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Cholera Toxin and Membrane Gangliosides: Binding and Adenylate Cyclase Activation in Normal and Transformed Cells

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Summary. A virally transformed, ganglioside GM_1-deficient cell line binds 2% of the cholera toxin (choleragen) bound by the parent line and is less responsive to choleragen with respect to adenylate cyclase stimulation. This biological response is maximal when 10% of choleragen-binding sites in the transformed line, or 0.5% in the parent line, are occupied. In contrast, in isolated fat cells saturation of binding and adenylate cyclase stimulation are seen at very similar concentrations.

Incubation of ganglioside GM_1 with intact cells increases choleragen binding (defined here as ganglioside incorporation) in the transformed cell line but does not enhance the biological response to choleragen. Stimulation of adenylate cyclase is enhanced in isolated fat cells, however, by exogenous ganglioside GM_1. The binding and cyclase response in fat cells can be reduced by the addition of the inactive analog and competitive antagonist, choleragenoid, and there is recovery of the enzyme response and binding upon subsequent addition of exogenous GM_1. Failure of enhancement in the transformed cell line is explained by the presence of a five- to tenfold excess of binding sites over the number required for the full biological effect of choleragen. Cells with a large excess of toxin receptors are relatively refractory to the blocking effects of choleragenoid on biological responses. Notably, untransformed cells, which contain large quantities of toxin receptor, cannot incorporate exogenously added ganglioside GM_1. These findings suggest the possible existence in the cytoplasmic membrane of specific molecular structures, present in finite and limited number, for recognizing and accepting ganglioside molecules exposed to the external medium.

Viral transformation of cultured cells usually leads to a marked loss of complex glycolipids including ganglioside GM_1[1] (Brady & Fishman, 1974; Hakomori, 1975), the natural receptor for cholera toxin (choleragen)

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1 *Abbreviations:* KRB, Krebs-Ringer's bicarbonate; GM_1, galactosyl-N-acetylgalactosaminyl-N-acetylneuraminyl-galactosyl-glucosyl ceramide; GD_{1a}, N-acetylneuraminyl-galactosyl-N-acetylgalactosaminyl-N-acetylneuraminyl-galactosylglucosylceramide; cyclic AMP, cyclic adenosine 3′:5′-monophosphate; MEM, minimal essential medium.

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(Cuatrecasas, 1973*b*,*c*; Holmgren, Lonnroth & Svennerholm, 1973; King & van Heyningen, 1973). In addition to demonstrating quantitative alterations in cell-surface characteristics, choleragen is a useful probe of the biologic function of ganglioside GM_1. This glycolipid binds to choleragen and facilitates transmission of the biologic action of the choleragen molecule to the cell membrane, resulting in irreversible stimulation of adenylate cyclase, probably as a result of direct association of 36,000 mol wt A subunit of choleragen with adenylate cyclase (Bennett, O'Keefe & Cuatrecasas, 1975; Sahyoun & Cuatrecasas, 1975). The marked, sustained elevation of intracellular concentrations of cyclic AMP characteristic of choleragen stimulation results in alterations in cellular function (Bennett & Cuatrecasas, 1975*a*) and proliferation (Froehlich & Rachmeler, 1972), and in some cases differentiation (O'Keefe & Cuatrecasas, 1974), of cultured cells. The present studies reconfirm the usefulness of choleragen in demonstrating alterations in ganglioside GM_1 on the cell surface and analyze the relationship of binding to biological effect (adenylate cyclase stimulation) of choleragen in a virally transformed, ganglioside GM_1-deficient (Brady & Fishman, 1974) line and its untransformed parent.

In the present studies the binding and adenylate cyclase activation are compared in fat cells, a ganglioside GM_1-deficient Kirsten murine sarcoma virus transformed line, and the parent line, which contains chemically measurable quantities of ganglioside GM_1 (Brady & Fishman, 1974). Saturation of choleragen binding and of adenylate cyclase activation in fat cells occurs at very similar choleragen concentrations. The transformed line binds 10-fold more choleragen, and the parent line more than 100-fold more choleragen, than is required for full adenylate cyclase activation. Incubation of cells with exogenous ganglioside GM_1 enhances the adenylate cyclase response to choleragen in fat cells, but not in either cell line. Analysis of these data and of results of competition studies with the inactive choleragen derivative, choleragenoid, provides an explanation why choleragenoid fails to inhibit the choleragen response in the two cell lines, but not in fat cells. These studies confirm the proposition (Bennett *et al.*, 1975) that the large number of choleragen binding sites, far in excess of those required for maximal biological stimulation by choleragen, are equivalent in conveying the biological function of choleragen within the membrane. Such quantitative studies allow prediction of the likelihood of alteration by ganglioside GM_1 of choleragen sensitivity as well as of blocking by choleragenoid of choleragen effects. The relevance of the experimental finding of "spare" recep-

tors in this model system to studies in which the quantity of membrane binding sites may be experimentally manipulated is discussed.

Materials and Methods

Eagle's MEM, glutamine, trypsin (1:250), and fetal calf serum were from Microbiological Associates. Collagenase, neuraminidase, and staphylococcal nuclease were from Worthington Biochemical Corporation, and bovine serum albumin (fraction *V*) was from Armour Pharmaceuticals. Ganglioside GM_1 was from Supelco. 2′,3′-isopropylidine adenosine was from Aldrich. Carrier-free [^{32}P]orthophosphoric acid and [^{3}H]cyclic AMP (22 Ci/mmole) were purchased from New England Nuclear, and carrier-free [^{125}I]NaI was obtained from Union Carbide. 5′ ATP, 5′ GTP, and phosphoenol pyruvate were from Sigma, and pyruvate kinase was from Boehringer.

A *Balb/C* 3*T*3 mouse fibroblast line and a derivative line transformed with Kirsten strain murine sarcoma virus, *KBalb/C* 3*T*3, were a gift of Dr. S. Aaronson and were free of mycoplasma by bacteriologic criteria at the initiation of these studies. Cells were grown in 75 cm^2 Falcon flasks in Eagle's Minimal Essential Medium (MEM) with Earle's balanced salts and 2 mM glutamine with 10% (*Balb/C* 3*T*3) or 1% (*KBalb/C* 3*T*3) fetal calf serum at 37° in 5% CO_2 without antibiotics. Cells were split with 0.05% trypsin-versene or suspended for experiments with versene alone.

Fat cells were prepared from 100 g male Sprague-Dawley rats by the method of Rodbell (Rodbell, 1964), washed 3 times, and suspended in Krebs-Ringer's bicarbonate – 1% albumin gassed with 10% CO_2–90% air.

Epidermal growth factor was purified from male mouse submaxillary glands by the method of Savage and Cohen (Savage & Cohen, 1972) to electrophoretic homogeneity on SDS gels.

Choleragen and choleragenoid purified by the method of Finkelstein and LoSpalluto (Finkelstein & LoSpalluto, 1970) were obtained from Dr. C.E. Miller, SEATO Cholera Research Program. For competitive binding experiments with cultured cells, choleragenoid was repurified as described below.

Choleragen was labelled with ^{125}I by the chloramine-T method of Hunter and Greenwood (Hunter & Greenwood, 1962) as described previously (Cuatrecasas, 1973*c*) with specific activity of 9–21 Ci/g. Binding assays were performed (Cuatrecasas, 1973*c*) by incubating samples of cells ($0.4–2.0 \times 10^5$) in 0.2 ml Hanks' balanced salt solution with 0.1–0.5% albumin for 20 min at 24° in plastic 12×75 mm tubes followed by dilution with 3 ml ice-cold buffer containing 0.1% albumin and immediate filtering on a multiple manifold filtration apparatus under reduced pressure with EAWP "Millipore" cellulose acetate filters. Control tubes contained in addition 10–50 μg unlabelled choleragen added 10 min before labelled material and showed no increase over binding seen in the absence of cells; i.e., excess unlabelled choleragen displaces virtually all label from cells, and radioactivity bound to filters accounted for all nonspecific binding. Consequently, experimental radioactivity bound minus that bound to filters was defined as specific binding in some experiments.

[α^{32}P]ATP was synthesized from ^{32}P orthophosphoric acid by the method of Symons (Symons, 1968) and purified on DEAE cellulose by elution with a water – 4 M formic acid, 0.4 M triethylamine formate gradient at 4°. Adenylate cyclase assays were performed as previously described (Bennett *et al.*, 1975; Bennett & Cuatrecasas, 1975*a*). Briefly, cells were suspended with versene, resuspended in 5.0 mM Tris-HCl with 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$, pH 8.0, and broken (Brinkman Polytron, setting #3, 45 sec) in the presence of 0.5 μg/ml purified staphylococcal nuclease (to prevent clumping by nucleic acids) at 4°. After centrifugation at $40,000 \times g$ for 20 min at 4°, the pellet was resuspended and

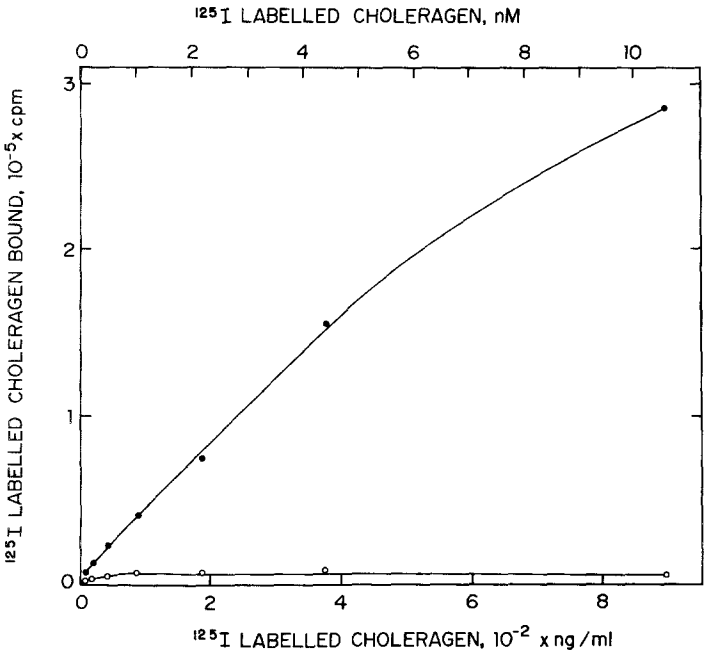

Fig. 1. Comparison of binding of ^{125}I-labelled choleragen to *Balb/C* 3T3 and *KBalb/C* 3T3 cells. Monolayers were suspended with versene, washed twice and resuspended in Hanks'-0.1% albumin buffer at 10^6 cells per ml, and assayed as in *Materials and Methods*. *Balb/C* 3T3-●-, *KBalb/C* 3T3 -○-

50–100 μg protein were incubated for 12 min at 30° with 2×10^6 cpm [α^{32}-P]-ATP in an incubation mixture containing 0.5 mM Mg^{++}-ATP, 0.1 mM Mg^{++}-GTP, 10 mM aminophylline, 5 mM $MgCl_2$, 5 mM phosphoenolpyruvate, and 50 μg pyruvate kinase in 50 mM Tris-HCl, pH 8.0, in a final volume of 0.1 ml. The reaction was terminated by boiling for 1 min and [^{32}P]cyclic AMP eluted from 1 g neutral alumina with 2 ml 25 mM Tris-HCl, pH 8.0, using 2×10^4 cpm [^{3}H]cyclic AMP to determine recovery (approximately 85%). Samples were diluted in Bray's solution and counted in a liquid scintillation counter. Blanks using boiled membranes gave 20–50 cpm per 10^6 cpm ATP. Each point was assayed in triplicate.

Protein was determined by the method of Lowry (Lowry *et al.*, 1951) using bovine serum albumin as standard.

Results

Binding of Choleragen and Stimulation of Adenylate Cyclase

Maximal ^{125}I-labelled choleragen binding to the transformed cell is not more than 2 to 5% of that of the parent line (Fig. 1), consistent

Table 1. Comparison of binding and biological effects of choleragen[a]

Cell type	Binding		Adenylate cyclase stimulation			Per cent occupancy at maximum stimulation
	Saturation (ng/ml)	Molecules bound at saturation (per cell)	K_a	Saturation (ng/ml)	Molecules bound at saturation (per cell)	
KBalb/C 3T3	80	$7.5–10 \times 10^4$	0.5	3	10^4	10
Balb/C 3T3	1000[b]	$4.5–6 \times 10^{6}$[b]	0.1	1	2.5×10^4	0.5
Rat adipocyte	80	2×10^4	30	50–100	2×10^4	75–100

[a] Binding and adenylate cyclase assays were performed as in *Materials and Methods.* Data are from separate binding and cyclase assays and are combined from several experiments to represent an average. Data on isolated rat adipocytes taken in part from Cuatrecasas (1973*a–d*).

[b] Full saturation could not be observed experimentally. Values are those experimentally determined at the highest ligand concentration used and are presented for purposes of comparison.

with the finding that the transformed cell lacks ganglioside GM_1 by chemical and enzymatic assays (Brady & Fishman, 1974). As has been noted previously, the sensitivity of the toxin as a probe of ganglioside GM_1 is several orders of magnitude greater than these assays (Hollenberg *et al.*, 1974) and can detect as few as 10^3 molecules per cell. Half-maximal binding is seen at about 2×10^{-10} M choleragen (20 ng per ml) in the transformed *KBalb/C 3T3* line. An end point for the binding curve is not readily seen in the untransformed cell line. The difficulty in full saturation may be a result of the presence of cell surface gangliosides which bind choleragen poorly but nevertheless contribute to the overall binding curve. Similar difficulties in saturation have been observed in membrane preparations from rat liver (Cuatrecasas, 1973*c*). These putative low affinity binding sites are probably not biologically significant, since gangliosides other than GM_1, which bind choleragen poorly, are not biologically effective receptors when inserted into cells (Fishman, Moss & Vaughan, 1976). The concentrations of cells must be adjusted appropriately for binding studies, since at higher *Balb/C 3T3* cell concentrations most of the choleragen in freshly iodinated preparations is extracted from the medium, and saturation cannot be seen experimentally. The *Balb/C 3T3* cells bind at least 5×10^6 molecules per cell, while the *KBalb/C 3T3* cells bind only about 10^5 (Table 1). Choleragen binding was unchanged in *KBalb/C 3T3* cells grown in the presence of 10% fetal calf serum.

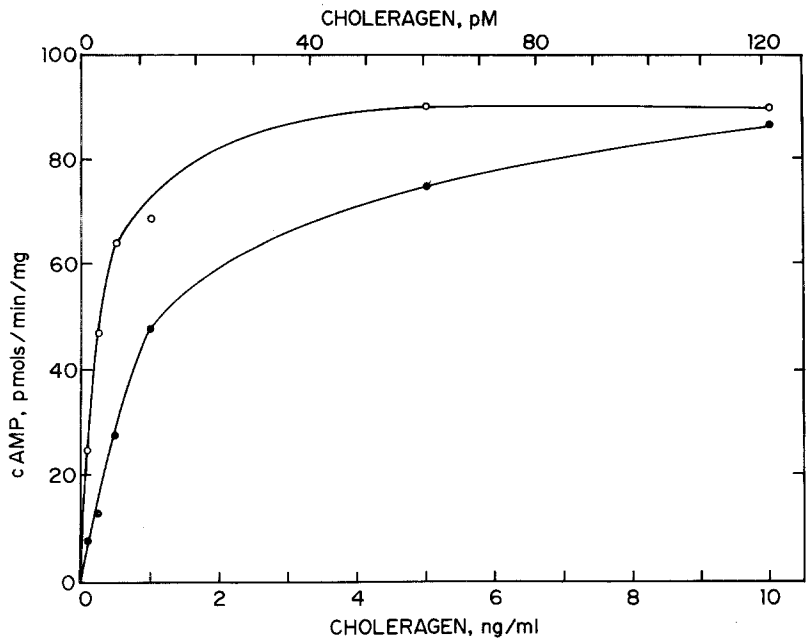

Fig. 2. Comparison of adenylate cyclase stimulation by choleragen in *Balb/C* 3*T*3 and *KBalb/C* 3*T*3 cells. Monolayers ($5–10 \times 10^6$ cells) were suspended with versene, washed twice, and resuspended in 10 ml MEM-0.1% albumin, incubated (3 hr, 37°, 10% CO_2) with various concentrations of choleragen, and assayed for adenylate cyclase activity as in *Materials and Methods*. Ordinate is pmoles cAMP produced per min per mg protein of crude particulate fraction. *Balb/C* 3*T*3 -o-, *KBalb/C* 3*T*3 -●-

Despite marked differences in choleragen binding, there is a relatively small but reproducible difference in sensitivity to choleragen as measured by stimulation of adenylate cyclase (Fig. 2). Half-maximal stimulation of the enzyme in membrane pellets from cells incubated with choleragen for 3 hr is seen at about 10^{-12} M for the parent *Balb/C* 3*T*3 and at 5×10^{-12} M for the transformed *KBalb/C* 3*T*3 line. As has been noted previously in another cell line (Hollenberg *et al.*, 1974), the parent cells, which bind larger amounts of choleragen, are more sensitive than the transformed cells, but the difference is quite small in comparison to the difference in binding. Parallel choleragen concentration dependent fluoride inhibition of adenylate cyclase is seen in both lines. DNA synthesis in the untransformed line, as in cultured human fibroblasts, is extremely sensitive to choleragen. Uptake of [^{3}H]thymidine by the parent line is maximally inhibited at 10^{-13} M choleragen in synchronized cultures stimulated to divide by epidermal growth factor (data not shown). Since the transformed line does not show altered growth characteristics in

response to choleragen, added cyclic AMP or dibutyryl cyclic AMP, theophylline, or any combination of these, it was not possible to compare the effects of choleragen on growth. Morphologic changes consisting of increased refractility and "rounding up" also seen in other cell lines (O'Keefe & Cuatrecasas, 1974; Donta, King & Sloper, 1973) are seen in both lines 15–30 min after exposure to choleragen. These effects are not seen with choleragenoid and probably reflect elevation of intracellular cyclic AMP concentrations.

Failure of Enhancement of Choleragen Effect in Cultured Cells by Ganglioside

It has been shown previously that exogenous ganglioside added to cells before choleragen and then removed from the medium by washing can enhance choleragen-stimulated lipolysis in isolated fat cells (Cuatrecasas, 1973*b*). Also, previous comparison of low-passage cultured cells which show higher choleragen binding with high-passage or virally transformed cells suggested that the relative number of receptors may be related to sensitivity to choleragen (Hollenberg *et al.*, 1974). Recently, Moss *et al.* (Moss *et al.*, 1976) have enhanced the sensitivity of cultured cells to choleragen with exogenous GM_1. Neuraminidase, which converts ganglioside GD_{1a} to GM_1, has been shown to increase the response of cultured adrenal tumor cells to choleragen (Haksar, Maudsley & Peron, 1975). Extensive efforts were made to increase the sensitivity of the transformed *KBalb/C 3T3* cell line to choleragen by insertion of ganglioside GM_1. GM_1 deficient, transformed cells (*KBalb/C 3T3*, Brady & Fishman, 1974) incubated with ganglioside GM_1 show markedly increased binding of choleragen depending on concentration of cells and choleragen (Fig. 3), but do not show altered rate or extent of adenylate cyclase stimulation when measured at $1^1/_2$ hr (incomplete stimulation) or 3 hr (stimulation essentially complete) using either maximally or submaximally stimulating concentrations of choleragen. Neither the K_m for ATP nor basal adenylate cyclase activity were altered by ganglioside preincubations. Incubation of *KBalb/C 3T3* cells with neuraminidase results in significantly increased binding of choleragen (Fig. 4), but adenylate cyclase activation shows no change in K_a or maximum activity in neuraminidase-treated cells (data not shown).

Comparison of data from binding and adenylate cyclase stimulation (Table 1) provides an explanation for these findings. Maximum biological

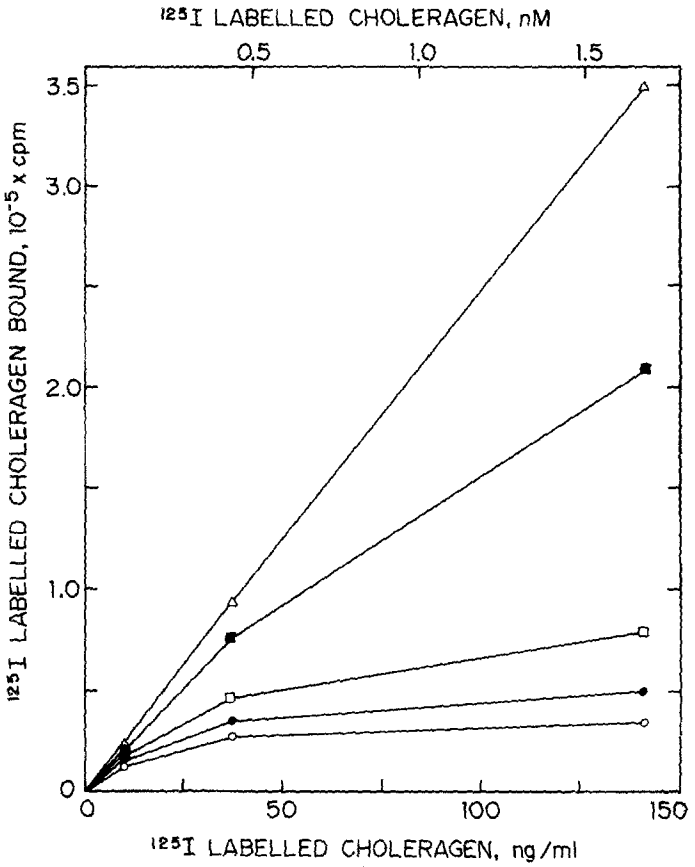

Fig. 3. Enhancement of binding of ^{125}I-labelled choleragen to *KBalb/C 3T3* cells by exogenous GM_1 ganglioside. Cells in monolayer were suspended, washed (Hanks'-0.1% albumin buffer), and incubated (3 hr, 37°) with various concentrations of ganglioside GM_1. Samples were washed 3 times to remove ganglioside and assayed for binding as in *Materials and Methods*. Control, -○-; ganglioside GM_1 concentrations: 0.1 μg/ml, -●-; 0.5 μg/ml, -□-; 2.5 μg/ml, -■-; 10 μg/ml, -△-

effects have been observed previously in amphibian erythrocytes and in cultured melanoma cells at concentrations of choleragen 4–10% of those required for saturation of binding. Similarly, maximal activity is seen at about 10 ng per ml choleragen in the *KBalb/C 3T3* line and saturation of binding at approximately 80 ng per ml. Thus, with only 10–20% of choleragen receptors occupied, activation is complete after 3 hr incubation, indicating that a 5- to 10-fold excess of binding sites must be present over those required for full stimulation. This discrepancy is markedly accentuated in the parent line, in which there is a 150- to 300-fold excess of binding sites observed at near-saturation of binding

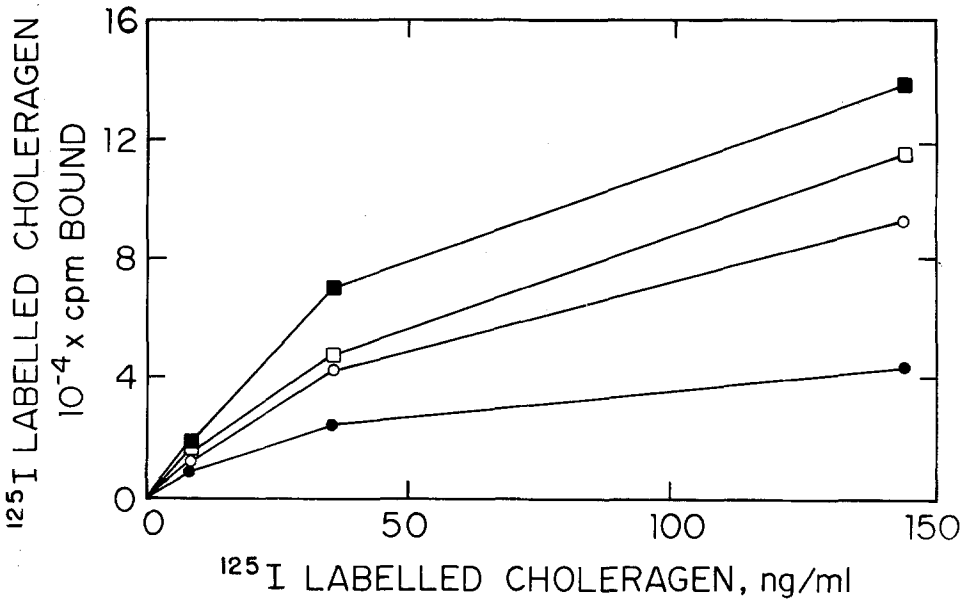

Fig. 4. Enhancement of binding of ^{125}I-labelled choleragen to *KBalb/C 3T3* cells by neuraminidase. Cells in monolayer were suspended, washed, and incubated (1 hr, 37°) with various concentrations of neuraminidase in MEM-0.1% albumin. Samples were then washed twice, resuspended, and assayed for binding as in *Materials and Methods*. Control, -●-. Neuraminidase concentrations: 10 μg/ml, -○-; 50 μg/ml, -□-; 100 μg/ml, -■-

(approximately 1 μg per ml choleragen) over those required for full biological effects (3–6 ng per ml). The addition of exogenous receptors would not be expected to alter sensitivity to choleragen in a cell line having more binding sites than are required for maximal stimulation, i.e., showing maximum adenylate cyclase stimulation at a fraction of total receptor occupancy.

Enhancement of Adenylate Cyclase Stimulation by Choleragen in Fat Cells

Isolated fat cells from 100-gram rats require approximately equal concentrations of choleragen for binding saturation and maximal stimulation of lipolysis (Cuatrecasas, 1973*a*, *c*). Enhancement of binding by exogenous ganglioside GM_1 is readily demonstrated, and washing of cells followed by addition of choleragenoid, the biologically inactive derivative of choleragen, abolishes this enhancement (not shown). Cells whose choleragen binding has been diminished to 10% of control by choleragenoid will bind 6-times control amounts after washing and subsequent incuba-

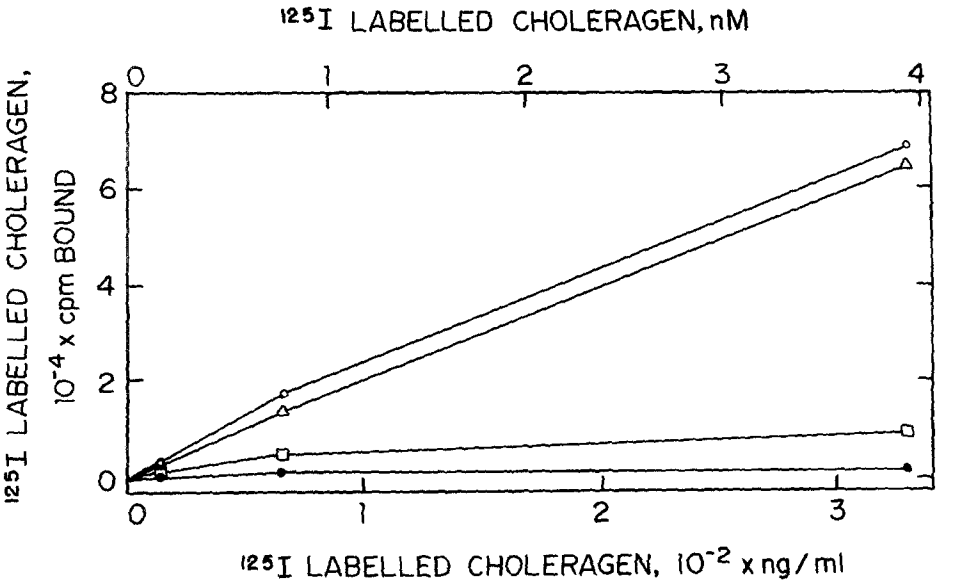

Fig. 5. Reconstitution of choleragen binding sites in isolated rat fat cells by exogenous GM_1 ganglioside after blocking of intrinsic receptors by choleragenoid. Cells from the epididymal fat pads of 4 rats were divided into 4 samples, incubated (20 min, 37°) with (2 samples) or without (2 samples) choleragenoid (1 μg/ml), and washed 3 times. All samples were then incubated (20 min, 37°) with (2 samples, 1 previously incubated with choleragenoid) or without GM_1 ganglioside (10 μg/ml), washed 3 times, and assayed for binding of ^{125}I-choleragen as in *Materials and Methods*. Control cells received no additions. Control, -□-; choleragenoid only, -●-; GM_1 only, -○-; choleragenoid followed by GM_1, -△-

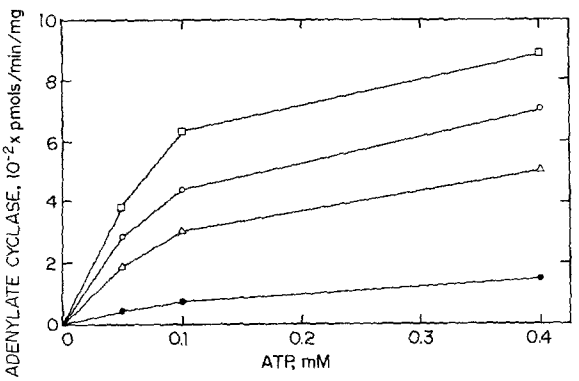

Fig. 6. Reconstitution of functional choleragen receptor by exogenous GM_1 after blocking of intrinsic receptor by choleragenoid. Fat cells from 10 rats were distributed into 5 vials and sequentially incubated with or without 20 ng/ml choleragenoid (15 min, 24°), 20 μg/ml GM_1 ganglioside (30 min, 37°), and 100 ng/ml choleragen (3 hr, 37°), and assayed for adenylate cyclase as in *Materials and Methods* with various concentrations of ATP. Cells were washed 2 to 3 times between additions and resuspended in MEM-0.1% albumin buffer. Control cells received no additions but were treated and washed similarly. Control, -●-; choleragenoid followed by choleragen, -△-; choleragenoid followed by GM_1 and then by choleragen, -○-; choleragen only, -□-

tion with GM_1, a 30-fold increase (Fig. 5). Since blocking of choleragen binding in fat cells is reflected in decreased lipolysis in response to choleragen (Cuatrecasas, 1973*d*), and since there is a nearly 1:1 ratio of binding sites to sites required for activation (as measured by lipolysis), it was expected that reconstitution of binding sites in fat cells by exogenous ganglioside would be accompanied by an increase in the rate of adenylate cyclase stimulation by choleragen.

In separate experiments (Fig. 6) using lower ligand concentrations appropriate for measuring biological responses, preincubation with choleragenoid followed by washing diminishes adenylate cyclase stimulation; furthermore, the subsequent incubation of such cells with ganglioside GM_1 (followed by washing) enhances the choleragen response. While the binding of choleragen is readily reduced by the high concentrations of choleragenoid (1 μg/ml) used in the binding experiments (Fig. 5), only partial reduction of adenylate cyclase activity is seen with the lower concentrations (20 ng/ml) used in the biological experiments to prevent carryover of choleragenoid during washes. Enhancement of binding is complete (Fig. 5) after 3 hr incubation of cells with ganglioside but there is only partial recovery in biological experiments (Fig. 6) at the shorter times necessary to ensure cell viability. Any changes in responsiveness are controlled by similar treatment of all samples (Fig. 6). That the exogenous receptor for choleragen functions effectively as a biological receptor for choleragen is confirmed by direct measurement of adenylate cyclase (Fig. 6) as well as of lipolysis (Cuatrecasas, 1973*b*).

Competition for Binding and Blocking of Adenylate Cyclase Stimulation by Choleragenoid

Because of the vast excess of receptors for choleragen in the cultured lines over the number required for adenylate cyclase activation, it is very difficult to block the biologic effect of choleragen in these cells. Even *KBalb/C* 3*T*3 cells, which bind relatively small amounts of choleragen, show at least a fivefold excess of receptors. In earlier experiments with fibroblasts, in which excess binding sites are found experimentally, a 50-fold excess of choleragenoid in the medium failed to block the biologic response, while a 1000-fold excess decreased adenylate cyclase stimulation significantly (Bennett *et al.*, 1975). As has been proposed (Bennett *et al.*, 1975), these data exclude the possibility of a subset of biologically effective binding sites with increased affinity for choleragen

or other special characteristics and indicate that the receptors are equivalent with respect to the biological effect of the choleragen. Thus, if 90% of the binding sites are blocked, the remaining 10%, which are adequate in number for full adenylate cyclase stimulation, nullify the effect of choleragenoid. Paradoxically, choleragenoid, a specific antagonist with no biologic activity[2] and with binding affinity equal to that of the active choleragen (Cuatrecasas, 1973*d*; Holmgren, Lindholm & Lonnroth, 1974), will appear to be ineffective experimentally if the quantitative estimates of binding saturation and binding sites required for full biologic activity are not considered. In the *Balb/C* 3*T*3 line, for example, 99% (of approximately 4×10^6 sites) can theoretically be blocked without altering the capacity of the cells to be fully activated by the remaining 1% (approximately 4×10^4 sites). Quantitative comparisons are shown in Table 1.

This concept is supported experimentally by comparison in the same experiment of binding and activation in *KBalb/C* 3*T*3 cells previously incubated with choleragenoid. Measurement of both binding and adenylate cyclase stimulation in the same cell population can be achieved with biologically active ^{125}I-labelled choleragen and indicates a marked discrepancy between competition for binding and blocking of adenylate cyclase stimulation (Fig. 7). Only with relatively low concentrations of choleragen and a 40- to 1000-fold excess of choleragenoid can the biological response be substantially blocked, and binding sites must be blocked by 95 to 99% to produce significant inhibition of adenylate cyclase stimulation by toxin.

The ability of very minimal, trace amounts of choleragen which contaminate most choleragenoid preparations to be expressed in biological experiments, even in the presence of a vast excess of the specific inhibitor, is also explained by these findings. Such effects are regularly seen with choleragenoid in cultured cells or *in vivo* but rarely with isolated fat cells, which do not contain a marked excess of binding sites. Inhibition of stimulation of DNA synthesis in cultured fibroblasts and in *Balb/C* 3*T*3 cells is seen with 10^{-13} M choleragen and is readily produced with most choleragenoid preparations. Repeated passage of choleragenoid through Sephadex G-50 columns at low pH permits separation of the A subunit of mol wt 36,000 from the dissociated B subunit, which can be reassociated by neutralization of the eluate (LoSpalluto & Finkelstein, 1972; Lonnroth & Holmgren, 1973). Similar purification is attained by eluting

2 Residual biological activity in choleragenoid preparations must be removed by repeated purification as described below.

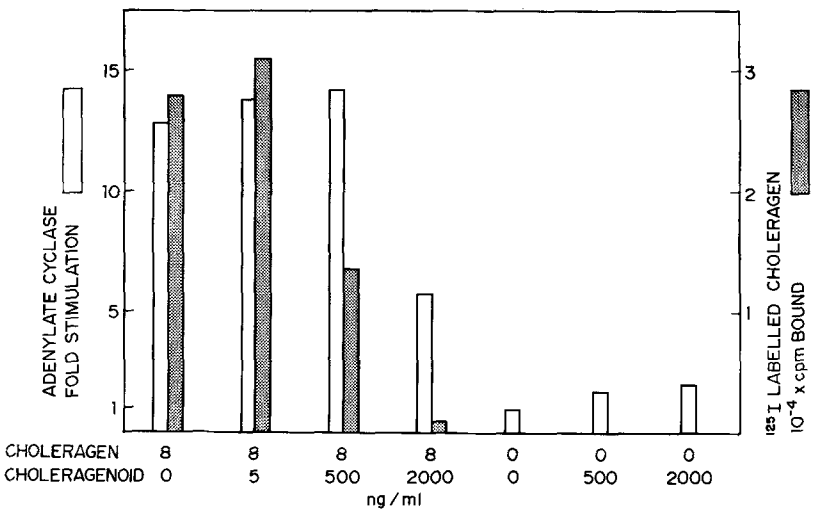

Fig. 7. Comparison of blocking by choleragenoid of binding and adenylate cyclase stimulation by ^{125}I-labelled choleragen in *KBalb/C 3T3* cells. In one experiment, monolayers were washed, suspended with versene, and incubated (30 min, 37°) with or without choleragenoid and then with or without 5 ng/ml ^{125}I-labelled choleragen (3 hr, 37°) in 5 ml MEM-0.1% albumin. Cells were assayed for binding (0.5 ml) by dilution and filtration or for adenylate cyclase (4.5 ml) as in *Materials and Methods*

the binding subunit with acid from ganglioside-Sepharose columns (Cuatrecasas *et al.*, 1973) loaded with choleragenoid, followed by removing contaminating ganglioside by passage through Sephadex G-25, neutralization, and repetition of this process 3–6 times. Choleragenoid prepared in this manner failed to inhibit [^{3}H]thymidine incorporation in epidermal growth factor-stimulated fibroblasts at 2×10^{-8} M, while inhibition from standard choleragenoid preparations is seen at 10^{-11} M. This suggests that the contamination in usual preparations is of the order of 0.1–1%. Similar inhibition could be demonstrated and reconstituted when the highly repurified choleragenoid was mixed with small amounts of pure choleragen (data not shown).

Discussion

These studies extend the earlier demonstration of enhancement of lipolysis by exogenous gangliosides in fat cells (Cuatrecasas, 1973*b*) by showing enhancement of an earlier effect of choleragen, the activation of adenylate cyclase, and by showing that such enhancement is still

demonstrable when endogenous receptors have been blocked previously by choleragenoid. The receptor is effectively reconstituted by addition of a known molecular species, ganglioside GM_1, in a cell which has largely lost its ability to bind choleragen, with restitution of biological effectiveness.

Evidence has been presented demonstrating that, although only a small proportion of sites are required to fully activate adenylate cyclase, a very much larger number of choleragen binding sites exist which are equivalent to those sites with respect to the biological action of choleragen. In isolated fat cells and in the transformed cell line, there is no subset of binding sites with special biological capability, since a 50-fold excess of a specific competitive inhibitor (i.e., choleragenoid) does not prevent activation (Fig. 7), and exogenous ganglioside GM_1 functions as effectively as the natural receptor. Very large (200- to 1000-fold) excesses of choleragenoid are required for inhibition in the untransformed *Balb/C* 3*T*3 line and in some human fibroblasts (Bennett *et al.*, 1975) while in the *KBalb/C* 3*T*3 line inhibition is seen with a 40-fold molar excess (Fig. 7) over choleragen. The number of choleragen molecules bound per cell is critical with regard to biological activation. It is notable that despite very large differences in the total number of binding sites among several cell types, the total number of cell bound choleragen molecules required for full cyclase activation is similar in a variety of cell types including the *Balb/C* 3*T*3 and *KBalb/C* 3*T*3 lines, isolated rat fat cells (Cuatrecasas, 1973*c*), cultured melanoma cells (O'Keefe & Cuatrecasas, 1974), and amphibian erythrocytes (Bennett & Cutrecasas, 1975*b*).

There is evidence that the A subunit of choleragen may combine directly with adenylate cyclase (Bennett *et al.*, 1975; Sahyoun & Cuatrecasas, 1975) to form a permanently activated integral membrane structure which may persist for as long as 14 days in cultured cells (O'Keefe & Cuatrecasas, 1974). If it is assumed that one choleragen activates one adenylate cyclase molecule, the similarity of the data from diverse cell types suggests that a similar number of adenylate cyclase molecules is found in a wide variety of cell types despite a marked variation in the number of choleragen binding sites as well as despite large differences in the basal levels, specific activities, or fluoride-stimulated activities of adenylate cyclase in such cells. The marked differences in surface glycolipid in these different cell types, as reflected in choleragen binding, are not characteristic of adenylate cyclase activation, which requires similar amounts of cell bound choleragen in diverse cell types.

Although incorporation of exogenous gangliosides into membranes,

as measured by the criterion of enhanced toxin binding, has been demonstrated in avian and amphibian erythrocytes, isolated rat adipocytes, and in a number of types of cultured cells, enhancement of choleragen binding after incubation of cells with ganglioside GM_1 was not demonstrable in the untransformed *Balb/C* 3*T*3 line or in cultured human fibroblasts in which binding was already very high. Control experiments in which cells were serially diluted with respect to choleragen demonstrated that this was not a result of complete extraction of choleragen from the medium, which can be observed at high cell concentrations and can produce artifactual saturation of choleragen binding. Incubation of *Balb/C* 3*T*3 cells with ganglioside at 37°, 41°, and 45° for various times did not enhance incorporation as measured by choleragen binding; this may have resulted from failure of insertion or inaccessibility of inserted ganglioside. The maximum number of choleragen molecules bound by *Balb/C* 3*T*3 cells was very similar to the number bound by *KBalb/C* 3*T*3 cells after maximum ganglioside GM_1 incorporation, suggesting that physical constraints within the membrane may have limited incorporation to a maximum of about 4 to 6×10^6 molecules per cell (Table 1). Similar maxima were seen in spontaneously transformed *TAL/N* or virally transformed *SVS AL/N* mouse cell lines and also in a human fibroblast line which showed incorporation of ganglioside (data not shown). All these results suggest that ganglioside incorporation may not be a simple partitioning of the molecule into the lipid bilayer of membranes, but may rather reflect the presence of limited numbers of specific acceptor macromolecules within the membranes. Choleragen can be used to follow the fate of natural or incorporated ganglioside over a period of days (O'Keefe & Cuatrecasas, 1977) or months (Manuelidis & Manuelidis, 1976) without the possibility of radiation damage in labelled cells that would occur with radioactive ganglioside.

If the findings with ganglioside GM_1 and its biologic effector in the membrane, adenylate cyclase, can be generalized, glycolipids may vastly outnumber the putative membrane effector molecules in other situations as well, especially since glycolipids are readily inserted into the membranes of intact cells from plasma (Marcus & Cass, 1969), where the concentration and supply of certain of these may be great; in such situations the insertion may not be controlled entirely by the cell unless some mechanism exists in the cell for specifically limiting the acceptance of the exogenous glycolipids. Unlike large glycoprotein hormone receptors, which are presumably only synthesized and assembled by the cell in which they reside, numbers of exposed glycolipids may be limited

only by physical constraints on the surface membrane or possibly by specific acceptor proteins (i.e., receptors) for these exogenously present glycolipids. Thus, binding sites for tetanus toxin (Clowes, Cherry & Chapman, 1972), viruses (Haywood, 1974), glycoprotein hormones (Kurosky *et al.*, 1972), interferon (Besancon & Ankel, 1974) and other biologically potent ligands which bind gangliosides may be subject to the same considerations as described here for gangliosides and choleragen.

If "spare" receptors are present, alteration of the number of binding sites (e.g., by addition of glycolipids) may not alter the experimental results, especially for complexes of very high affinity, such as exists for choleragen. This consideration could be important in efforts to assess the role of glycolipids in the growth and behavior of cells in culture and could explain in part the ineffectiveness of added glycolipids in most studies of this kind despite occasional positive findings (Laine & Hakomori, 1973).

The assessment of marked differences in the amounts of ligand required for saturation of a biological effect in comparison with binding may allow predictions of experimental behavior. As in the *KBalb/C* 3*T*3 line, if the ratio: (molecules of ligand bound at saturation of binding)/(molecules bound at saturation of biological effect) is $\gg 1$, it is expected that (i) further enhancement of binding, e.g., by addition of receptors, will not increase sensitivity as measured by the biological effect and (ii) that competition by a specific inhibitor may be difficult to demonstrate. Conversely, if blocking by a competitor (i.e., competitive antagonist) is readily seen experimentally, and if sensitivity to a ligand is enhanced by increased numbers of receptors, it is unlikely that "spare" receptors are present. Studies using exogenous ganglioside to enhance the response of rabbit gut loops to choleragen (Holmgren *et al.*, 1975), which are consistent with this prediction, showed that choleragenoid inhibits choleragen action in control loops but not in loops preincubated with GM_1. Choleragenoid competes effectively in isolated cells which show very little binding of choleragen (Cuatrecasas, 1973*d*; Bennett & Cuatrecasas, 1975; Gill & King, 1975).

The biological effect under study must be in close proximity, or closely linked, to the binding function at the membrane, since intracellular magnification of a protracted but submaximal effect at the membrane may over given time periods produce maximal stimulation of an observed, far-removed biological effect (e.g., DNA synthesis).

It has been demonstrated that choleragen stimulation of another GM_1-deficient cell line can be enhanced by exogenous ganglioside (Fish-

man *et al.*, 1976; Moss *et al.*, 1976). It is anticipated that this line would show a marked deficiency of GM_1 such that the concentration of choleragen required for saturation of binding is less than that required for full stimulation after GM_1 insertion, and that stimulation with or without insertion of small amounts of GM_1 would be easily blocked by choleragenoid.

The basis for the delay between choleragen binding (complete in about 10 min) and adenylate cyclase activation has not been elucidated. It has been shown that choleragen is at least bivalent (Craig & Cuatrecasas, 1975), and the likelihood of 4 to 6 B subunits per toxin molecule suggests that it may be multivalent. It was suggested that multivalent binding may be slow and might induce the dissociation of the toxin into its two major subunits (A and B). If the lag is a reflection of the time required for the A subunit to dissociate from B subunits and subsequently become incorporated into or traverse the membrane (Bennett *et al.*, 1975), it is possible that in cells preincubated with ganglioside, the larger numbers of available membrane GM_1 molecules would speed the dissociation reaction and produce a lag period shorter than that of cells having binding sites blocked by choleragenoid. This finding is consistent with a proposed mechanism of choleragen action (Bennett *et al.*, 1975) in which diffusion of the A subunit within the two dimensional plane of the membrane leads to physical association of the A subunit with adenylate cyclase and the activation of the enzyme. This may be analogous to a proposed mechanism of action of hormones (Bennett *et al.*, 1975; Bennett & Cuatrecasas, 1977; Cuatrecasas & Hollenberg, 1976). The role of the reported enzymatic activity of choleragen (Gill, 1975; Moss *et al.*, 1977) in this scheme is unknown.

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