to those containing approximately 7×10^6 molecules per cell of exogenous G_{M2} or G_{M3}. Cells containing exogenous G_{M1} bound [^{125}I]choleragen (Moss, Manganiello, Fishman and Vaughan, unpublished observations).

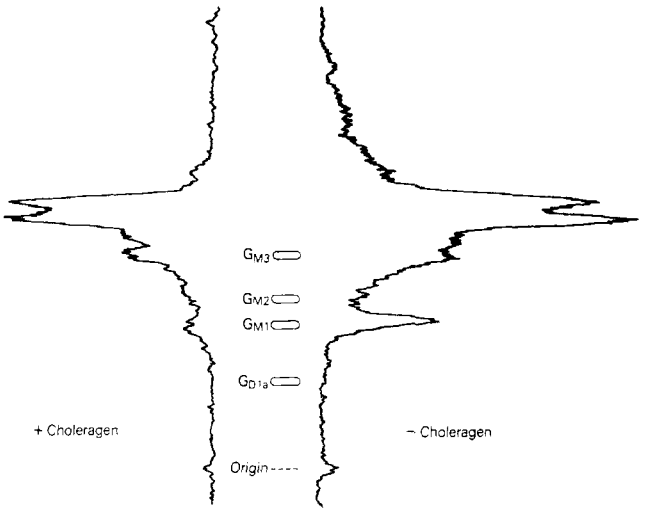

FIGURE 6: Effect of choleragen on labeling of exogenous G_{M1} bound to NCTC 2071 cells by galactose oxidase and NaB^3H_4. NCTC 2071 cells were incubated for 18 h with 2 nmol of G_{M1} per dish, and then washed and treated as described in the legend to Figure 4. Gangliosides were isolated and chromatographed, and the chromatogram was radioscanned as described under Methods.

eragen binding to and sensitivity of ganglioside-enriched cells (Cuatrecasas, 1973b; Gill and King, 1975; Holmgren et al., 1975; Moss et al., 1976). We have demonstrated recently that G_{M1}-deficient transformed mouse fibroblasts that are choleragen insensitive become responsive to the toxin upon binding exogenous G_{M1} (Moss et al., 1976) and that other gangliosides are less effective or ineffective (Fishman et al., 1976). Specific sugar components of the oligosaccharide portion of the ganglioside are apparently the critical determinants of choleragen binding. We have now examined directly the nature of the interaction of choleragen with membrane-bound G_{M1} by evaluating the effects of the toxin on the accessibility of certain sugar residues to chemical and enzymatic modification.

Galactose oxidase oxidizes the C-6 position of some galactosyl and *N*-acetylgalactosaminyl residues in isolated glycolipids (Bradley and Kanfer, 1964) and the oxidized residues can be reduced subsequently with NaB^3H_4 (Suzuki and Suzuki, 1972). The surface glycolipids of intact erythrocytes (Steck and Dawson, 1974; Gahmberg and Hakomori, 1973a) and of mouse and hamster fibroblasts (Gahmberg and Hakomori, 1973b; 1975) have been labeled by this technique. In the intact human fibroblasts, labeling of gangliosides with NaB^3H_4 was minimal in cells that had not been treated with galactose oxidase. Incorporation of ^3H depended on the time of exposure to galactose oxidase and the length of the oligosaccharide portion of the ganglioside. The human fibroblasts contain twice as much G_{M2} as G_{M1} (Fishman et al., 1977), but the *N*-acetylgalactosaminyl residue of G_{M2} was not readily oxidized by galactose oxidase. Similar difficulty was encountered in oxidizing free G_{M2} (Suzuki and Suzuki, 1972). Of the total G_{M1} in the human fibroblasts, a maximum of about 10% or approximately 8×10^5 molecules of G_{M1} per cell was apparently accessible to galactose oxidase. These fibroblasts bound maximally approximately 5×10^5 molecules of choleragen per cell. In the fibroblasts that had bound choleragen, G_{M1} was essentially completely protected from galactose oxidase but oxidation of other glycolipids was little affected. Similarly, in the ganglioside-deficient NCTC 2071 cells that had taken up G_{M1} from the incubation medium, choleragen prevented enzymatic oxidation of the incorporated exogenous G_{M1}.

TABLE IV: Effect of Choleragen on Chemical and Enzymatic Oxidation of Exogenous G_{M1} Bound to NCTC 2071 Cells.[a]

Oxidant	^3H in G_{M1} (dpm/mg of cell protein) 10^{-3}	
	−Choleragen	+Choleragen
None[b]	23	21
$NaIO_4$	118	81
Galactose Oxidase	160	20

[a] NCTC 2071 cells were incubated for 18 h with 2 nmol of G_{M1} per dish (Moss et al., 1976), and then washed and incubated for 2 h with or without choleragen (10 μg/mL). The cells were washed and treated with $NaIO_4$ or galactose oxidase and NaB^3H_4. ^3H in G_{M1} was determined from radioscan of the ganglioside. [b] No radioactive peak corresponding to G_{M1} was observed in the radioscan; therefore, the area corresponding to G_{M1} was scraped from the chromatogram for radioassay. (cpm were converted to dpm based on the counting efficiency, 10%, which was obtained by chromatographing, scraping, and counting [^3H]G_{M1} of known radioactivity as described under Methods.)

Sodium periodate and NaB^3H_4 have been used to label the sialic acid residues of soluble glycoproteins (Van Lenten and Ashwell, 1971) and surface glycoproteins of erythrocytes (Liao et al., 1973). When exposure to periodate was limited, choleragen reduced the labeling of G_{M1} in human fibroblasts by 70%. Choleragen did not, however, prevent oxidation of G_{M1} in cells exposed to periodate for prolonged periods. Exogenous G_{M1} bound to NCTC 2071 cells could also be partially protected from periodate oxidation by choleragen. The observation that choleragen was less effective in preventing periodate oxidation of sialic acid than it was in blocking the enzymatic oxidation of galactosyl residues could reflect the relatively small size of the periodate molecule. It could also be related to the nature and extent of the molecular interaction of choleragen with G_{M1}. From the evidence presented here and prior reports demonstrating that asialo-G_{M1} and G_{D1b} interact relatively poorly with choleragen (Holmgren et al., 1973, Craig and Cuatrecasas, 1975), it seems probable that the sialic acid moiety of G_{M1} does play a part in choleragen binding.[5] The studies reported here clearly demonstrate that the portions of the G_{M1} molecule specifically important for choleragen binding, the terminal galactosyl and the sialyl residues, are accessible in the cell membrane to external oxidants and can be shielded from their action by choleragen.

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[5] Choleragen interacted with G_{M2}, asialo-G_{M1}, or G_{D1b} incorporated into liposomal model membranes. This was demonstrated by the binding of [^{125}I]choleragen to the liposomes and the small, but significant, protective effect of choleragen on the labeling of these glycolipids by galactose oxidase and NaB^3H_4 (Fishman, Moss, Richards, and Alving, unpublished observations).

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Use of a Fluorescent Probe to Determine the Viscosity of LM Cell Membranes with Altered Phospholipid Compositions†

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ABSTRACT: The phospholipid composition of LM cells grown in tissue culture was altered by substituting ethanolamine for choline in the growth medium. The plasma membrane isolated from cells grown in medium containing ethanolamine for 83 h had a sixfold increase in the ratio of phosphatidylethanolamine to phosphatidylcholine, the two major phospholipid classes. This was accompanied by small changes in other lipid components of the membrane. There was also a sixfold increase in the amount of triacylglycerols and alkyldiacylglycerols which were not associated with the membrane fraction of the cell. No significant changes occurred in the lipid composition of cells during growth in choline containing medium. The viscosity of plasma membranes was studied in whole cells and isolated membranes using the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene. Plasma membranes isolated from ethanolamine-supplemented cells had greater viscosities than membranes isolated from choline-supplemented cells. When whole cells were labeled with the fluorescent probe, the opposite trend in the apparent membrane viscosity was observed. This was due primarily to the probe penetrating into nonmembranous neutral lipids rather than remaining localized in the surface membrane of the cells. Since the enthanolamine-supplemented cells contained more low viscosity neutral lipids, the whole cells gave an apparently lower viscosity as compared with choline-supplemented cells. Thus, measurements carried out on whole cells gave an inaccurate determination of the viscosity of the surface membrane.

Many methods have been used to study the role of lipids in problems of membrane structure and function. One approach has been to manipulate the lipid composition of cells growing under defined conditions (for a review, see Silbert, 1975). This approach, which has largely involved alterations in the fatty acid composition of *E. coli* and *A. laidlawii,* has recently been extended to animal cells in tissue culture (Wisnieski et al., 1973; Williams et al., 1974; Horwitz et al., 1974; Ferguson et al., 1975). Changes in the fatty acid composition in these cells have been shown to alter lectin binding and agglutination (Horwitz et al., 1974; Rittenhouse et al., 1974) and virus infection (Li et al., 1975).

In comparison with the studies on the properties of fatty acids, relatively little has been done with respect to the properties of phospholipid polar head groups in membranes. In order to study the effect of the phospholipid head group in mammalian cell membranes, techniques have been developed

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