# Synthesis and Uptake of Gangliosides by Choleragen-Responsive Human Fibroblasts†

Peter H. Fishman,* Joel Moss, and Vincent C. Manganiello

ABSTRACT: Human fibroblasts, cultured in medium containing 10% fetal calf serum, responded dramatically to choleragen with an increase in cyclic adenosine monophosphate content to greater than 48 times basal levels. Analysis of these cells for gangliosides indicated that the major ganglioside was *N*-acetylneuraminylgalactosylglucosylceramide ($G_{M3}$) with trace amounts ($\leq$100 pmol/mg of protein) of other gangliosides including $G_{M1}$, the putative choleragen receptor. Although the cells contained three glycosyltransferases required for ganglioside synthesis, the *N*-acetylgalactosaminyltransferase activity necessary for the conversion of $G_{M3}$ to more complex gangliosides was not detected. When the cells were grown in medium containing [$^{14}$C]galactose or *N*-acetyl[$^{3}$H]mannosamine, however, all of the gangliosides became labeled, indicating that the cells can synthesize complex gangliosides. Although fetal calf serum contains gangliosides including $G_{M1}$, [$^{3}$H]$G_{M1}$ was taken up poorly from the growth medium and uptake at the rate observed could have accounted for <2% of the $G_{M1}$ content of the cells. When the cells were incubated in chemically defined medium containing [$^{3}$H]$G_{M1}$ at the concentrations present in fetal calf serum, rapid uptake of the ganglioside occurred and the total $G_{M1}$ content of the cells increased threefold in <3 h. Thus, although the cells are capable of binding exogenous gangliosides, the gangliosides in fetal calf serum are in a form not readily available to the cells.

Work by several investigators has indicated that the effects of choleragen on vertebrate cells result from activation of adenylate cyclase (Finkelstein, 1973). The initial step involves binding of choleragen to cell-surface receptors presumed to be the monosialoganglioside $G_{M1}$[1] (Cuatrecasas, 1973; Holmgren et al., 1973; King and van Heyningen, 1973). Although earlier reports indicated that cultured human fibroblasts had hexosamine-containing gangliosides (Callahan et al., 1970; Kolodny et al., 1973), in more recent studies, the only gangliosides found in such cells were $G_{M3}$ and $G_{D3}$ (Dawson et al., 1972; Bach et al., 1975). We found, as did Hollenberg and Cuatrecasas (1973), that human fibroblasts respond dramatically to choleragen. These cultured cells are grown in medium which contains fetal calf serum which has been reported to contain gangliosides, including $G_{M1}$ (Yogeeswaran et al., 1970). In order to further clarify the role of $G_{M1}$ in the response of human fibroblasts to choleragen, we studied the ganglioside content of these cells as well as the synthesis of endogenous gangliosides and the uptake of exogenous gangliosides by these cells.

## Experimental Procedure

*Cells and Cell Culture.* Human diploid fibroblasts from an apparently normal male (Manganiello and Breslow, 1974) were grown in Eagle's medium supplemented with Earle's salts, 10% fetal calf serum (North American Biological), 2 mM glutamine, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (referred to as "complete medium") in 100-mm plastic petri dishes as previously described (Manganiello and Breslow, 1974). All experiments were carried out 5 days after initiation of subcultures when the density was approximately $5 \times 10^6$ cells/dish. Where indicated, the cells were incubated in chemically defined medium (NCTC 135). Binding of exogenous [$^{3}$H]$G_{M1}$ was determined as previously described (Moss et al., 1976).

*Ganglioside Isolation and Analysis.* Cells from confluent cultures were washed three times with 5 mL of phosphate-buffered saline at 0–4 °C, scraped in 2 mL of the same buffer, and collected by centrifugation for 10 min at 1000*g*. The pellet was extracted with 20 volumes of chloroform–methanol (2:1, v/v) for 30 min at 25 °C and then with 10 volumes of chloroform–methanol (1:2, v/v) for 30 min at 45 °C. The extracts were combined and chloroform was added until the ratio of chloroform to methanol was 2:1. The solution was partitioned with 1/5 volume of water and the lower organic phase was washed eight times with theoretical upper phase, chloroform–methanol–water (3:48:47, v/v). The combined upper phases were taken to dryness. The residue was dissolved in 0.5 mL of chloroform–methanol–water (60:30:4.5, v/v) and desalted on a column (0.5 g) of Sephadex G-25 superfine (Pharmacia) which was equilibrated and eluted with the same solvent (Fishman et al., 1972). A total of 5.5 mL was used to elute the gangliosides. Using radiolabeled gangliosides, recoveries with this procedure ranged from 84% ($G_{D1a}$) to 96% ($G_{M2}$). Individual gangliosides were separated by thin-layer chromatography (Fishman et al., 1974), detected with resorcinol reagent and quantified by densitometry (Brady and Mora, 1970). Radioactive gangliosides extracted from cells cultured in medium containing 50 $\mu$Ci per dish of [$^{14}$C]galactose (60 mCi/mmol from Amersham/Searle) or 1 mCi per dish of *N*-acetyl[$^{3}$H]mannosamine (3.8 Ci/mmol from New England Nuclear) were detected with a thin-layer radioscanner (Brady and Mora, 1970). Where indicated, gangliosides were treated with Vibrio cholera neuraminidase (Behringwerke AG)

† From the Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, and the Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20014. *Received October 4, 1976.*

[1] Abbreviations used are: $G_{M1}$, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)galactosylglucosylceramide; $G_{M2}$, *N*-acetylgalactosaminyl-(N-acetylneuraminyl)galactosylglucosylceramide; $G_{M3}$, *N*-acetylneuraminylgalactosylglucosylceramide; $G_{D3}$, *N*-acetylneuraminyl-*N*-acetylneuraminylgalactosylglucosylceramide; $G_{D1a}$, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl) galactosylglucosylceramide; GL-2, galactosylglucosylceramide; cAMP, cyclic adenosine 3′,5′-monophosphate.

TABLE I: Effect of Choleragen on Intracellular cAMP Accumulation in Human Fibroblasts.[a]

| Choleragen (ng/mL) | cAMP (pmol/mg of protein) | Fold Stimulation |
|---|---|---|
| 0 | 10 | |
| 2 | 65 | 6.5 |
| 20 | 194 | 19 |
| 200 | 417 | 42 |
| 2000 | 478 | 48 |

[a] Fibroblasts were incubated for 90 min at 30 °C in Hank's medium at the indicated choleragen concentrations. The medium was aspirated and cAMP was isolated and assayed as described previously (Moss et al., 1976). cAMP levels represent the average of duplicate determinations. Cholera toxin was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by Dr. R. Finkelstein of the University of Texas Southwestern Medical School, Dallas, Texas, and supplied to us by Dr. Carl E. Miller, NIAID.

TABLE II: Ganglioside Composition of Human Fibroblasts.[a]

| Ganglioside | pmol/mg of Protein | | | |
|---|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 | Mean ± SD |
| $G_{M3}$ | 3130 | 3100 | 3050 | 3093 ± 40.4 |
| $G_{M2}$ | 43 | 123 | 136 | 101 ± 50.4 |
| $G_{D3}$ | 55 | 53 | 93 | 68 ± 22.5 |
| $G_{M1}$ | 46 | 41 | 52 | 46 ± 5.5 |
| $G_{D1a}$ | 69 | 99 | 58 | 75 ± 21.2 |

[a] Human fibroblasts were grown in complete medium until confluent. For each experiment, cells from five dishes were washed with phosphate-buffered saline, scraped from the dishes, and collected by centrifugation. Following extraction of the cells with chloroform–methanol solutions and partitioning of the extract with water, the ganglioside fraction was desalted on Sephadex G-25 and separated by thin-layer chromatography. Gangliosides were visualized with resorcinol reagent and quantified by densitometry as described under Experimental Procedure.

by incubation with 0.1 mL of the enzyme solution for 20 h at 37 °C.

*Glycosyltransferase Assays.* Activities of four glycosyltransferases involved in ganglioside biosynthesis were assayed essentially as previously described (Fishman et al., 1972, 1974). For sialyltransferase I activity, the incubation conditions were as follows: 10 nmol of GL-2, 25 nmol of CMP-[$^{14}$C]sialic acid (9 $\mu$Ci/$\mu$mol from New England Nuclear), 37.5 $\mu$g of cardiolipin, 375 $\mu$g of Cutscum, 2.5 $\mu$mol of sodium cacodylate buffer (pH 6.0), and 100 $\mu$g of cell protein in a final volume of 25 $\mu$L; for *N*-acetylgalactosaminyltransferase: 50 nmol of $G_{M3}$, 10 nmol of UDP-[$^{14}$C]GalNAc (14 $\mu$Ci/$\mu$mol from International Chemical and Nuclear), 200 $\mu$g of Nonidet NP-40, 2.5 $\mu$mol of sodium cacodylate (pH 7.0), 1 $\mu$mol of $MnCl_2$, and 50 $\mu$g of cell protein in a final volume of 50 $\mu$L; for galactosyltransferase: 10 nmol of $G_{M2}$, 50 nmol of UDP-[$^{14}$C]Gal (6 $\mu$Ci/$\mu$mol from New England Nuclear), 100 $\mu$g of Triton CF-54, 50 $\mu$g of Tween 80, 2.5 $\mu$mol of sodium cacodylate buffer (pH 7.0), 1 $\mu$mol of $MnCl_2$, and 250 $\mu$g of cell protein in a final volume of 50 $\mu$L; for sialyltransferase II: 25 nmol of $G_{M1}$, 25 nmol of CMP-[$^{14}$C]sialic acid, 50 $\mu$g of Triton CF-54, 25 $\mu$g of Tween 80, 2.5 $\mu$mol of sodium cacodylate buffer (pH 6.3), and 100 $\mu$g of cell protein in a final volume of 25 $\mu$L. Assays for sialyltransferase II were incubated for 1 h and all others for 2 h at 37 °C. Labeled reaction products were isolated by Sephadex column chromatography as previously described (Fishman et al., 1972). For sialyltransferase II assays, the columns were eluted with an additional 5 mL of solvent to improve recovery of [$^{14}$C]$G_{D1a}$ (Fishman et al., 1976).

## Results

*Response of Human Fibroblasts to Choleragen.* Incubation of human fibroblasts with choleragen for 90 min produced a concentration-dependent increase in their cAMP content (Table I); at high concentrations of the toxin, cAMP levels were elevated 48-fold over basal levels.

*Ganglioside Composition of Human Fibroblasts.* As reported by Dawson et al. (1972), the human fibroblasts contained four major neutral glycosphingolipids: glucosylceramide, lactosylceramide, trihexosylceramide, and globoside. When the cells were grown in medium containing [$^{14}$C]galactose for 5 days, all of these glycolipids became radiolabeled (data not shown). The ganglioside composition of the fibroblasts is shown in Figure 1 (lane 4). The major ganglioside was

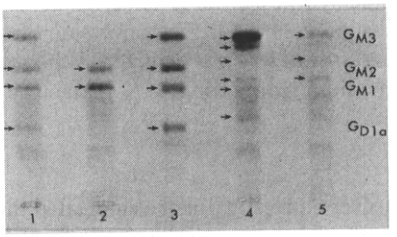


FIGURE 1: Thin-layer chromatogram of gangliosides extracted from human fibroblasts grown in complete medium. Gangliosides were isolated from the cells, separated by thin-layer chromatography, and visualized with resorcinol reagent as described under Experimental Procedures. Lane 1, ganglioside standards incubated with heat-inactivated neuraminidase; lane 2, same as lane 1 but incubated with active neuraminidase; lane 3, ganglioside standards; lane 4, ganglioside fraction from human fibroblasts (top two arrows indicate $G_{M3}$, next arrow $G_{M2}$, next $G_{M1}$, next $G_{D3}$, and last arrow $G_{D1a}$); lane 5, same as lane 4 but incubated with neuraminidase. Arrows indicate resorcinol-positive bands.

$G_{M3}$. There were trace amounts of gangliosides with mobilities similar to those of $G_{M2}$, $G_{M1}$, $G_{D3}$, and $G_{D1a}$ on the thin-layer chromatogram.[2] Following treatment of the extracted gangliosides with Vibrio cholera neuraminidase, which hydrolyzed 94% of the $G_{M3}$, gangliosides corresponding to $G_{M2}$ and $G_{M1}$ were still present (Figure 1, lane 5). Both of these gangliosides are resistant to neuraminidase (see lane 2, Figure 1). There was, in fact, an increase in $G_{M1}$, since, under these conditions, $G_{D1a}$ was converted to $G_{M1}$ (see lane 2, Figure 1). The ganglioside composition of the human fibroblasts is summarized in Table II. Dawson et al. (1972) found 1.09 nmol of $G_{M3}$/mg of dry weight in cultured human skin fibroblasts, which, assuming 40% of the dry weight is protein, would correspond to 2.7 nmol/mg of protein. Bach et al. (1975) reported 6.18 nmol of lipid-bound sialic acid per mg of protein in their human fibroblasts. In both of these reports, values for $G_{D3}$ were much higher than those that we have found.

*Glycosyltransferase Activities in Human Fibroblasts.* We measured the activities of four glycosyltransferases involved in ganglioside biosynthesis (Fishman et al., 1972). Activities of three of these enzymes were present in cell homogenates but there was no detectable UDP-GalNAc:$G_{M3}$ *N*-acetylgalac-

[2] Although the structures of these gangliosides have not been determined, evidence presented in this paper and the following paper in this issue (Moss et al., 1977) is consistent with these structures. Thus, for convenience, the indicated nomenclature is used to identify the gangliosides from human fibroblasts.

TABLE III: Ganglioside Glycosyltransferase Activities in Cultured Human Fibroblasts.[a]

| Glycosyltransferase | Reaction | Transferase Act. Expt 1 | Expt 2 | Expt 3 |
|---|---|---|---|---|
| Sialyltransferase I | GL-2 → $G_{M3}$ | 166 | 216 | 288 |
| *N*-acetylgalactosaminyl-transferase | $G_{M3}$ → $G_{M2}$ | 0 | 0 | |
| Galactosyltransferase | $G_{M2}$ → $G_{M1}$ | 219 | 208 | |
| Sialyltransferase II | $G_{M1}$ → $G_{D1a}$ | 1217 | 795 | 1898 |

[a] Human fibroblasts from confluent monolayers (five dishes for each experiment) were washed, disrupted, and assayed for glycosyltransferase activities as described under Experimental Procedure. Mean activities from duplicate assays are expressed as pmol of $^{14}C$-labeled product formed per mg of protein per h from exogenous acceptors.

tosaminyltransferase activity (Table III). Synthesis of $G_{M2}$ in homogenates could not be detected with either endogenous or exogenous acceptors using a sensitive thin-layer chromatographic assay (Fishman et al., 1975). All attempts to detect activity by varying pH, detergents, and substrate concentrations were unsuccessful.[3]

*Ganglioside Synthesis in Culture.* In order to further explore the origin of the gangliosides in the human fibroblasts, the cells were cultured in medium containing either [$^{14}C$]galactose or *N*-acetyl[$^{3}H$]mannosamine (Brady and Mora, 1970; Simmons et al., 1975). With either of these precursors, all of the gangliosides isolated from the fibroblasts were labeled (Figure 2). As expected, most of the radioactivity was associated with $G_{M3}$. The small galactose-labeled peak which migrated above $G_{M3}$ appeared not to be a ganglioside, as it was not labeled in cells grown with *N*-acetyl[$^{3}H$]mannosamine. The radioactive material corresponding to $G_{D1a}$ was isolated from the chromatogram and further analyzed. The effect of neuraminidase on this material is shown in Figure 3. Following enzyme digestion, approximately 50% of the radioactivity of the *N*-acetyl[$^{3}H$]mannosamine-labeled material was released and the remainder migrated like $G_{M1}$ as expected, since *N*-acetylmannosamine is a precursor of sialic acid and neuraminidase releases one of the two sialic acids from $G_{D1a}$. The [$^{14}C$]galactose-labeled $G_{D1a}$ was only partially digested by neuraminidase, but, following treatment, a new peak corresponding to $G_{M1}$ appeared. When the labeled $G_{M3}$ was similarly analyzed, all of the radioactive product from [$^{14}C$]$G_{M3}$ treated with neuraminidase corresponded to GL-2. Neuraminidase treatment of [$^{3}H$]$G_{M3}$ released all of the radioactivity and yielded unlabeled GL-2. Thus, the fibroblasts can incorporate a specific radioactive precursor into both sialic acid moieties of $G_{D1a}$ and can incorporate radioactive galactose into the asialo portion of this molecule.

*$G_{M1}$ Uptake by Human Fibroblasts.* It is known that calf serum contains gangliosides (Yogeeswaran et al., 1970) and the fetal calf serum used in our studies contained all of the gangliosides found in the cells including 0.5 nmol of $G_{M1}$/mL (data not shown). The possibility existed, therefore, that some portion of the gangliosides found in the cells was derived from the serum. We had previously demonstrated that cultured mouse fibroblasts could bind exogenous [$^{3}H$]$G_{M1}$ (Moss et al., 1976). When the human fibroblasts were incubated in complete medium containing [$^{3}H$]$G_{M1}$, uptake of the ganglioside was minimal (<2% of the $G_{M1}$ content of the cells in 24 h). In contrast, when the cells were incubated in chemically defined medium (NCTC 135), there was rapid uptake of the [$^{3}H$]$G_{M1}$ (Table IV). Approximately $25 \times 10^6$ molecules of [$^{3}H$]$G_{M1}$ were bound per cell by 3 h with no increase on further incubation even though only a small percentage of the exogenous $G_{M1}$ had been taken up by the cells. [$^{3}H$]$G_{M1}$ was recovered from the cells as shown in Figure 4 and no other radioactive gangliosides were detected. Analysis of radioactivity and $G_{M1}$ content in fibroblasts incubated for 3 h with [$^{3}H$]$G_{M1}$ indicated that 90% of the radioactivity remained as [$^{3}H$]$G_{M1}$, and there was excellent agreement between the increment in $G_{M1}$ content measured chemically and the bound [$^{3}H$]$G_{M1}$ (Table V). Thus, in the presence of fetal calf serum, there was minimal uptake of [$^{3}H$]$G_{M1}$, whereas, in the absence of serum, [$^{3}H$]$G_{M1}$ was taken up by the fibroblasts and their $G_{M1}$ content was increased approximately three- to fourfold.[4]

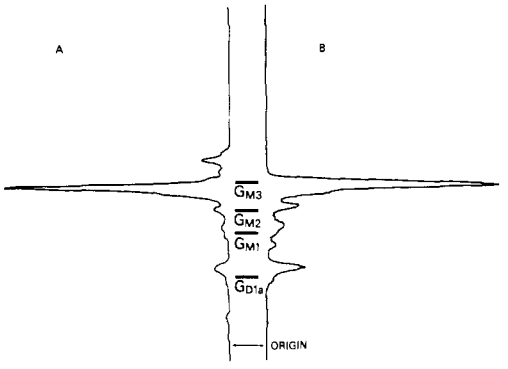


FIGURE 2: Radioscans of thin-layer chromatograms of gangliosides isolated from human fibroblasts grown with radiolabeled precursors. Cells were cultured for 24 h in complete medium containing 50 μCi per dish of [$^{14}C$]galactose or 1 mCi per dish of *N*-acetyl[$^{3}H$]mannosamine. Gangliosides were isolated, chromatographed, and detected as described under Experimental Procedures. (A) Radioscan of $^{14}C$-labeled gangliosides; (B) radioscan of $^{3}H$-labeled gangliosides.

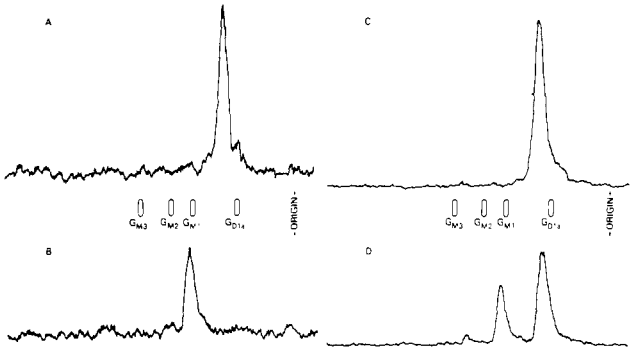


FIGURE 3: Effect of neuraminidase on radioactive $G_{D1a}$ derived from human fibroblasts. Material corresponding to [$^{3}H$]- or [$^{14}C$]$G_{D1a}$ was isolated from the thin-layer chromatogram described in Figure 2 by scraping the silica gel from the plate and eluting the material from the gel with chloroform–methanol–water (10:10:3, v/v). Portions of the labeled material were incubated with active or heat-inactivated neuraminidase, desalted on Sephadex, and rechromatographed as described under Experimental Procedures. [$^{3}H$]$G_{D1a}$ incubated with heat-inactivated (A) or active (B) neuraminidase. [$^{14}C$]$G_{D1a}$ incubated with heat-inactivated (C) or active (D) neuraminidase.

[3] No *N*-acetylgalactosaminyltransferase activity was observed in several other human fibroblast lines derived from skin biopsies or amniotic fluid; in all of these cells, the other glycosyltransferase activities were detected.

[4] Fetal calf serum had a similar effect on the uptake of [$^{3}H$]$G_{M1}$ by $G_{M1}$-deficient transformed mouse fibroblasts. The cells bound 100 times more $G_{M1}$ in the absence of serum than in its presence (Fishman, Moss and Manganiello, manuscript submitted).

TABLE IV: Uptake of [$^3$H]$G_{M1}$ by Human Fibroblasts Incubated in Serum-Free Medium.[a]

| Time (h) | [$^3$H]$G_{M1}$ Content (molecules bound/cell × $10^{-6}$) |
|---|---|
| 0 | 1.6 |
| 3 | 23.9 |
| 5 | 27.8 |
| 10 | 25.8 |
| 24 | 24.6 |

[a] Fibroblasts grown in dishes in complete medium were washed three times with NCTC 135 medium. Each dish of cells was then incubated at 37 °C with 10 mL of NCTC 135 medium containing 2.7 nmol of [$^3$H]$G_{M1}$ (22 mCi/mmol), which was prepared as previously described (Moss et al., 1976). At the indicated times, the medium was removed and the cells were washed four times with 4 mL of phosphate-buffered saline. The cells were scraped in the same buffer and the dishes were washed twice with the buffer. The cells and washes were combined and centrifuged and the precipitate was dissolved in 0.2 mL of 1 M NaOH. Duplicate aliquots were analyzed for protein and radioactivity. Cell numbers were calculated from the protein value, 1 mg of protein being equivalent to 2.88 × $10^6$ cells. Less than 10% of the total exogenous $G_{M1}$ was taken up by the cells.

TABLE V: Recovery and Quantification of [$^3$H]$G_{M1}$ Taken up by Human Fibroblasts.[a]

| Analysis | Addition to Medium: None | Addition to Medium: [$^3$H]$G_{M1}$ |
|---|---|---|
| Total protein (mg) | 5.8 | 6.1 |
| $^3$H in ganglioside fraction (cpm) | | 5389[b] |
| [$^3$H]$G_{M1}$ (pmol/mg of protein) | | 107[c] |
| $G_{M1}$ (pmol/mg of protein) | 45 | 159 |
| Net increase in $G_{M1}$ (pmol/mg of protein) | | 114 |

[a] Human fibroblasts were incubated for 4 h in NCTC 135 medium with or without [$^3$H]$G_{M1}$ (1 nmol/dish; 14.9 $\mu$Ci/$\mu$mol) as described in Table IV. The cells were harvested, washed, and extracted with chloroform–methanol; the residues were dissolved in 1 M NaOH and analyzed for protein and radioactivity; and the extracts were partitioned into ganglioside and nonganglioside fractions as described under Experimental Procedure. Samples of each fraction were analyzed for radioactivity and the remainder of the ganglioside fractions were subjected to thin-layer chromatography. Following radioscanning of the chromatogram, (see Figure 4), the gangliosides were visualized with resorcinol reagent and quantified by densitometry. [b] Represents 90% of total radioactivity found in the cells. [c] Calculated from the specific activity of the added [$^3$H]$G_{M1}$.

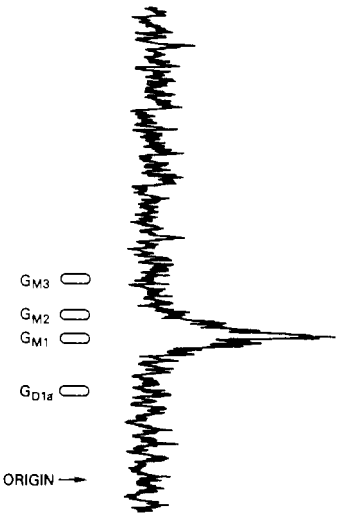


FIGURE 4: Recovery of [$^3$H]$G_{M1}$ from human fibroblasts. Human fibroblasts were incubated for 4 h in NCTC 135 medium (serum-free) containing 14 nmol of [$^3$H]$G_{M1}$ (15 mCi/mmol) (Moss et al., 1976). The cells were washed, harvested, and extracted as described in Table II. The extract was desalted on a small column of Sephadex G-25 and chromatographed as described under Experimental Procedures. The chromatogram was then scanned for radioactivity.

## Discussion

A wide variety of vertebrate cells respond to choleragen (Finkelstein, 1973). The initial step in the action of toxin is the binding of the B subunits to the membrane receptor which is presumably the monosialoganglioside, $G_{M1}$ (Cuatrecasas et al., 1973; Lönnroth and Holmgren, 1973). This conclusion is based on the ability of $G_{M1}$ to inhibit choleragen binding and action (Cuatrecasas, 1973; Holmgren et al., 1973; King and van Heyningen, 1973), the enhancement of toxin binding and/or effects after incubation of cells or membranes with $G_{M1}$ (Cuatrecasas, 1973), and the correlation between $G_{M1}$ content and choleragen response in transformed mouse fibroblasts (Hollenberg et al., 1974) and intestinal cells (Holmgren et al., 1975). We have recently reported that $G_{M1}$-deficient mouse fibroblasts that are unresponsive to choleragen when grown in chemically defined medium become responsive after binding exogenous $G_{M1}$ (Moss et al., 1976). After binding comparable amounts of $G_{M3}$, $G_{M2}$, or $G_{D1a}$, the cells remained unresponsive (Fishman et al., 1976).

Many cells contain the enzymes necessary for synthesizing gangliosides but other cells do not (Fishman, 1974). Cultured human fibroblasts are very responsive to choleragen (Table I, and Hollenberg and Cuatrecasas, 1973) and the presence of hexosamine-containing gangliosides in such cells has been reported (Callahan et al., 1970; Kolodny et al., 1973). However, more detailed analyses indicated that cultured human fibroblasts were deficient in gangliosides more complex than $G_{M3}$ and $G_{D3}$ (Dawson et al., 1972; Bach et al., 1975). The human fibroblasts studied here contained relatively small amounts (≤100 pmol/mg of protein) of gangliosides that corresponded to $G_{M2}$, $G_{M1}$, and $G_{D1a}$. The differences between our findings and observations of others (Dawson et al., 1972; Bach et al., 1975) may reflect differences in the source of the cells, age of the cells in culture,[5] or methodology. Our procedure for isolation of gangliosides did not include dialysis during which losses could occur (Kanfer and Spielvogel, 1973).

Although the cells contained three of the glycosyltransferase activities involved in ganglioside biosynthesis, they apparently lacked the *N*-acetylgalactosaminyltransferase essential for $G_{M2}$ synthesis. However, when grown in the presence of radioactive sugars, the cells incorporated radioactivity into both the inner and terminal sialic acids of $G_{D1a}$ as well as into the asialotetrahexosylceramide portion of the molecule. They also incorporated radioactivity into $G_{M1}$ and $G_{M2}$. In terms of the known pathway for ganglioside biosynthesis (Fishman, 1974), this means that the cells which contained 222 pmol/mg of protein of $G_{M2}$, $G_{M1}$, and $G_{D1a}$ were capable of synthesizing these gangliosides from $G_{M3}$. With a doubling time of 24 h, the cells would require for synthesis of this amount of gangliosides an *N*-acetylgalactosaminyltransferase activity of approximately 10 pmol (mg of protein)$^{-1}$ h$^{-1}$. This level of activity

[5] We recently observed a similar ganglioside content and incorporation of radioactive precursors into these gangliosides in a newly established line of human skin fibroblasts (three subcultures from the original tissue).

might not have been detected in the assay used. It is also possible that less than optimal assay conditions, hydrolysis of product in the assay, lability of the enzyme, or release of an inhibitor during cell lysis accounts for our failure to detect any enzyme activity.

The $G_{M1}$ content of the human fibroblasts, although relatively small, nevertheless represents $8 \times 10^6$ molecules per cell.[6] It seems likely that this amount is ample to account for the responsiveness of these cells to choleragen, since in $G_{M1}$-deficient transformed mouse fibroblasts uptake of only $10^5$ molecules of exogenous $G_{M1}$ per cell was sufficient to confer maximal sensitivity to choleragen (Moss et al., 1976). Despite a large, though limited, capacity for binding exogenous $G_{M1}$ ($25 \times 10^6$ molecules per cell) from serum-free medium, the human fibroblasts took up relatively little $G_{M1}$ from medium containing serum. It would appear, therefore, that $G_{M1}$ may be bound to proteins or other constituents of serum and in that form is not readily available for uptake by cells. This might mean that in vivo the bulk of the gangliosides found in specific cells were synthesized in those cells. On the other hand, the ability of the cultured human fibroblasts to bind $G_{M1}$ from serum may not be representative of the situation in other types of cells and in certain instances even a relatively low rate of uptake from plasma could contribute significantly to the ganglioside pool.[7] In any case, it appears that the choleragen-sensitive cultured human fibroblasts contain $G_{M1}$, the putative choleragen receptor, and a majority of the ganglioside is synthesized de novo by these cells.

Acknowledgments

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[6] Based on the binding of [$^{125}$I]choleragenoid, Bennett et al. (1975) estimated that cultured human fibroblasts contained between 0.5 and 1 $\times 10^6$ receptors per cell. Our own studies with [$^{125}$I]choleragen indicated $5 \times 10^5$ receptors per cell (Moss et al., 1977).

[7] When the $G_{M1}$-deficient mouse cells were grown in medium containing 10% fetal calf serum, they became responsive to choleragen. As indicated in footnote 3, these cells in the presence of serum bind small amounts of [$^3$H]$G_{M1}$ which are apparently sufficient to confer sensitivity to choleragen. Using a surface labeling procedure, we could detect $G_{M1}$ on the mouse cells grown with fetal calf serum (Fishman, Moss, and Manganiello, manuscript submitted).