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Cautionary Note on the Use of the B Subunit of Cholera Toxin as a Ganglioside GM1 Probe: Detection of Cholera Toxin A Subunit in B Subunit Preparations by a Sensitive Adenylate Cyclase Assay

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The use of the B subunit of cholera toxin, a protein that binds specifically to ganglioside GM1, has provided a new paradigm for studying physiological functions of ganglioside GM1. The B subunit inhibited the growth of rat glioma C6 cells that had been pretreated with ganglioside GM1. In some preparations of the B subunit, the inhibition was independent of adenylate cyclase activation and was due to the binding of the B subunit to ganglioside GM1 inserted onto the cell surface. However, in other preparations of the B subunit, there was an additional inhibitory effect due to small contaminations with the A subunit, which caused increases in intracellular cyclic adenosine monophosphate (cAMP) levels and concomittant growth inhibition. This vanishingly small contamination with the A subunit could not be detected by conventional protein sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis but could be measured utilizing a sensitive adenylate cyclase activation assay. Thus caution must be used to ensure that any biological effects of the B subunit are not due to contaminating A subunit and are due solely to the binding of the B subunit to ganglioside GM1 exposed on the cell surface. This is especially important in cyclic nucleotide-sensitive systems.

Key words: rat glioma C6 cells, B subunit of cholera toxin, cyclic adenosine monophosphate, cAMP

Research on the biological functions of gangliosides, sialic acid-containing glycosphingolipids, has accelerated in recent years due to the discovery of their profound effects when administered exogenously [reviewed in 2–5]. Several laboratories have

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CT, cholera toxin; IBMX, 1-methyl-3-isobutylxanthine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Ganglioside nomenclature follows Svennerholm [1].

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convincingly demonstrated that exogenous gangliosides can alter the growth properties of a variety of cell types [2,3] and also have neuritogenic and neuronotrophic effects both in vivo and in vitro [4–7]. The monosialoganglioside GM1, in particular, has been shown to alter cellular proliferation, to cause differentiation of neuronal cultures, to enhance sprouting of regenerating peripheral nerves, and to accelerate repair of central nervous system (CNS) damage [2–7]. These findings led to the contention that the effect of exogenous ganglosides reflects the function of their endogenous counterparts. To gain insight into the putative role of GM1, a different approach was recently developed [8,9]. The B subunit of cholera toxin (CT), which is pentavalent and binds solely to ganglioside GM1 on the cell surface [8,10], was used as a ganglioside-specific probe to induce cell proliferation in quiescent, nontransformed mouse 3T3 fibroblasts [11,12] and in resting thymocytes [8,13]. In contrast, the B subunit inhibits the growth of *ras*-transformed 3T3 cells as well as rapidly dividing normal 3T3 cells [11]. The finding that a bimodal response to the B subunit could be observed in the 3T3 cells just by varying their state of growth suggests that endogenous gangliosides may be bimodal regulators of both positive and negative signals for cell growth.

Recently, others have extended the use of the B subunit to evaluate the role of membrane GM1 in the regulation of growth in different cell types, such as astroglial cells [14], rat glioma C6 cells [15,16], and rat thyroid FRTL-5 cells [17]; to examine the immunomodulatory properties of ganglioside GM1 [18]; and to study the functions of endogenous ganglioside GM1 in neurite regeneration in vitro [19–21].

It is important to establish that the biological effects of the B subunit are due entirely to interaction with ganglioside GM1 and not to activation of adenylate cyclase resulting from contamination with the A subunit of CT. The A subunit is an enzyme that can catalytically activate adenylate cyclase by adenosine diphosphate (ADP)-ribosylation of the stimulatory G protein [22]. Extremely small amounts of the A subunit can therefore have significant effects on cells, which are sensitive to changes in cyclic adenosine monophosphate (cAMP) levels.

Rat glioma C6 cells seem to be especially suitable for the detection of vanishingly small amounts of the A subunit. The major advantages of C6 cells are that they are nearly devoid of ganglioside GM1, that the level of membrane-associated GM1 can be manipulated by the functional insertion of exogenous GM1 [10,23], and that they have an adenylate cyclase system that is very sensitive to the A subunit [10,23]. Furthermore, in a recent study, I showed that pure preparations of the B subunit inhibited DNA synthesis in rapidly dividing rat glioma C6 cells in which the level of membrane-bound GM1 was increased by pretreatment either with exogenous ganglioside or with butyrate, which increased de novo biosynthesis of GM1 [15]. However, I have now found, as shown here, that some preparations of the B subunit give a much greater growth inhibition, which corresponds with increased adenylate cyclase activity due to a small contamination ($\leq$0.05%) with the A subunit. The small contamination could only be detected with the very sensitive assay used in these studies and caution must be taken in the use of the B subunit as a tool to study ganglioside function.

MATERIALS AND METHODS

Materials

The B or binding subunit of CT was purchased from List Biological Laboratories (Campbell, CA) and from Schwarz/Mann Biotech (Cleveland, OH). CT was from List

Biological Laboratories and was iodinated using the chloramine-T method [24]. GM1 was purified as described previously [25] from bovine brain gangliosides after treatment with *Vibrio cholerae* neuraminidase [EC 3.2.1.18], which hydrolyzes polysialogangliosides to GM1. Asialo-GM1 ($GgOSe_4Cer$) was prepared by heating GM1 for 2 h at 80°C in 0.1 N HCl to hydrolyze all the sialic acid residues. The asialo-GM1 solution was desalted on Sephadex G-25 columns and separated into neutral (asialo GM1) and acidic fractions (GM1) on DEAE-Sephadex as described previously [25]. The neutral fraction (solvent A) was analyzed by thin-layer chromatography on silica gel with the solvent system chloroform:methanol:water (70:22:3, v/v). Only asialo-GM1 was detected by spraying the plates with orcinol, and no gangliosides were detected when the thin-layer chromatogram was treated with resorcinol reagent. [^{3}H]thymidine (55 Ci/mmol) was purchased from ICN (Irvine, CA). Adenosine triphosphate (ATP), guanosine triphosphate (GTP), and IBMX were from Sigma Chemical Co. (St. Louis, MO). DMEM was from Advanced Biotechnologies Inc. (Silver Spring, MD).

Cell Lines and Cell Cultures

Rat glioma C6 cells, obtained from the American Type Culture Collection, were used between passages 40 and 50 and were grown as described previously [26]. For measurement of DNA synthesis and cAMP accumulation and for in situ binding studies, the cells were seeded 3–4 days before an experiment and were grown on multicluster plastic tissue culture dishes (24 × 16 mm wells from Costar, Cambridge, MA) in DMEM containing 5% FCS [26].

Measurement of DNA Synthesis in C6 Cells

At a density of 7.5×10^5 cells/well, the cells were washed with DMEM to remove residual serum. Glycolipids were dissolved in sterile distilled water by warming at 37°C for 1 h. The concentrated glycolipid solutions then were diluted with serum-free medium and added to the cells to give a final concentration of 0.2 μM. After incubation of the cells for 16 h at 37°C, the medium was removed and the cells were washed with DMEM and treated with the indicated reagent. After 20 h, the cells were pulsed with 0.5 μCi of [^{3}H]thymidine for 4 h. The incorporation of radioactivity into trichloroacetic acid-insoluble material was measured as described [11].

Assay of cAMP and Adenylate Cyclase

Adenylate cyclase activity was determined by the change in intracellular cAMP in intact cells and by the direct assay of adenylate cyclase activity in the cell lysates. Accumulation of cAMP by the cells was determined essentially as described previously [15,26]. Briefly, cells were incubated in serum-free medium buffered with 25 mM Hepes (pH 7.4) and supplemented with 0.5 mM IBMX, a phosphodiesterase inhibitor, and 0.01% bovine serum albumin. After incubations with various concentrations of CT and the B subunit for 2 h, cAMP was extracted from the cells with 0.1 M HCl and measured by radioimmunoassay [15,26]. The precipitated proteins remaining in the dish were dissolved in 0.2 M NaOH and measured [27]. Values are the means of triplicate determinations, and standard errors were routinely less than 10% of the mean. For the adenylate cyclase method, the cells were cultured in 25 cm^2 flasks, treated with various agents as described in the text, washed with cold phosphate-buffered saline (PBS), detached, and lysed in 2 mM Tris HCl (pH 7.4)/1 mM EDTA by sonication. Adenylate

TABLE I. Effect of the B Subunit of Cholera Toxin (CT) on DNA Synthesis and Iodinated CT Binding in Rat Glioma C6 Cells†

Culture treatment	[^{3}H]thymidine incorporation (% of none)			^{125}I-CT bound (fmol/mg protein)
	None	Concanavalin A	B subunit	
Control	100 ± 3	103 ± 5	97 ± 4	34 ± 4
GM1	96 ± 6	97 ± 7	72 ± 6*	2,240 ± 200
Asialo-GM1	104 ± 5	105 ± 6	106 ± 5	35 ± 7

†Cells were cultured for 16 h without or with GM1 (0.2 μM) or with asialo-GM1 (0.2 μM), washed with DMEM, and incubated in the absence or in the presence of Con A (1 μg/ml) or B subunit from source 1 (1 μg/ml) for 20 h. [^{3}H]thymidine incorporation was determined as described in Materials and Methods. The results are from five different experiments. The data are expressed as percentages of the none values obtained in the absence of any reagent. Iodinated CT binding was assayed in duplicate cultures by incubation with 20 nM iodinated CT for 1 h at 37°C. Nonspecific binding was determined by the addition of 0.2 μM unlabeled CT to the assay. All values were corrected for nonspecific binding and are the means of quadruplicate determinations.

*Statistically significant (Student's t test), $P < 0.01$ compared to untreated cells.

cyclase activity was measured as described previously in the presence of 50 μM GTP [28].

Measurement of Specific Binding of Iodinated CT

Binding of iodotoxin to cells in situ was measured as described elsewhere [26]. Briefly, cells were incubated with iodinated CT (0.1–20 nM) in 0.25 ml of serum-free medium buffered with 25 mM Hepes (pH 7.4) and supplemented with 0.1% bovine serum albumin. After 1 h at 37°C, the medium was removed, and iodinated CT bound to the cells was determined. Nonspecific binding was determined by the addition of 0.2 μM unlabeled CT to the assay.

RESULTS

Effect of the B Subunit From Different Sources on DNA Synthesis in Rat Glioma C6 Cells

In agreement with previous studies [15,16], the B subunit of CT had no effect on the proliferation of control C6 cells. However, prior treatment of C6 cells with 0.2 μM GM1, which caused a 60-fold increase in iodinated CT binding, rendered the cells sensitive to the B subunit (Table I). The antiproliferative activity of the B subunit was not associated with any cytotoxic effects; the number of viable cells, determined by trypan blue exclusion, was always more than 95%. The inhibitory effect of the B subunit appears to be specific in that concanavalin A, a lectin that can bind to cell-surface glycoconjugates, had no effect on the growth of the control or GM1-treated C6 cells (Table I). Furthermore, treatment of the cells with 0.2 μM asialo GM1, which does not increase CT binding, did not render the cells responsive to the B subunit (Table I). The experiments described above were all performed with B subunit obtained from source 1.[1] In other studies, investigators have used the B subunit obtained from another source [8,13,14,16–21]. Therefore, it was of interest to compare the effects of these preparation

[1]There are only two commercial sources for pure B subunit of cholera toxin. Source 1 is Schwarz/Mann Biotech (Cleveland, OH). Source 2 is List Biological Laboratories (Campbell, CA).

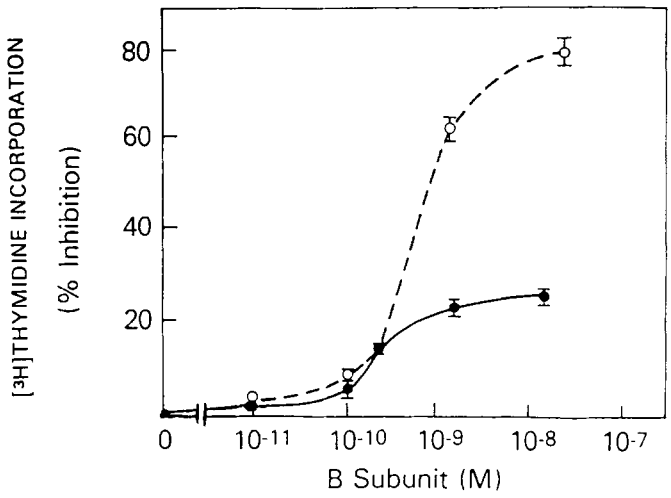

Fig. 1. Effect of the B subunit from different sources on DNA synthesis in rat glioma C6 cells. Cells treated with GM1 (0.2 μM) for 16 h were exposed to the various concentrations of the B subunit obtained from either source 1 (●) or source 2 (O) and assayed for [^{3}H]thymidine incorporation. The results are expressed as the percent inhibition ± SD compared to cells not exposed to the B subunit.

on DNA synthesis in C6 cells treated with GM1. Surprisingly, the B subunit from source 2 was a much more potent inhibitor of DNA synthesis (Fig. 1). At the low concentration of 1.7 nM, the B subunit from source 1 gave only 20% inhibition; that from source 2 gave 60% inhibition.[2]

Effect of the B Subunit From Different Sources on Binding of Iodinated CT

A possible explanation for the different potencies of the B subunit obtained from the two sources is that the preparations differ in their binding to GM1 on the cell surface. The dose-dependent binding of iodinated CT to GM1-treated cells correlated closely with the dose-dependent inhibition of DNA synthesis induced by the B subunit obtained from source 1 (Fig. 2). Furthermore, inhibition of the binding of iodinated CT to C6 cells by unlabeled B subunit from the two sources showed exactly the same concentration dependence (data not shown). The half-maximal inhibition values for both preparations of B subunit were 0.4 nM. This result indicates that the different effects on DNA synthesis by the two preparations of the B subunit were not due to differences in their binding capacities or affinities to GM1 on the cells.

Effect of the B Subunit From Different Sources on cAMP Levels

The proliferation of rat glioma C6 cells is inhibited by agents that increase intracellular cyclic nucleotide levels [29,30]. Since the A subunit of CT activates adenylate cyclase by ADP-ribosylation of the stimulatory G protein [22], it was therefore possible that the higher inhibitory activity of the B subunit from source 2 was due to some contamination of this preparation with the A subunit. Both preparations of

[2]Similar results were obtained using the B subunit obtained from Sigma Chemical Co. (St. Louis, Mo.), which is also a product of List Biological Laboratories.

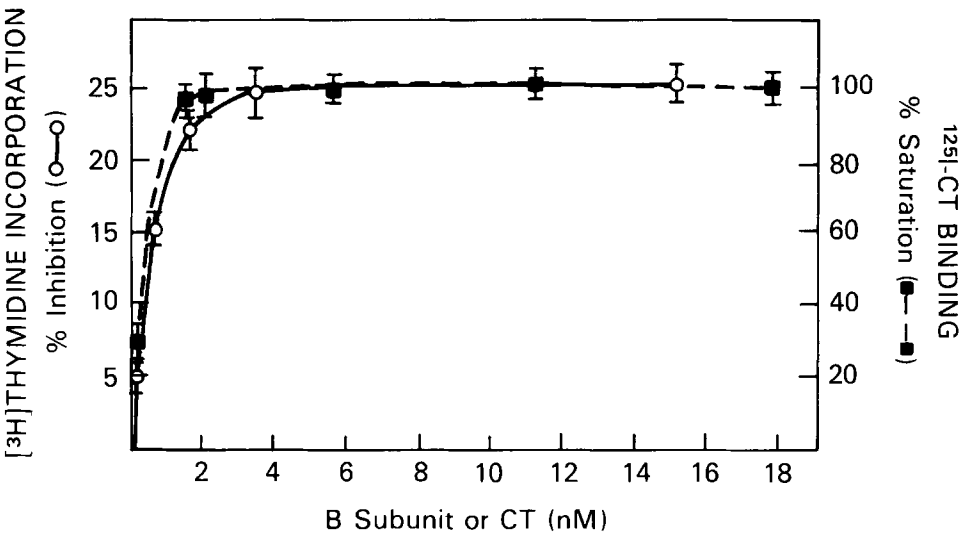

Fig. 2. Dose-dependent effect of the B subunit on DNA synthesis and the binding of iodinated CT in rat glioma C6 cells. Cells treated with GM1 (0.2 μM) for 16 h were exposed to various concentrations of B subunit obtained from source 1 and assayed for [^{3}H]thymidine incorporation (O). The results are expressed as the percent inhibition ± SD compared to cells not exposed to the B subunit. Specific iodinated CT binding (■) was measured in duplicate cultures treated with iodinated CT as described in Materials and Methods. The data are expressed as percent of maximum specific ^{125}I-CT binding.

TABLE II. Effect of the B Subunit From Different Sources and Cholera Toxin on cAMP Production*

Addition concentration (nM)	cAMP accumulation (pmol/mg)		
	B subunit (source 1)	B subunit (source 2)	CT
None	24 ± 4	25 ± 3	24 ± 2
0.1	25 ± 5	23 ± 1	375 ± 42
1.7	25 ± 4	29 ± 5	948 ± 87
17	27 ± 6	82 ± 12	1,107 ± 103

*Cultures of C6 cells were treated with GM1 (0.2 μM) for 16 h. The cells were then washed with DMEM and incubated at 37°C with the indicated concentrations of CT or the B subunit obtained from either source in DMEM/Hepes containing IBMX (0.5 mM). Cellular content of cAMP was assayed after 2 h by radioimmunoassay as described in Materials and Methods. The values are the means ± SD (n = 3).

the B subunit appeared to be holotoxin-free by silver staining of SDS-PAGE; there was no detectable Mr 22,000 band of the A1 subunit (data not shown). However, this method cannot detect a contaminant of ≤0.1%. To exclude the possibility of contamination by small amounts of the A subunit, the ability of the B subunits obtained from the different sources to cause accumulation of cAMP in C6 cells was tested. The B subunit obtained from source 1 did not induce any significant increases in cAMP levels in GM1-treated C6 cells. In contrast, the B subunit from source 2 seemed to be contaminated with the A subunit; the highest concentration led to slightly increased cAMP levels (Table II). In comparison, intact CT at the same concentration caused a 50-fold increase in cAMP production in the same experiment (Table II).

Effect of the B Subunit From Different Sources on Adenylate Cyclase Activity

cAMP assays in cells are not accurate enough to detect small but significant changes in intracellular levels of cAMP, whereas direct measurements of adenylate cyclase activity can greatly increase the sensitivity. This is probably due to residual phosphodiesterase activity as well as extrusion of cAMP into the medium, especially after prolonged incubation. Therefore, to be able to measure very small effects on the level of intracellular cAMP, the B subunit-mediated activation of adenylate cyclase was measured directly. The B subunit obtained from source 1, even at concentrations up to 17 nM, did not activate the cyclase in GM1-treated C6 cells (Fig. 3), whereas the B subunit from source 2 had a detectable effect on the activation of adenylate cyclase at a concentration as low as 1.7 nM and caused 2.2-fold activation of adenylate cyclase at a concentration of 17 nM (Fig. 3). This is the same concentration range of B subunit from source 2 that gave strong inhibition of DNA synthesis. In comparison, the whole CT, at concentrations as low as 1 and 10 pM, activated adenylate cyclase 1.8- and 3.3-fold, respectively (Fig. 4A). According to this assay, this preparation of the B subunit obtained from source 2 contains A subunit at a level of 0.05%. It should be noted that the cAMP assay is not sensitive enough to detect 1 pM CT, since the minimum concentration of CT that caused a significant increase in cAMP levels in these cells was 10 pM (data not shown). Measurement of adenylate cyclase after a 24 h incubation with CT, instead of 6 h, increased the sensitivity of the assay even more (compare Fig. 4A and B). Under these conditions, even a concentration of CT as low as 0.1 pM could be detected. It is clear from Figure 4B that the B subunit from source 1, even at a concentration 100,000 times higher than the minimal concentration of CT that can be detected, had no effect on adenylate cyclase (Fig. 4B). Thus this preparation of the B subunit seems to be free of any detectable adenylate cyclase-activating A subunit; this adenylate cyclase

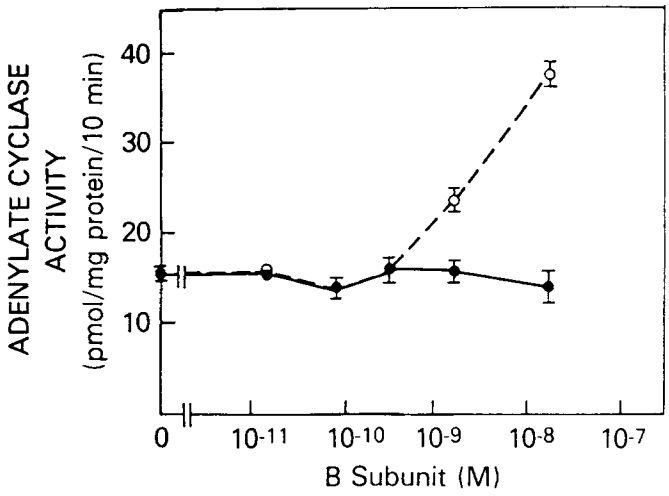

Fig. 3. Effect of the B subunit from different sources on adenylate cyclase activation. Cultures of C6 cells were treated with GM1 (0.2 μM) for 16 h, washed with DMEM, and incubated at 37°C in medium with the various concentrations of the B subunit obtained from either source 1 (●) or source 2 (○). Adenylate cyclase activity was measured after 6 h as described in Materials and Methods.

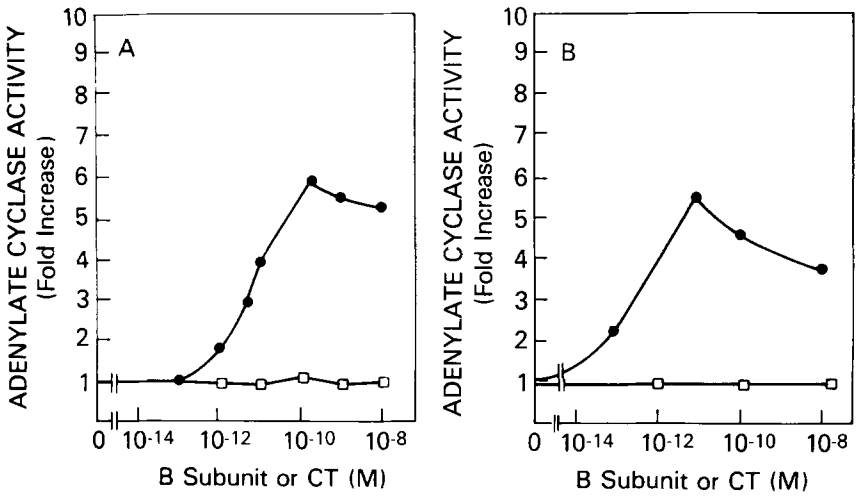

Fig. 4. Effect of CT and the B subunit on adenylate cyclase activation. Cultures of C6 cells were treated with GM1 (0.2 μM) for 16 h, washed with DMEM and incubated at 37°C in medium with the indicated concentrations of CT (●) or the B subunit obtained from source 1 (□). Adenylate cyclase activity was measured after 6 (**A**) or 24 h (**B**). The data are expressed as -fold increase relative to the untreated controls and the variability was less than 5%.

assay could have detected a contaminating amount of A subunit in the B subunit preparations of ≤0.006%.

DISCUSSION

Exogenously added gangliosides alter the growth and induce differentiation of a variety of cell types [reviewed in 2–6]. These observations led to the suggestion that the effects of exogenous gangliosides mimic the functions of their endogenous counterparts. However, the relationship between these observations and the function of endogenous gangliosides in the process of cellular differentiation and proliferation remains ill defined. To explore a potential function for endogenous gangliosides, the B subunit of CT was used as a probe to interact specifically with cell-surface ganglioside GM1 [8,9,11]. The B subunit stimulated DNA synthesis in resting thymocytes [8,13] and in quiescent, nontransformed mouse 3T3 fibroblasts [10,11] but inhibited the growth of *ras*-transformed 3T3 cells as well as 3T3 cells during a rapid growth phase [11]. Recently, the use of the B subunit of CT has been extended to the study of role of GM1 in many other systems [14–21,31,32].

In agreement with previous studies [15,16], it was found in this study that the growth-inhibitory effects of some preparation of the B subunit on GM1-treated C6 cells were clearly independent of any effects on changes in cAMP levels or adenylate cyclase activity. Furthermore, the extent of binding of the B subunit to the cells correlated exactly with the biological effects. However, it is clear from this study that in some preparations of the B subunit there is an additional inhibitory effect due to increases in cAMP levels. This is due to a very small contaminant of the A subunit, which could not be detected by conventional protein analysis. Even with the less sensitive cAMP assay, the B subunit from source 2 seems to be contaminated with the A subunit, since the

highest concentrations lead to increased cAMP levels (Table II). More convincingly, this was demonstrated by the sensitive method of measuring adenylate cyclase activity (Fig. 3). No activation of adenylate cyclase was observed previously in rat thymocytes with the B subunit obtained from this source [8,13]. This apparently conflicting result is most likely due to the presence of a less sensitive adenylate cyclase system in thymocytes compared to that in C6 cells. A concentration of holotoxin of 10^{-8} M is required to activate adenylate cyclase threefold in rat thymocytes [8], whereas only 10^{-11} M holotoxin is required for the same degree of activation in C6 cells (Fig. 4A).

Possible contamination of the B subunit with vanishingly small amounts of the A subunit was not of critical importance in studies of rat thymocytes, since increases in the level of cAMP and the B subunit have opposite effects on thymocyte proliferation [8]. However, in systems in which both increases in intracellular levels of cAMP and the B subunit cause changes in the same direction, precautions must be taken to ensure that any effect of the B subunit is not due to an increase in cAMP levels caused by a trace of the A subunit. At present, the most sensitive method of measuring contamination of the B subunit of CT with the A subunit is to determine adenylate cyclase activity after long exposure times, which increases the sensitivity of the assay (compare Fig. 4A and B).

The elucidation of the relationship between exogenous gangliosides and the function of endogenous gangliosides in cells is a crucial prerequisite for the understanding of their function. The use of the B subunit has provided a new paradigm for studying ganglioside GM1 function. However, caution must be taken to ensure that any effects are not due to contaminating A subunit and are due solely to the binding of the B subunit to the oligosaccharide moieties of ganglioside GM1 exposed on the cell surface.

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REFERENCES

1. Svennerholm L: In Florkin M, Stotz FH (eds): "Comprehensive Biochemistry." Oxford: Pergamon Press, 1970, Vol. 18, pp 201–215.
2. Hakomori S: Annu Rev Biochem 50:733, 1981.
3. Hakomori S: Cancer Res 45:2405, 1985.
4. Ledeen RW: J Neurosci Res 12:147, 1984.
5. Ledeen RW: Trends Neurosci 169, 1985.
6. Yates AJ: Neurochem Pathol 5:309, 1986.
7. Roisen FJ, Bartfeld H, Nagele R, Yorke G: Science 214:577, 1981.
8. Spiegel S, Fishman PH, Weber RJ: Science 230:1283, 1985.
9. Spiegel S: In Ledeen RW, Hogan EL, Tettamanti G, Yates AJ, Yu RK (eds): "New Trends in Ganglioside Research: Neurochemical and Neuroregenerative Aspects." Padova: Fidia Research Series, Liviana Press, 1988, Vol. 14, pp 405–421.
10. Fishman PH: J Membrane Biol 69:85, 1982.
11. Spiegel S, Fishman PH: Proc Natl Acad Sci USA 84:141, 1987.
12. Spiegel S, Panagiotopoulos C: Exp Cell Res 177:414, 1988.
13. Dixon SJ, Stewart D, Grinstein S, Spiegel S: J Cell Biol 105:1153, 1987.
14. Facci L, Skaper SD, Favaron M, Leon A: J Cell Biol 106:821, 1988.
15. Spiegel S: Biochim Biophys Acta 969:249, 1988.

16. Skaper SD, Facci L, Favaron M, Leon A: J Neurochem 51:688, 1988.
17. Tetsumoto T, Takada K, Amino N, Miyai K: Biochem Biophys Res Commun 157:605, 1988.
18. Campbell KS, Munson AE: J Pharmacol Exp Ther 242:895, 1987.
19. Doherty P, Walsh FS: J Neurochem 48:1237, 1987.
20. Doherty P, Mann DA, Walsh FS: J Neurochem 49:1676, 1987.
21. Ledeen RW, Cannella MS, Roisen FJ: In Rahmann H (ed): "Gangliosides and Modulation of Neuronal Function." Berlin: Springer-Verlag, 1986, pp 491–499.
22. Gilman AG: Cell 36:577, 1984.
23. Fishman PH: J Membrane Biol 54:61, 1980.
24. Cuatrecasas P: Biochemistry 12:3547, 1973.
25. Pacuszka T, Duffard RO, Nishimura RN, Brady RO, Fishman PH: J Biol Chem 253:5839, 1978.
26. Spiegel S: Biochemistry 24:5947, 1985.
27. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
28. Spiegel S, Kassis S, Wilchek M, Fishman PH: J Cell Biol 99:1575, 1984.
29. Daniels MP, Hamprecht B: J Cell Biol 63:691, 1974.
30. Sharma SK, Raj ABJ: J Neurosci Res 17:135, 1987.
31. Feuerstein N, Spiegel S, Mond, JJ: J Cell Biol 107:1629, 1988.
32. Spiegel S: J Biol Chem 264:6766, 1989.