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Cholera Toxin and Cell Growth: Role of Membrane Gangliosides

(adenylate cyclase/cAMP/glycosyltransferases/DNA synthesis)

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ABSTRACT **The binding of cholera toxin to three transformed mouse cell lines derived from the same parent strain, and the effects of the toxin on DNA synthesis and adenylate cyclase activity, vary in parallel with the ganglioside composition of the cells. TAL/N cells of early passage, which contain large quantities of gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a}, as well as the glycosyltransferases necessary for the synthesis of these gangliosides, bind the most cholera toxin and are the most sensitive to its action. TAL/N cells of later passage, which lack chemically detectable G_{M1} and G_{D1a} and which have no UDP-Gal:G_{M2} galactosyltransferase activity, are intermediate in binding and response to the toxin. SVS AL/N cells, which lack G_{M2} in addition to G_{M1} and G_{D1a} and which have little detectable UDP-GalNAc:G_{M3} *N*-acetylgalactosaminyltransferase activity, bind the least amount of toxin. The SVS AL/N cells are the least responsive to inhibition of DNA synthesis and stimulation of adenylate cyclase activity by cholera toxin. Gangliosides (especially G_{M1}), which appear to be the natural membrane receptors for cholera toxin, may normally have important roles in the regulation of cell growth and cAMP-mediated responses.**

Cholera toxin (choleragen), the principal exoenterotoxin of *Vibrio cholerae*, is an oligomeric protein (molecular weight, approx. 100,000) (2, 7) which acts at the plasma membrane to exert its biological effect (3–7). The diverse actions of the toxin (2, 8–13) can be attributed to its ability to stimulate specifically the membrane-localized enzyme, adenylate cyclase (EC 4.6.1.1), thereby raising intracellular levels of cAMP. It is presumably by this mechanism that choleragen acts as a potent inhibitor of mitogen-induced DNA synthesis in human fibroblasts (14). Also, stimulation of adenylate cyclase probably explains the toxin's ability to inhibit mitogenic transformation of lymphocytes (15), to stimulate steroidogenesis in adrenal cells (16, 17), and to induce differentiation in melanoma cells (18). In all cells so far studied, choleragen is nontoxic by several criteria (14–17) and the action of choleragen is observed to persist for at least two weeks in culture (18).

It has been observed that gangliosides can interact specifically with choleragen so as to block the binding of the toxin to cell membranes (3–7, 19–23). In particular, G_{M1} is a highly potent inhibitor of choleragen binding; G_{D1a} and G_{M2} are at least 50-fold, and G_{M3} at least 250-fold less effective than G_{M1} (4, 20). In fat cells it has been possible to enhance the binding of toxin as well as the biological response to the toxin by incorporating exogenous ganglioside G_{M1} into the cell membrane, strongly suggesting that ganglioside G_{M1} is the natural membrane receptor for choleragen (4).

Considerable evidence indicates that tumor cell membranes differ from those of normal cells. In addition to differences in the agglutinability of cells by plant lectins (24–26), transformed cells exhibit incomplete synthesis of carbohydrate chains in membrane glycoproteins (27–29) and glycolipids (30–33), and increased synthesis of a sialofucopeptide (34, 35).

The membrane complement of gangliosides in a particular cell can be altered dramatically in association with virus- or carcinogen-induced transformation. Mouse cells transformed by simian virus 40 (SV40), polyoma, or Moloney sarcoma viruses possess markedly reduced amounts of the gangliosides G_{M2}, G_{M1}, and G_{D1a} as well as UDP-GalNAc:G_{M3} *N*-acetylgalactosaminyltransferase activity, the enzyme involved in the synthesis of these glycolipids (36, 39). Similar changes have been observed in hamster cells after transformation by SV40, polyoma, and chemical carcinogens (37). Mouse cells transformed by Kirsten sarcoma virus (38) or chemicals and x-irradiation (unpublished) lose gangliosides G_{M1} and G_{D1a} concomitant with reduced UDP-Gal:G_{M2} galactosyltransferase activity. In addition, extensive culturing of spontaneously transformed cell lines can lead to alterations in glycolipid composition and metabolism (40, 41). Cells with differences in their membrane gangliosides exhibit different growth characteristics *in vitro*, and different abilities to generate tumors *in vivo*. There has been considerable speculation as to the possible role of complex membrane glycolipids in the processes of transformation and neoplasia. Since choleragen, which interacts specifically with ganglioside G_{M1} (3–7, 19–23), can affect the *in vitro* growth characteristics of cells in culture (16–18) and can inhibit DNA synthesis in normal cells (14, 15), it is of interest to study the effects of choleragen on transformed cells which may have diminished quantities of ganglioside G_{M1}.

The present report examines the interaction of choleragen with three transformed cell lines derived from an epithelial line of inbred AL/N mouse embryo kidney (39, 42–44) which differ in ganglioside contents: spontaneously transformed TAL/N, subcultured less than sixty times ($P < 60$); TAL/N, subcultured more than 200 times ($P > 200$) and SVS AL/N, an SV40 transformed line. Measurements of the specific binding of choleragen are described in parallel with measurements of (*a*) the inhibitory action of choleragen on DNA synthesis and (*b*) the stimulatory action of choleragen on membrane adenylate cyclase activity. The present studies elucidate further the function of gangliosides in the action of cholera

Abbreviations: The nomenclature of Svennerholm (ref. 1) is used to designate the gangliosides: G_{M3}, *N*-acetylneuraminylgalactosylglucosylceramide; G_{M2}, *N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{M1}, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; G_{D1a}, *N*-acetylneuraminylgalactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosylceramide; MEM, minimal essential medium; TBS, 0.1 M Tris·HCl buffered saline (pH 7.4); SV40, simian virus 40.

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toxin and suggest that gangliosides may play an important role in the regulatory mechanism for cell growth.

MATERIALS AND METHODS

Cells. Cells used in earlier studies (39, 42–44) were propagated in Eagle's minimal essential medium (MEM) supplemented with penicillin and streptomycin (50 μg of each per ml), 10% fetal calf serum, and gassed with 5% CO_2 in air. Pleuropneumonia-like organisms were not detected prior to initiating these studies. All experiments were done on confluent monolayers at high density.

Assay of Glycosyltransferase Activities and Quantitation of Gangliosides. Cells were harvested by scraping in 0.1 M Tris·HCl-buffered saline, pH 7.4 (TBS), and glycosyltransferase activities were determined in crude homogenates essentially as described previously (38, 44) with conditions optimized (Table 2). The source of the ganglioside acceptors, G_{M3} and G_{M2}, has been described (44). UDP-[^{14}C]GalNAc (14 μCi/μmole) and UDP-[^{14}C]Gal (254 μCi/μmole) were obtained from ICN and NEN, respectively. Nonradioactive UDP-Gal was from Sigma.

Gangliosides were extracted from the various cell lines as described previously (42, 43), were separated by thin-layer chromatography on silica gel G coated glass plates, developed with chloroform–methanol–0.25% aqueous $CaCl_2$ (60:35:8, v/v/v) and quantitated by densitometry after detection with resorcinol reagent. The identity of gangliosides so isolated from TAL/N and SVS AL/N cells has been previously determined (39, 42, 43). Radioactive gangliosides isolated from cells grown for 4 days in media containing 1.5 μCi/ml of [^{14}C]galactose (22 Ci/mmole, from ICN), were detected by radiometric scanning of thin-layer plates, and quantitated by liquid scintillation counting.

Effect of Cholera Toxin on DNA Synthesis. Confluent 5-cm diameter monolayers were washed once with TBS, and 2 ml of TBS containing increasing amounts of cholera toxin was added. After 30 min, the solution was replaced with 5 ml of standard medium, and at 24 hr, 5 μCi of [*methyl*-^{3}H]thymidine (NEN, 14 Ci/mmole) was added to each plate for 30 min. Cells were harvested by scraping in 2 ml of 10% trichloroacetic acid, washed by centrifugation three times in 5% trichloroacetic acid, and dissolved in 1 M NaOH for radioactivity and protein analysis. No visible change in viability (phase contrast microscopy) is observed up to 2 days after treatment with choleragen and cells can be replated with good efficiency after treatment with choleragen.

Binding of ^{125}I-Labeled Choleragen. Binding studies on the various AL/N cells were performed with ^{125}I-labeled choleragen as described for fat cells and membranes (3–7), fibroblasts (14), and erythrocytes (13). Cells at high density in 75-cm^2 T-flasks were harvested intact by gentle scraping in Hank's balanced salt solution, pH 7.4, containing 0.1% (w/v) bovine albumin. Cholera toxin (lot nos. 0172 and 1071), purified by the method of Finkelstein and LoSpalluto (45) was from Dr. C. E. Miller, SEATO Cholera Research Program, and from Dr. R. A. Finkelstein.

Adenylate Cyclase Activity. Adenylate cyclase activity was measured in confluent cell monolayers by a modification (13, 46) of the method of Pohl *et al.* (47) using neutral alumina columns (1 × 4 cm) to isolate cAMP (48, 49). After 4.5-hr exposure to choleragen in serum-free MEM, at 37°, cells were harvested by scraping in hypotonic buffer ($MgCl_2$, 0.2 mM; $CaCl_2$, 0.2 mM; *Staphylococcus aureus* nuclease, 1 μg/ml; Tris·HCl, 5 mM, pH 8). Membrane aliquots (0.1–0.2 mg of protein) obtained after homogenization (Brinkmann Polytron) and centrifugation (40,000 × *g* for 30 min at 0°) were resuspended at 0° in 50 mM Tris·HCl, pH 8, and were assayed for adenylate cyclase activity during a 12-min reaction at 33° in a mixture (volume 0.1 ml) containing: [α-^{32}P]ATP [1.2 mM, 60 cpm/picomole, (13, 46)], $MgCl_2$ (5 mM), aminophylline (5 mM), phospho*enol*pyruvate (5 mM), and pyruvate kinase (60 μg/ml). Enzyme activity in duplicate samples, corrected for chromatographic recovery, for the activity of pre-boiled membranes, and for the production of cAMP in the absence of toxin is expressed (Fig. 3) as picomoles of cAMP formed per min per mg of membrane protein.

TABLE 1. *Ganglioside composition of transformed AL/N mouse cell lines, nmoles of sialic acid/mg of protein**

Cell line	Ganglioside G_{M3}	G_{M2}	G_{M1}	G_{D1a}
TAL/N ($P < 60$)	0.85 ± 0.12	0.45 ± 0.05	0.09 ± 0.05	0.80 ± 0.22
TAL/N ($P > 200$)	0.76 ± 0.13	1.95 ± 0.26	N.D.	N.D.
SVS AL/N	1.05 ± 0.10	N.D.	N.D.	N.D.

N.D. indicates not detectable; lower limit of detection was 0.2 nmoles of lipid-bound sialic acid or 0.04 nmoles/mg of protein for analysis of 5 mg.

* Values are the mean ± SEM of triplicate determinations performed on cells harvested 20 doublings apart.

RESULTS

Ganglioside Content and Glycosyltransferase Activities of Transformed Cells. The ganglioside contents of the three cell lines differ substantially (Table 1). In agreement with previous studies (39, 42–44), TAL/N ($P < 60$) has a full complement of gangliosides. However, SVS AL/N cells have only G_{M3} and TAL/N ($P > 200$) cells contain only G_{M3} and G_{M2}. The absolute contents of gangliosides of these cell lines has been observed to change somewhat during serial passage, as indicated by differences in previously reported values (42, 43); the present data, obtained on two separate occasions approximately 20 doublings apart, are close to recently reported values (43). When cells were grown in a medium containing [^{14}C]galactose, only labeled G_{M3} (20,000 cpm/nmole) was detected in SVS AL/N. TAL/N ($P > 200$) cells contained labeled G_{M3} (17,000 cpm/nmole) and G_{M2} (23,000 cpm/nmole); there were 380 cpm/mg of cell protein migrating in the position of G_{D1a}, which radioactivity could correspond to 19 picomoles of G_{D1a} per mg of cell protein.

The glycosyltransferase activities (Table 2) are consistent with the apparent absence of certain gangliosides in SVS AL/N and TAL/N ($P > 200$) cells. SVS AL/N cells have low activities of the two transferases necessary for the synthesis of G_{M2} and G_{M1} (44); TAL/N ($P > 200$) cells, which do not synthesize chemically detectable G_{M1}, have less than 2% of the galactosyltransferase activity found in early passage TAL/N cells, which are capable of synthesizing both G_{M2} and G_{M1}.

Binding of [^{125}I]-Labeled Cholera Toxin. All three AL/N cell lines bind different but appreciable amounts of ^{125}I-labeled choleragen (Fig. 1). At cell concentrations of about 2 × 10^6 per ml, the plateau of the binding isotherm is not reached even at relatively high radioligand concentrations (200–300 ng/ml) (Fig. 1B). At lower ^{125}I-labeled choleragen concentrations (Fig. 1A), where the biological effects are manifest, the differ-

TABLE 2. *Glycosyltransferase activities in transformed AL/N cell lines*

Cell line	Glycosyltransferase activity: Endogenous*	+ Acceptor*	Difference*	Activity†
	G_{M3} + UDP-GalNAc → G_{M2} + UDP			
TAL/N, $P < 60$	507	3848	3341	877
TAL/N, $P > 200$	194	4938	4744	1245
SVS AL/N	629	880	251	66
	G_{M2} + UDP-Gal → G_{M1} + UDP			
TAL/N, $P < 60$	725	3215	2490	610
TAL/N, $P > 200$	602	644	42	10
SVS AL/N	583	683	100	25

For *N*-acetylgalactosaminyltransferase assays performed as previously described (44), reaction mixtures (50 μl) contained 75 μg of cell protein, 50 nmoles of G_{M3} (when added), 10 nmoles of UDP-[^{14}C]GalNAc (14 μCi/μmole), 2.5 μmole of Na cacodylate (pH 7.2), 1 μmole of $MnCl_2$, and 200 μg of NP-40 (Shell Oil Co.) and were incubated 2 hr at 37°. For galactosyltransferase assays, reaction mixtures (50 μl) contained 250 μg of cell protein, 10 nmoles of G_{M2} (when added), 50 nmoles of UDP-[^{14}C]Gal (3 μCi/μmole, 2.5 μmoles of Na cacodylate (pH 5.8), 1 μmole of $MnCl_2$, 100 μg of Triton CF-54, and 50 μg of Tween 80 and were incubated 3 hr at 37°.

* cpm, average of three separate experiments, corrected for zero time controls.

† pmoles/mg of protein per hr.

ences in binding between the three cell lines are especially apparent. TAL/N ($P < 60$) cells, which contain detectable amounts of all four gangliosides, bind the most choleragen. TAL/N cells ($P > 200$), which possess only (as detected chemically) G_{M3} and G_{M2}, bind somewhat lower amounts of the toxin. Although G_{M2} has a 50- to 1000-fold lower affinity for choleragen (4, 20), the relative increase in G_{M2} content of TAL/N ($P > 200$) may possibly compensate for the lack of G_{M1} and may account for the binding of nearly as much choleragen as do the parent TAL/N ($P < 60$) cells. In contrast, a much lower amount of ^{125}I-labeled choleragen is bound to SVS AL/N cells, which contain no detectable (by radiochemical methods) higher gangliosides.

These results suggest that the binding of choleragen, with its known specificity for G_{M1}, may be a much more sensitive indicator than direct chemical estimates of the presence of G_{M1} in the plasma membrane. Thus, to test for the presence of G_{D1a}, which itself competes poorly if at all with G_{M1} for choleragen binding, cells were treated for 1 hr at 37° with *Clostridium perfringens* neuraminidase [1.1 unit/mg (Worthington), 25 μg/ml in complete growth medium], which can remove terminal sialic acid residues from G_{D1a} to generate G_{M1} (50, 51). Following neuraminidase digestion, the binding capacity for choleragen (10 ng/ml) of SVS AL/N cells (2×10^5 per ml) is increased from 34 pg per 10^5 cells to 150 pg per 10^5 cells; the binding of TAL/N ($P < 60$) cells is increased from 150 pg to 573 pg per 10^5 cells. These results suggest the presence of G_{D1a} in SVS AL/N cells, despite the inability to detect this glycolipid by more conventional chemical methods. The binding of choleragen appears to be restricted mainly to the plasma membrane, since the binding of choleragen by SVS AL/N cells disrupted by sonication after neuraminidase digestion is the same as the binding by intact cells (above).

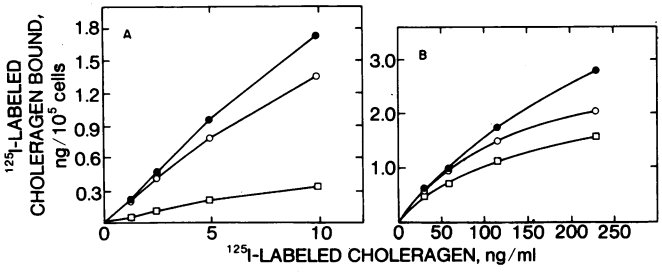

FIG. 1. Binding of cholera toxin to transformed AL/N cells. Choleragen binding was measured as outlined in Methods and described previously (3–7). ●, TAL/N, $P = 46$; ○, TAL/N, $P = 269$; □, SVS AL/N. (A) Binding to 8×10^4 cells in 0.4 ml of buffer. (B) Binding to 3.2×10^5 cells in 0.2 ml of buffer.

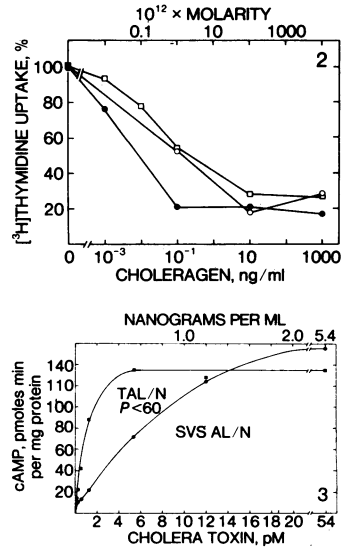

FIG. 2. Inhibition of thymidine incorporation by cholera toxin. Results are expressed as the percent of the incorporation of radioactivity measured in the absence of choleragen (50,000–100,000 cpm/mg of protein for all three cell lines). ●, TAL/N ($P < 60$); ○, TAL/N ($P > 200$); □, SVS AL/N.

FIG. 3. Cholera toxin-stimulated adenylate cyclase activity of transformed AL/N cells. SVS AL/N (●); and TAL/N ($P < 60$) (■).

Effect of Cholera Toxin on DNA Synthesis. In agreement with previous experiments with human fibroblasts (14), choleragen is a potent inhibitor of DNA synthesis in transformed mouse AL/N cells. Inhibition of DNA synthesis requires only a brief exposure of cells to choleragen, in harmony with the extraordinary rapidity and high affinity of toxin binding (3–6). Maximal inhibition of DNA synthesis (80%) in TAL/N ($P < 60$) cells occurs by exposure to choleragen (100 ng/ml in TBS) for 10 min and then replacement of the buffer with fresh growth medium; treatment with choleragen for 60 min does not lead to a greater inhibition. The effect of choleragen (10 ng/ml) on TAL/N ($P < 60$) can be abolished by the simultaneous addition of ganglioside G_{M1} (1 nmole/ml) followed by washing the cells at 30 min. The inhibition of DNA synthesis is specific, as incorporation of [^{3}H]leucine is unaffected.

The DNA synthetic response at 24 hr is diminished by choleragen in all three cell lines (Fig. 2). TAL/N ($P < 60$) cells are most sensitive to choleragen action; maximal suppression of thymidine incorporation occurs with 0.1 nM choleragen. The inhibition of thymidine incorporation appears to be somewhat greater for TAL/N ($P > 200$) than for

SVS AL/N at 0.1 and 10 ng/ml of choleragen. Of the three cell lines, the TAL/N ($P > 200$) cells would thus appear to be intermediate in their sensitivity to choleragen. The marked decrease (one to two orders of magnitude) in the sensitivity to choleragen of TAL/N ($P > 200$) and SVS AL/N cells compared with TAL/N ($P < 60$) cells correlates well with the relative lack of G_{M1} in the former two cell lines.

Effects of Cholera Toxin on Adenylate Cyclase. It is very likely that all of the biological effects of choleragen are mediated by its action on adenylate cyclase (2–13). Direct measurements of membrane adenylate cyclase were performed 4.5 hr after exposure of cell monolayers to increasing amounts of choleragen (Fig. 3). The full effect of the toxin on adenylate cyclase activity is observed at 3 hr, and increases only slightly (3% per hr) thereafter for as long as 7 hr after the exposure of cells to choleragen. The adenylate cyclase activity of SVS AL/N cells is stimulated half-maximally by approximately 6 pM choleragen, at which concentration the adenylate cyclase activity of TAL/N ($P < 60$) cells is already maximally stimulated (Fig. 3). The half-maximal concentrations for stimulation of TAL/N ($P < 60$) cells is about 0.8 pM choleragen. It is pertinent that, in analogy with the effects on DNA synthesis (Fig. 2), the maximal stimulation achieved by high concentrations of choleragen does not differ appreciably for the two cell lines.

DISCUSSION

It is clear from the present study that cells with more complex gangliosides are more sensitive to the action of choleragen than cells that have little or no capacity for the synthesis of G_{M2}, G_{M1}, and higher gangliosides. The most striking contrast is observed between TAL/N ($P < 60$) cells, which possess readily detectable ganglioside G_{M1}, and SVS AL/N cells, which possess mainly G_{M3}. SVS AL/N cells have fewer membrane receptors for choleragen, which deficiency may account for the lower sensitivity of the cells toward the action of choleragen. In comparison, TAL/N ($P < 60$) cells can bind up to five times more choleragen; there is about a 10-fold greater sensitivity towards choleragen stimulation of adenylate cyclase and an even greater relative sensitivity of TAL/N ($P < 60$) cells toward the inhibitory action of choleragen on DNA synthesis.

It is tempting to attribute the different sensitivities toward choleragen action to one specific ganglioside. G_{M1} is the most likely candidate, since its affinity for choleragen is at least 50- (4) to 1000- (20) times greater than that of other gangliosides and since it is not detectable (chemically) in those cells (TAL/N ($P > 200$) and SVS AL–N) that bind less toxin and that are least sensitive to choleragen action. Furthermore, the activity of the galactosyltransferase responsible for the biosynthesis of G_{M1} from G_{M2} is virtually undetectable in the TAL/N ($P > 200$) and SVS AL/N cells (Table 2).

It is pertinent that, despite our inability to detect ganglioside G_{M1} chemically, and despite the apparent absence of the enzyme responsible for the biosynthesis of this ganglioside, SVS AL/N cells can nevertheless bind significant quantities of choleragen and are still capable of responding to the toxin. These apparent discrepancies are most readily explained by the differences in the sensitivities of the methods used to detect the gangliosides. By the chemical method it is possible to detect about 10^6 molecules of ganglioside G_{M1} per cell, whereas with ^{125}I-labeled cholera toxin (about 1 Ci/μmole) fewer than 10^3 molecules per cell can be detected easily. The residual binding of the toxin to the "ganglioside-deficient" cells probably reflects the presence of small amounts of G_{M1}, which could arise from the persistence of a very small quantity of the appropriate galactosyltransferase, despite its apparent absence (Table 2). Since the "loss" of this enzyme is selective and not accompanied by a fall in the sialyl-transferase enzyme (40, 41, 44), a small degree of activity could lead to the biosynthesis of ganglioside G_{D1a}, probably in quantities exceeding those of G_{M1}. The substantial enhancement in toxin binding following neuraminidase digestion, which should convert G_{D1a} to G_{M1} (50, 51), supports this view. Alternatively, it is possible that small quantities of G_{M1} and G_{D1a} present in these cells could be derived from the serum in the culture medium [plasma contains substantial quantities of gangliosides (52)]. Since exogenous gangliosides can incorporate spontaneously into cells so as to bind toxin and to lead to biological responses (3–6), it will be important to examine transformed cells cultured either in the absence of serum or with ganglioside-free serum. The possibility must also be considered that, despite evidence to the contrary (3–7, 19–23), a ganglioside besides G_{M1} may also serve as a biological receptor for choleragen under certain circumstances.

As in other cell types (13, 14, 18), the biological effects of the toxin are maximal when only a small proportion of the total binding sites for the toxin are occupied. Maximal activation of adenylate cyclase activity in TAL/N ($P < 60$) cells occurs when about 3% of the toxin binding sites are occupied; the corresponding figure for the SVS AL/N cells is about 10% (fewer than 8000 molecules bound per cell). Further, since as described in other systems (14, 18), maximal activation of adenylate cyclase is not required to inhibit maximally DNA synthesis, this biological response exhibits an even greater sensitivity to choleragen than does adenylate cyclase activity. Maximal effects (on adenylate cyclase activation) probably depend only on binding a number of toxin molecules that approaches the number of adenylate cyclase molecules§. Under these conditions it is not the maximal response that will be affected by decreasing the total number of binding sites, but instead the sensitivity or the "apparent" affinity of the response to the toxin. Thus, the differences in the dose–response relationship (Figs. 2 and 3) are not likely to reflect differences in true affinities (3–6, 13) but differences in the number of binding sites.

The differences in the ability of choleragen to stimulate adenylate cyclase can be correlated with observed differences in the growth characteristics of TAL/N ($P < 60$) and SVS AL/N cells. Increasing numbers of studies suggest an important role for cAMP in the regulation of growth in mammalian cells (53–55), in general with lower levels of cAMP associated with rapid cell growth or a lower degree of "contact inhibition." Fewer data are available concerning the specific enzymes (adenylate cyclase, phosphodiesterase) responsible for the altered steady-state levels of cAMP (56). SVS AL/N cells have three times, and TAL/N ($P < 60$) cells about two times, the saturation density of the parent NAL/N cells (42, 43). Since the adenylate cyclase of SVS AL/N cells is relatively [compared with TAL/N, ($P < 60$)] insensitive to the stimulus of choleragen, it is tempting to propose that the activation of the cyclase might also be relatively insensitive to the as yet unknown "physiologic" stimuli. A lower

§ It has been proposed (13, 58) from kinetic studies that the toxin–receptor complex, which is initially not associated with adenylate cyclase, subsequently associates stoichiometrically with adenylate cyclase to form a very stable complex.

steady state concentration of cAMP might therefore obtain so as to permit a saturation density higher than that of TAL/N ($P < 60$) cells. It was noted in the present study that addition of choleragen either to SVS AL/N or to TAL/N ($P < 60$) cells yields cultures that appear more "contact inhibited."

The present study thus not only emphasizes the possible importance of membrane gangliosides themselves, particularly G_{M1}, but also suggests that gangliosides may play an important role in the responsiveness of adenylate cyclase to normal stimuli and in the control of cell growth. It is possible, for example, that specific gangliosides may assist directly in the binding of factors in the serum or medium, or they may assist indirectly in the processes by which these factors modulate regulatory enzymes such as adenylate cyclase in the cell membrane. Alternatively, cell surface gangliosides may themselves serve as highly specific sites (or cell-bound "hormones") which are recognized by specific "receptors" (analogous to choleragen) on the surface of the same or separate cells, the association of which may lead to stimulation of adenylate cyclase and thus to the accompanying alterations in cell growth. Although in either scheme the gangliosides would play a key role in the initial cell surface event, the complete biological response would depend on the integrity of all of the subsequent steps. It could therefore be anticipated that cell transformation could result not only from alterations in membrane gangliosides but also from totally different changes such as deficiencies or dysfunctions of adenylate cyclase or cAMP-dependent protein kinases. Transformed cell lines with no apparent defects in glycolipid composition (39), or revertants with persistent abnormalities in gangliosides (37), offer opportunities for testing these hypotheses. The present studies suggest ways of examining the possible involvement of membrane glycolipids in the regulation of cell growth.

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