# Effect of serum on ganglioside uptake and choleragen responsiveness of transformed mouse fibroblasts

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**Abstract** NCTC 2071 cells, transformed mouse fibroblasts, did not respond to choleragen when grown in chemically defined medium. When grown in medium containing 10% fetal calf serum, however, the cells accumulated cyclic AMP upon exposure to the toxin. Gangliosides isolated from the fetal calf serum were as effective as whole serum in inducing choleragen responsiveness in the cells. The putative choleragen receptor, the monosialoganglioside $G_{M1}$, could not be detected by chemical analysis in cells exposed to serum. $^3$H-Labeled $G_{M1}$ was detected in these cells, however, following sequential exposure to galactose oxidase and sodium borotritide. Thus, uptake of minute amounts of $G_{M1}$ from serum by these cells sensitized them to choleragen.

**Supplementary key words** cyclic AMP · adenylate cyclase · glycolipids · membrane receptors · cell surface labeling · galactose oxidase

We recently reported that chemically transformed mouse fibroblasts (NCTC 2071 cells), when grown in chemically defined medium in the absence of serum, did not respond to choleragen (1). We attributed the lack of response to the inability of these cells to synthesize the choleragen receptor, the monosialoganglioside $G_{M1}$ (2). These fibroblasts did bind exogenous $G_{M1}$, however, and subsequently, did respond to choleragen (1). Other exogenous gangliosides were ineffective or much less effective in inducing a response to the toxin (2).

In this paper we report that NCTC 2071 cells grown in medium with fetal calf serum become sensitive to choleragen. Serum contains gangliosides including $G_{M1}$ (3, 4); however, studies with human fibroblasts demonstrated that the $G_{M1}$ was not readily available to these cells (5). Since prior work on the choleragen responsiveness of cultured cells was performed with cells grown in medium containing serum (6), we decided to quantify the $G_{M1}$ uptake and choleragen responsiveness of the cells grown with serum.

## EXPERIMENTAL PROCEDURES

NCTC 2071 cells were obtained from the American Type Culture Collection and grown in completely synthetic NCTC 135 medium as previously described (1). Where indicated, fetal calf serum (North American Biologicals, Miami, FL) was added to Eagle's minimal essential medium. The determination of cyclic AMP and the preparation of [$^3$H]$G_{M1}$ (4.37 Ci/mmol) by oxidation with galactose oxidase and reduction with NaB[$^3$H]$_4$ have been described (1). $G_{M1}$ obtained from Supelco, Inc., Bellefonte, PA, was purified further by thin-layer chromatography and was at least 99% pure.

### Isolation of serum gangliosides

Gangliosides were isolated from 30-ml batches of fetal calf serum according to Yu and Ledeen (4) with the following modifications. Instead of dialyzing the crude ganglioside fraction, it was dissolved in 4 ml of chloroform–methanol 2:1 (v/v) and 0.2 ml of 0.1 M EDTA (pH 7) and desalted on a column of 2.5 g of Sephadex G-25 superfine, which was eluted with 20 ml of solvent as previously described (7). Similarly, fraction B from the DEAE–Sephadex column was taken to dryness, treated with base (1 ml of 0.6 M NaOH in methanol and 2 ml of chloroform)

Abbreviations: $G_{M1}$, galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide; $G_{M3}$, *N*-acetylneuraminylgalactosylglucosylceramide; $G_{D1a}$, *N*-acetylneuraminyl-galactosyl-*N*-acetylgalactosaminyl-[*N*-acetylneuraminyl]-galactosylglucosylceramide; PBS, phosphate-buffered saline (pH 7.4); MEM, Eagle's minimal essential medium; [$^3$H]$G_{M1}$, $^3$H-labeled $G_{M1}$.

TABLE 1. Effect of fetal calf serum on the response of NCTC 2071 cells to choleragen

| Medium | Cyclic AMP Content | |
|---|---|---|
| | −Choleragen | +Choleragen |
| | *pmol/mg protein* | |
| NCTC 135 | 4.1[b] | 6.4[b] |
| MEM + 10% fetal calf serum[a] | 4.9 | 19.0 |

[a] NCTC 135 medium containing 10% fetal calf serum was toxic to the cells.

[b] The effect of choleragen on cAMP was not statistically significant ($P > 0.1$) in this experiment; this control has been repeated in more than 10 experiments.

Cells were grown in NCTC 135 medium until confluent and the medium was replaced with either NCTC 135 medium or Eagle's minimal essential medium (MEM) plus 10% fetal calf serum. After 24 hr, the cells were washed twice with Hanks medium and incubated in 10 ml of Hanks medium with or without 10 μg of choleragen for 3 hr. Cyclic AMP content was determined as described previously (1) and is the mean of triplicate determinations.

for 1 hr at 37°C, neutralized with 0.15 ml of 4 M acetic acid, and desalted on Sephadex as described above.

### Cell surface labeling

Serum gangliosides bound to the cells were labeled by incubating the cells with galactose oxidase (137 units/mg from Worthington Biochemical Corp., Freehold, NJ) (2, 8). Each dish of cells was incubated with 30 units of galactose oxidase in 2.5 ml of PBS for 2 hr, washed twice with 5 ml of PBS, and the cells from 30 dishes were collected and incubated with 3 mCi of $NaB[^3H]_4$ (6 Ci/mmol from Amersham/Searle, Arlington Heights, IL) in 0.5 ml of PBS for 10 min. The cells were washed three times with PBS.

### Isolation of labeled gangliosides

Gangliosides were isolated from the cells (1) and further purified on small (100 mg) columns of Unisil (4). The gangliosides were separated by thin-layer chromatography on silica gel 60 coated glass plates (E. Merck, Darmstadt, Ger.) in the solvent system chloroform–methanol–0.25% $CaCl_2$ 60:35:8 (v/v/v), detected by radioscanning and visualized with resorcinol reagent (7, 9). Radioactivity was determined by calculating the areas under the peaks of the radioscans and comparing these values with those obtained from known amounts of $[^3H]G_{M1}$ applied to the chromatograms (8). $G_{M1}$ was determined by densitometry; the chromatograms were scanned at 580 nm with a Zeiss chromatogram scanner equipped with a recorder. The areas under the peaks of the recorder tracing were compared to values obtained from known amounts of $G_{M1}$ applied to the chromatograms (10).

## RESULTS

As shown in **Table 1**, NCTC 2071 cells, following incubation overnight in medium containing 10% fetal calf serum, responded to choleragen; intracellular cyclic AMP content increased 5-fold in 3 hr. Cells not exposed to serum remained unresponsive to the toxin. The fetal calf serum used in these experiments contained gangliosides and the two gangliosides corresponding to $G_{M1}$ were the predominant ones resistant to bacterial neuraminidase (**Fig. 1**). Gangliosides extracted and purified from 1 ml of fetal calf serum (containing 7.4 nmol of ganglioside-bound sialic acid and 0.1 nmol of $G_{M1}$) were as effective as that amount of serum in sensitizing NCTC 2071 cells to choleragen (**Table 2**). On chemical analysis of cells grown with serum, however, no $G_{M1}$ was detected.

Exogenous $G_{M1}$ bound to NCTC 2071 cells can be oxidized by galactose oxidase and the oxidized $G_{M1}$ can be reduced subsequently with $NaB[^3H]_4$ (8). Using this technique, radioactivity was incorporated into the ganglioside fraction of cells grown with serum and a peak of radioactivity corresponding to $G_{M1}$ was observed after separation of the ganglioside fraction by thin-layer chromatography (**Fig. 2**). No labeled $G_{M1}$ was detected by this technique in NCTC 2071 cells grown in the absence of serum (2). Based on the sp act of the $NaB[^3H]_4$, we calculated that approximately 80,000 molecules of $G_{M1}$ per cell were accessible to

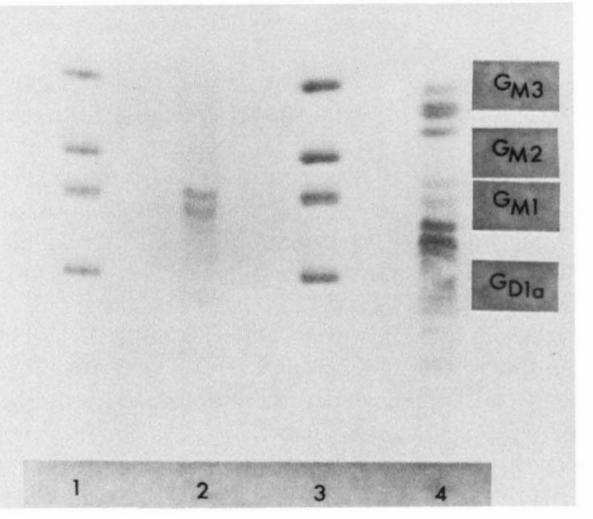


**Fig. 1.** Pattern of gangliosides isolated from fetal calf serum and effect of neuraminidase on serum gangliosides. Gangliosides were extracted from fetal calf serum, purified, separated by thin-layer chromatography, and visualized with resorcinol as described in Experimental Procedures. Where indicated, gangliosides were incubated with 0.1 ml of Vibrio cholera neuraminidase solution (Behring Diagnostics, Sommerville, NJ) for 18 hr. Lanes 1 and 3, ganglioside standards as indicated; lane 2, gangliosides from 5 ml of serum after neuraminidase treatment; lane 4, gangliosides from 5 ml of serum.

galactose oxidase. Since previous studies indicated that NCTC 2071 cells bound greater amounts of exogenous $G_{M1}$ from chemically defined medium (1), it appeared that the gangliosides in serum were not readily taken up by the cells.

When cells were incubated in medium with 10% serum (which represents ~ 100 pmol of $G_{M1}$ per dish) or in the absence of serum plus 30 nM $G_{M1}$ (300 pmol per dish) both supplemented with [$^3$H]$G_{M1}$ (2.5 pmol per dish), $G_{M1}$ was detected chemically only in the cells incubated without serum (Table 3). [$^3$H]$G_{M1}$ was recovered from both sets of cells but it represented a smaller percentage of the total radioactivity associated with the serum-treated cells than the cells incubated without serum. The sp act of the [$^3$H]$G_{M1}$ recovered from the cells not exposed to serum (30 $\mu$Ci/$\mu$mol) was similar to that added to the medium (36 $\mu$Ci/$\mu$mol) and the amount of $G_{M1}$ bound to the cells increased with time of incubation. Based on the chemical analysis and radioactivity recovered as $G_{M1}$, between 15 and 18% of the added $G_{M1}$ was recovered in the cells after incubation for 42 hr without serum. In the cells incubated with serum, the amount of [$^3$H]$G_{M1}$ (in dpm/mg of cell protein) decreased between 18 and 42 hr of incubation. After 42 hr, these cells contained less [$^3$H]$G_{M1}$ than did the cells incubated without serum. If the [$^3$H]$G_{M1}$ added to the medium had equilibrated with the $G_{M1}$ in the serum, the cells would have bound 90 pmol of $G_{M1}$ in 42 hr and this amount of $G_{M1}$ would have been detected chemically by our method.

## DISCUSSION

Gangliosides have been found in calf and bovine serum (3, 4) and the fetal calf serum used in our experiments contained two gangliosides identified as $G_{M1}$, based on their mobility on thin-layer chromatograms and their resistance to bacterial neuraminidase, which cleaves terminal sialic acid residues from $G_{M3}$ and $G_{D1a}$ but not from $G_{M1}$ (11). The two forms of $G_{M1}$ presumably differ due to the presence of *N*-acetylneuraminic or *N*-glycolylneuraminic acid (3, 4). The fetal calf serum used in our studies contained 7.4 nmol of ganglioside-bound sialic acid but only 0.1 nmol of $G_{M1}$ per ml. Yogeeswaran et al. (3), however, reported that $G_{M1}$ represented 25% of the ganglioside-bound sialic acid which was 7–7.6 nmol per ml in new born calf serum. The reasons for apparently large differences in $G_{M1}$ content are unclear.

Cells and isolated membranes can bind exogenous $G_{M1}$ (12–14). This bound $G_{M1}$ enhanced the cellular responsiveness to choleragen and increased choleragen binding. Recently, we demonstrated that the number of $G_{M1}$ molecules that must be bound per cell to induce responsiveness to choleragen is quite small (1, 2). Since most cultured cells are grown in medium containing serum which, as demonstrated here and elsewhere, has a high ganglioside content, uptake of $G_{M1}$ from the supplemented medium could modify the ganglioside composition of the cells

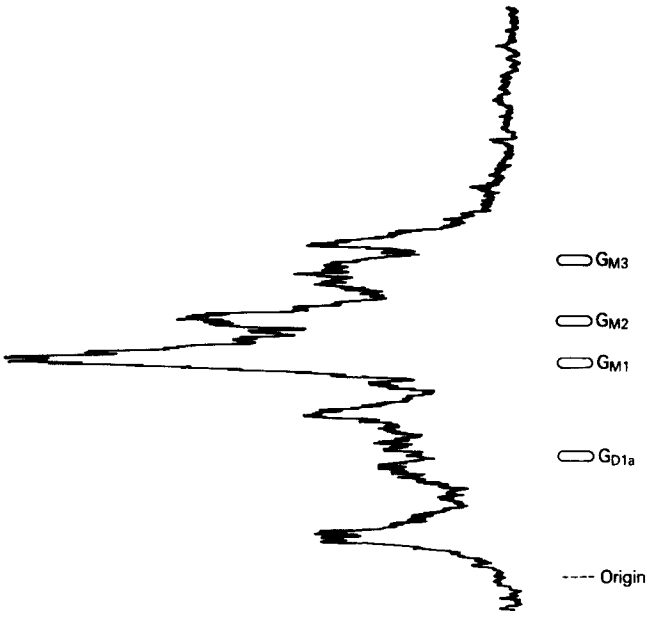


**Fig. 2.** Radioscan of thin-layer chromatogram of gangliosides isolated from NCTC 2071 cells labeled with galactose oxidase and NaB[$^3$H]$_4$. NCTC 2071 cells (30 dishes) were incubated for 18 hr in MEM containing 10% fetal calf serum, washed, and incubated with galactose oxidase and NaB[$^3$H]$_4$ as described in Experimental Procedures. Gangliosides were extracted from the cells, purified, and separated by thin-layer chromatography. Radioactivity was detected by radioscanning and standards were visualized by resorcinol reagent.

TABLE 2. Effect of serum and serum gangliosides on the response of NCTC 2071 cells to choleragen

| Medium | Cyclic AMP Content | |
|---|---|---|
| | −Choleragen | +Choleragen |
| | *pmol/mg protein* | |
| MEM + 10% serum | 2.1 ± 1 | 11.0 ± 1.9 |
| NCTC 135 + serum gangliosides | 0.3 ± 0.3[a] | 9.3 ± 1.9 |

[a] We have observed previously that, in NCTC 2071 cells incubated with gangliosides, the basal content of cAMP was lower than in cells not exposed to gangliosides (1,2).

Cells were grown to confluency in NCTC 135 medium, incubated with the indicated medium (10 ml per dish) for 24 hr, exposed to choleragen, and analyzed for cyclic AMP as described in Table 1. Gangliosides were extracted and purified from fetal calf serum as described in Experimental Procedures. Gangliosides isolated from 1 ml of serum were added to each dish of cells. Values are the mean ± SD of triplicate determinations.

TABLE 3. Effect of serum on uptake of [$^3$H]$G_{M1}$ by NCTC 2071 cells

| Additions to Medium | Incubation Time | Cell Protein | Radioactivity | | $G_{M1}$ Taken up by Cells | |
|---|---|---|---|---|---|---|
| | | | Total | [$^3$H]$G_{M1}$ | | |
| | *hr* | *mg* | *dpm* | *dpm* | *pmol* | *molecules/cell* ($\times 10^{-3}$) |
| 10% Serum | 18 | 4.1 | 18,100 | 11,950 | 1.2[a] | 63[b] |
| 10% Serum | 42 | 4.9 | 16,800 | 9,440 | 1.0[a] | 41[b] |
| 30 nM $G_{M1}$ | 18 | 3.5 | 6,350 | 5,000 | 74 | 4,400 |
| 30 nM $G_{M1}$ | 42 | 4.4 | 21,230 | 17,800 | 271 | 12,900 |

[a] No $G_{M1}$ was detected visually on the chromatogram; these values are calculated from the sp act of the [$^3$H]$G_{M1}$ (4.37 Ci/mmol) added to the medium.

[b] These values do not include the 80,000 molecules per cell detected by the galactose oxidase procedure.

NCTC 2071 cells were grown for 18 or 42 hr in MEM containing 10% fetal calf serum or NCTC 135 medium containing 30 nM $G_{M1}$; both media were further supplemented with 2.4 $\times 10^4$ dpm per dish of [$^3$H]$G_{M1}$ (4.37 Ci/mmol). Cells from five dishes were pooled for each determination. Gangliosides were extracted from the cells, purified, separated by thin-layer chromatography, analyzed for radioactivity by radioscanning, and quantified by densitometry as described in Experimental Procedures. Only one radioactive peak corresponding to $G_{M1}$ was observed as previously reported (2).

and thereby their responsiveness to choleragen. In the present study, we have examined the extent of $G_{M1}$ uptake by cells in the presence and absence of serum. In medium supplemented with serum most of the ganglioside was unavailable to the cells, but sufficient $G_{M1}$ could be bound to induce a choleragen response in unresponsive cells. We have previously observed a choleragen response with as few as 17,000 molecules of $G_{M1}$ per cell and a maximal response with $10^5$ molecules per cell (1). As gangliosides extracted from serum were as effective as whole serum, it is unlikely that other components in the serum are required to sensitize the cells to choleragen.

The relatively limited uptake of gangliosides from serum may be due to their association with serum proteins. As can be inferred from **Table 3**, exogenous [$^3$H]$G_{M1}$ did not equilibrate rapidly with gangliosides in the serum and although radioactive $G_{M1}$ was bound, no bound ganglioside was detected chemically. In contrast, cells incubated in defined medium bound ganglioside, and analysis of the cell-bound $G_{M1}$ showed that the [$^3$H]$G_{M1}$ had equilibrated with the exogenous unlabeled $G_{M1}$ in the medium.

Although little $G_{M1}$ was bound to NCTC 2071 cells grown in serum, it was clearly detectable using the galactose oxidase–sodium borotritide method of analysis. Previous studies with transformed mouse fibroblasts indicated that the ability of these cells to bind or respond to choleragen was correlated with the presence of and the ability to synthesize $G_{M1}$ (6). However, even transformed cells that were devoid of chemically detectable $G_{M1}$ and deficient in the requisite enzymes for $G_{M1}$ synthesis were able to bind and respond somewhat to the toxin. Hollenberg et al. (6) suggested that perhaps these cells were able to incorporate gangliosides from the serum in the culture medium and this is borne out by the data reported here.

We have recently shown that normal human fibroblasts can bind [$^3$H]$G_{M1}$ from medium containing serum (5). These cells can synthesize the ganglioside and respond to choleragen in the absence of added $G_{M1}$. Thus, the ability to bind exogenous ganglioside is not confined to cells deficient in ganglioside synthesis. The capacity of the human fibroblasts to bind ganglioside was, however, not unlimited; there appeared to be a maximal number of $G_{M1}$ molecules that could be bound per cell (5).

Finally, these and other studies clearly indicate that the amount of $G_{M1}$ per cell required for a choleragen response is below the limits of chemical detection. Thus, the absence of chemically detectable $G_{M1}$ in cells or membranes is not evidence against the participation of $G_{M1}$ as the choleragen receptor in such systems[1]. Similar considerations apply to the possible role(s) of gangliosides as receptors for other toxins, hormones, and interferon (15–18). The ability of serum gangliosides to modify the ganglioside content of cultured cells suggests that NCTC 2071 cells grown in defined medium may be the best available model for the investigation of gangliosides as membrane receptors.

[1] In a recent report, Kanfer et al. (19) suggested that $G_{M1}$ is not the choleragen receptor in fat cells. They based their conclusions on their inability to detect chemically any $G_{M1}$ in fat cell preparations even though they calculated that a maximum of $1.7 \times 10^5$ molecules of $G_{M1}$ per cell could have escaped detection.

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