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Mechanism of Action of Cholera Toxin: Effect of Receptor Density and Multivalent Binding on Activation of Adenylate Cyclase

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Summary. Choleragen (cholera toxin) activates adenylate cyclase in HeLa cells, which contain less than 15,000 toxin receptors per cell, in a time- and concentration-dependent manner. Activation is blocked by the addition of the oligosaccharide chain of the ganglioside G_{M1}, the receptor for the toxin. When the cells are preincubated with choleragen at 4 °C and then incubated with oligosaccharide at 37 °C, adenylate cyclase is activated less than 10%. When the preincubation phase is above 18 °C, adenylate cyclase becomes activated and the amount of activation depends on the time of preincubation. This inhibitory effect of the oligosaccharide is also observed with human lymphocytes and rat glial C6 cells but not with Friend erythroleukemic and mouse neuroblastoma N18 cells. The latter two cell lines have large numbers of toxin receptors, whereas the former two cell lines have few receptors. When the number of toxin receptors in HeLa and C6 cells is increased by treating the cells with G_{M1}, activation of adenylate cyclase by choleragen is no longer blocked by the oligosaccharide. The oligosaccharide has a corresponding effect on the displacement of bound ^{125}I-choleragen. When bound to cells at 4 °C, most of the radiotoxin is displaced from HeLa, C6, and lymphocytes but not from Friend, N18, or HeLa cells pretreated with G_{M1}. In untreated HeLa cells, dissociation of toxin-receptor complexes by the oligosaccharide depends on the time and temperature of complex formation; above 18 °C, the toxin rapidly becomes stably bound to the cells. The inhibitory effect of G_{M1} oligosaccharide is reversible, as, once it is removed, the small amount of toxin that remains bound can activate adenylate cyclase. These results are consistent with a model in which choleragen, which is multivalent, must bind to several G_{M1} molecules on the cell surface in order to subsequently activate adenylate cyclase. Lateral mobility of toxin-receptor complexes may be required only to achieve multivalent binding in cells with few receptors.

Cholera toxin (choleragen), which causes biological effects in a wide variety of vertebrate cells, contains two protomers, A and B. The B protomer consists of 5 identical peptide chains (Gill, 1977) and binds to specific plasma membrane receptors, ganglioside G_{M1}[1] molecules (Cuatrecasas, 1973*a*, *b*; Holmgren, Lonnroth & Svennerholm, 1973; King & van Heyningen, 1973; Moss et al., 1976*a*; Moss, Manganiello & Fishman, 1977). The A protomer is composed of two dissimilar polypeptides linked by a disulfide bond and causes the biological effects of the toxin by activating adenylate cyclase (Bennett et al., 1976; Fishman & Brady, 1976; Gill, 1977; van Heyningen, 1977). There is a characteristic lag (10–60 min) in the activation of adenylate cyclase in intact cells, whereas, under appropriate conditions, activation occurs immediately in broken cells (Gill, 1977). Extensive studies indicate that the activation process requires the A subunit of choleragen and NAD, and involves ADP-ribosylation of a guanyl nucleotide binding protein that presumably regulates adenylate cyclase (Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Johnson, Kaslow & Bourne, 1978).

Although the nature of the toxin receptor is known and certain aspects of the process of adenylate cyclase activation have been elucidated, little is known about the intervening steps between binding and acti-

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[1] Abbreviations used are: G_{M1}, galactosyl (β1–3)N-acetylgalactosaminyl(β1-4)[N-acetylneuraminyl(α2-3)]galactosyl(β1-4)glucosyl (β1-1)ceramide; HEPES, N-2-hydroxyethylpiperazine-N′-ethylsulfonic acid; MEM, minimal essential medium; cAMP, adenosine 3′:5′-monophosphate.

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vation. Choleragen causes patching and capping on lymphocytes (Revesz & Greaves, 1975; Craig & Cuatrecasas, 1975; Sedlacek et al., 1976), suggesting that the toxin is multivalent. Direct studies with the oligosaccharide moiety of G_{M1} indicate that each toxin molecule can bind up to 5 oligosaccharides, presumably one for each of the peptides in the B protomer (Sattler et al., 1977; Fishman, Moss & Osborne, 1978). Several models have been proposed to explain how the A protomer, which is initially on the external surface of the cell membrane, interacts with adenylate cyclase, which is on the internal surface of the membrane; these models invoke multivalent binding and lateral mobility of toxin-receptor complexes to varying degrees (Bennett et al., 1976; Fishman & Brady, 1976; van Heyningen, 1977; Gill, 1977). The present work describes the relative roles of these two processes and the influence of receptor density on choleragen activity.

Materials and Methods

Cells and Materials

HeLa cells, strain R, were propagated in Eagle's MEM supplemented with 10% fetal calf serum as described previously (Henneberry, Fishman & Freese, 1975). Friend erythroleukemic cells (line GM979) were propagated in suspension culture in Ham's F-12 medium plus 15% fetal calf serum (Fishman & Atikkan, 1979). Mouse neuroblastoma N18 and rat glial C6 cells were cultured in Dulbecco's modified MEM containing 10% and 5% fetal calf serum as described previously (Duffard et al., 1977; Fishman & Atikkan, 1979). Lymphocytes were isolated from human peripheral blood by the method of Boyum (1968) using Lymphoprep (Accurate Chemical & Scientific Corp., Hicksville, N.Y.). Choleragen was purchased from Schwarz/Mann, Orangeburg, N.Y. G_{M1} and G_{M1}-oligosaccharide were obtained as described previously (Fishman et al., 1978).

Activation of Adenylate Cyclase

Cells grown in monolayer were washed 3 times with Dulbecco's phosphate-buffered saline, detached from the culture vessels with a rubber policeman, and collected by centrifugation. The Friend erythroleukemic cells were washed and collected by centrifugation. The cells were suspended in MEM buffered with HEPES, pH 7.4, (Microbiological Associates, Rockville, Md.) containing 0.01% bovine serum albumin. Portions of the cell suspensions were incubated with and without choleragen, usually in a total volume of 1 ml at 37 °C for 2 hr unless otherwise indicated. Then 4 ml of ice-cold 0.154 M NaCl was added and the cells were collected by centrifuging at $600 \times g$ for 5 min. The cells were disrupted in 2 mM Tris-Cl, pH 7.4, at 4 °C using a Polytron tissue disrupter (5 sec at half maximum speed).

Assay of Adenylate Cyclase

Portions of the homogenate (approximately 100 µg of protein) were assayed for adenylate cyclase activity as described by Tallman, Smith and Henneberry (1977) with the following modifications. For HeLa and C6 cells, the incubations contained 1.5 mM ATP, 0.1 mM GTP, 80 mM Tris-HCl (pH 7.8), 6 mM $MgSO_4$, and 10 mM theophylline in a total volume of 100 µl; incubation temperature was 34 °C. For Friend cells and lymphocytes, the buffer was 50 mM HEPES (pH 7.4), and the temperature was 33 and 37 °C, respectively. For N18 cells, the incubations contained 2 mM ATP, 50 mM Tris-Cl (pH 7.4), 10 mM $MgCl_2$, and 10 mM theophylline; incubation temperature was 30 °C. Fluoride-activated adenylate cyclase was assayed in the presence of 10 mM NaF. After 10 min of incubation, the reactions were terminated by boiling for 2 min and portions were assayed for cAMP by a protein binding technique (Tallman et al., 1977). Determinations were made in triplicate and corrected for the amount of cAMP present in the homogenates (zero time control); standard deviations were less than 10% of the mean. Adenylate cyclase activity is expressed as pmol of cAMP formed per mg protein per 10 min.

Binding of ^{125}I-choleragen

Choleragen was radiolabeled with ^{125}I using the chloramine-T method as described by Cuatrecasas (1973a). Specific radioactivities ranged from 4.5 to 45 µCi per µg and between 60 and 90% of the radioactivity bound to an excess of cell membranes (Cuatrecasas, 1973*a*). Cells (10^3 to 10^6) were incubated with 2.5×10^{-10} M ^{125}I-choleragen in 0.2 ml of Tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin for 45 min at 37 °C. Then 1.5 ml of ice-cold buffer containing 1% bovine serum albumin was added to each tube (12×75 mm polystyrene from Falcon Plastics, Oxnard, Calif.), and the contents were filtered under vacuum on 25 mm Millipore EAWP filters (Cuatrecasas, 1973*a*). The filters were washed twice with the same buffer and were counted on a Beckman gamma 4000. Nonspecific binding was determined by preincubating the cells with 2×10^{-7} M unlabeled choleragen. Values are the mean of triplicate determinations and have been corrected for nonspecific binding; variation among the triplicates was less than 10%.

Binding of iodotoxin to cell membranes was determined as described above except the samples were filtered on EGWP filters (Cuatrecasas, 1973*a*). Cell membranes were prepared by homogenizing the cells in 10 volumes of 0.25 M sucrose-5 mM Tris-HCl (pH 7.8) with 15 strokes in a Dounce homogenizer. The homogenate was centrifuged at $1900 \times g$ for 15 min and the resulting supernatant was centrifuged at $40{,}000 \times g$ for 1 hr. The final pellet was resuspended in the same buffer and frozen at −20 °C until used. Recovery of surface toxin receptors was 44–55% and toxin binding per mg protein was enriched 15 to 20-fold over intact cells.

Results

Activation of Adenylate Cyclase in HeLa Cells by Choleragen

When HeLa cells were exposed to choleragen, adenylate cyclase activity increased after a lag period of 30 min and reached a maximum by 2 hr (Fig. 1 *A*). Adenylate cyclase activity in choleragen-treated cells was enhanced ~2-fold by GTP whereas fluoride-stimulated activity decreased; these effects have been reported by others (Bennett et al., 1976). Maximal activation occurred at 10^{-10} M choleragen and half-

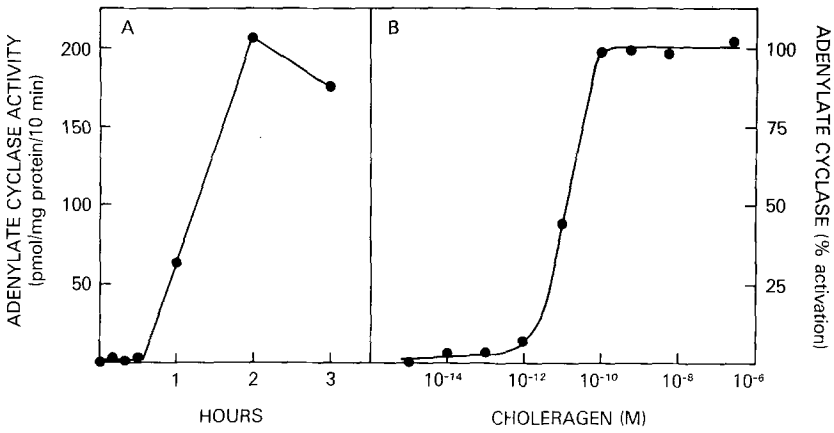

Fig. 1. Effect of incubation time and choleragen concentration on activity of adenylate cyclase in HeLa cells. (*A*) One ml portions of HeLa cells (5×10^6) suspended in MEM buffered with HEPES (pH 7.4) and containing 0.01% bovine serum albumin were incubated at 37 °C with 60 nM choleragen for the indicated times and then assayed for adenylate cyclase activity as described in Materials and Methods; activities have been corrected for basal activity. (*B*) Same as in (*A*) except the choleragen concentration was varied as indicated and the incubation time was 2 hr

maximal activation (K_a), at 10^{-11} M (Fig. 1 *B*). Under these latter conditions, each cell was exposed to 1000 molecules of toxin. Since HeLa cells contain less than 15,000 toxin receptors per cell (*see* below), it would appear that initially the choleragen molecules would be widely dispersed on the cell surface and all of their binding sites might not be occupied by receptors. We explored this possibility by preincubating the cells for 5 min with choleragen at 4 °C to reduce any lateral mobility of membrane components and then incubated the cells at 37 °C in the presence of the oligosaccharide portion of G_{M1}. Table 1 shows the oligosaccharide effectively blocked the activation of adenylate cyclase, but the amount of inhibition decreased as the cells were incubated longer at 37 °C. Even when the cells were preincubated with choleragen for 1 hr at 4 °C, the oligosaccharide was still effective. The inhibitory effect of the oligosaccharide was concentration-dependent; 100% inhibition was observed at 100 μM and 50% inhibition at 3.5 μM.

Effect of G_{M1}-Oligosaccharide on Choleragen Action with Other Cells

In order to determine if this effect was unique to HeLa cells, we tested several other cell lines (Table 2). The oligosaccharide blocked the activation of adenylate cyclase in human lymphocytes and rat C6 glial cells but not in Friend erythroleukemic or mouse N18 neuroblastoma cells. Even at different ratios of toxin to oligosaccharide, no inhibition was observed with these latter two cell lines. The major difference between the two classes of cell lines was the amounts of toxin that bound to them and thus the density of toxin receptors (Table 3). These varied from less than 7,000 molecules per glial cell to almost 4 million per neuroblastoma cell. The affinity of choleragen for the various cell lines appeared to be similar; half

Table 1. Effect of G_{M1}-oligosaccharide on activation of adenylate cyclase in HeLa cells by choleragen[a]

Time of incubation (hr)	Inhibition of choleragen-activated adenylate cyclase (%)
1	94[b]
2	77
3	56

[a] HeLa cells were incubated for 5 min at 4 °C with and without 12 nM choleragen in MEM buffered with HEPES (pH 7.4) and containing 0.01% bovine serum albumin. Then 20 μM G_{M1}-oligosaccharide was added to some of the samples and all were incubated at 37 °C for the indicated times. Adenylate cyclase was assayed as described in Materials and Methods. After the subtraction of basal activity, the % inhibition due to the oligosaccharide was calculated.

[b] When G_{M1}-oligosaccharide was added prior to choleragen, the inhibition at 1 hr was 88%.

Table 2. Effect of G_{M1}-oligosaccharide on activation of adenylate cyclase in various cells by choleragen[a]

Cell line	CT (nM)	G_{M1}-oligo (μM)	Hours at 37 °C	Adenylate cyclase activity	
				−oligo	+oligo
Human lymphocytes	120	50	1	180	0
Friend erythroleukemic	120	100	1	155	200
	12	100	1	197	158
	0.1	100	1	156	162
Mouse N18 neuroblastoma	60	20	2	53	58
	1	100	2	79	101
	0.1	50	2	64	89
Rat glial C6	1	100	2	107	11

[a] Cells were incubated at 4 °C with and without choleragen (CT) for 5 min at which time G_{M1}-oligosaccharide (oligo) was added and the cells were incubated at 37 °C. Adenylate cyclase activity was assayed as described in Materials and Methods. Activities have been corrected for basal activity and are expressed as pmol of cAMP per mg protein per 10 min.

Table 3. Density of choleragen receptors in various cell lines[a]

Cell line	Cell volume (μ^3)	^{125}I-choleragen bound			
		(fmol per 10^6 cells)	(Molecules cell)	(Molecules μ^2)	K_d ($\times 10^{-10}$ M)[b]
HeLa	1680	23.8	14,300	21	6.5
C6	550	11.4	6,870	21	5.2
Lymphocyte	165	44.4	26,700	184	6.8
Friend	330	500	302,000	1310	2.4
N18	1250	6230	3,750,000	6680	2.8

[a] Cell numbers and cell sizes were determined with a Coulter Model ZH cell counter and channelizer, and cell volumes and surface areas were calculated from the cell diameters, using latex beads of known diameters to standardize the instrument. Binding of ^{125}I-choleragen to the various cell lines was determined as described in Materials and Methods. The number of cells per incubation was varied to ensure that less than 20% of the total radioligand, was bound and the concentration of labeled toxin was increased until saturation was reached. Values were corrected for nonspecific binding which was determined in the presence of excess unlabeled choleragen.

[b] Represents the concentration of choleragen at which half-saturation was observed.

Table 4. Effect of increased G_{M1} content on inhibition of choleragen activation of adenylate cyclase by G_{M1}-oligosaccharide[a]

Cells and treatment		Adenylate cyclase activity			Choleragen receptors (fold increase)
	CT	−	+	+	
	oligo	−	−	+	
Exp. 1 (HeLa)					
MEM + 10% fetal calf serum		15.6	296	72.6	1
MEM		21.4	248	48.6	0.6
MEM + 1 μM G_{M1}		22.6	321	313	275
Exp. 2 (C6 Glial)					
MEM		9	105	23	1
MEM + 1 μM G_{M1}		31	163	161	78

[a] HeLa cells were cultured overnight and C6 cells were cultured 1 hr in the indicated medium. The cells were harvested, washed, and suspended in MEM buffered with HEPES (pH 7.4) and containing 0.01% bovine serum albumin. Portions (1 ml) were incubated at 4 °C for 10 min with and without choleragen (120 pM for HeLa and 10 nM for C6). Then, G_{M1}-oligosaccharide (20 nmol for HeLa and 100 nmol for C6) was added to some of the samples and all were incubated at 37 °C for 2 hr. Adenylate cyclase activity (pmol per mg protein per 10 min) and binding of ^{125}I-choleragen were determined as described in Materials and Methods.

maximal saturation (K_d) occurred at choleragen concentrations between 2.4 and 6.8×10^{-10} M[2].

We explored the effect of receptor density further by incubating HeLa and C6 cells with medium containing G_{M1} in order to increase the number of choleragen receptors (Moss et al., 1976*a*; Fishman, Moss & Manganiello, 1977). The oligosaccharide no longer inhibited the activation of adenylate cyclase when the number of toxin molecules bound per cell was increased 275-fold in HeLa and 78-fold in C6 (Table 4). Even when HeLa cells were incubated with 50 nM G_{M1} and the receptor density was increased 20-fold, the oligosaccharide was not effective (data not shown).

Effect of G_{M1}-Oligosaccharide on Choleragen Binding

When the various cell lines were preincubated at 4 °C with ^{125}I-choleragen and the incubated at 37 °C with G_{M1}-oligosaccharide, the bound toxin was displaced from some of the cell lines but not others (Table 5). The ability of the oligosaccharide to displace the labeled toxin correlated with its ability to inhibit the activation of adenylate cyclase as well as the density of toxin receptors on these cells. We were able to directly demonstrate this latter relationship with HeLa cells incubated with G_{M1}. As the concentration of G_{M1} and, thereby, the number of new toxin receptors per cell was increased, the ability of the oligosaccharide to displace bound choleragen decreased (Table 5). Table 5 also shows that displacement depended on the oligosaccharide concentration; 50% displacement occurred at 0.2 to 0.3 μM G_{M1}-oligosaccharide with untreated HeLa cells.

The ability of G_{M1}-oligosaccharide to displace ^{125}I-toxin from some but not other cells was not due simply to competition between the oligosaccharide and surface receptors for the toxin. When the iodotoxin was incubated at 4 °C with 10^6 HeLa or 2500 N18 cells (total number of binding sites per incubation was equivalent), 20 μM G_{M1}-oligosaccharide displaced 7-fold more bound toxin from the HeLa cells than from the N18 cells. This is further supported by the ability of the oligosaccharide, when added first, to block choleragen binding to N18 (data not shown; *see also* Fishman et al., 1978).

[2] Scatchard plots of the binding data were all curvilinear and indicated apparent positive cooperativity. For this reason, as well as those raised by Cuatrecasas (1973*a*), we expressed the relative affinities of choleragen binding to the various cells as the concentration of toxin required to half-saturate the available binding sites. In this regard, the concentration of unlabeled choleragen required to inhibit iodotoxin binding to HeLa cells by 50% was 5.7×10^{-10} M, a value very similar to that required for half-saturation (Table 3).

Table 5. Displacement of bound choleragen by G_{M1}-oligosaccharide from various cell lines[a]

Cell line	G_{M1}-oligosaccharide (μM)	% of bound choleragen displaced	Choleragen receptors (fold increase)
Neuroblastoma N18	50	2	
Friend erythroleukemic	100	30	
Human lymphocytes	100	78	
Rat glial C6	100	94	
HeLa	100	95	
	20	92	1
	1	66	
HeLa plus 10 nM G_{M1}[b]	20	63	6.3
100 nM G_{M1}	20	13	65
1 μM G_{M1}	20	0	275

[a] Cells were incubated with ^{125}I-choleragen for 10 min at 4 °C in Tris-buffered saline (pH 7.4) containing 0.1% bovine serum albumin. Then G_{M1}-oligosaccharide was added to some of the samples and all were incubated for 45 min at 37 °C. Binding was determined as described in Materials and Methods and was corrected for nonspecific binding.

[b] HeLa cells were cultured overnight in MEM containing G_{M1} as indicated.

Effect of Time and Temperature on Inhibition by G_{M1}-Oligosaccharide

When HeLa cells were preincubated with choleragen at 37 °C, the ability of G_{M1}-oligosaccharide to block the activation of adenylate cyclase was rapidly lost (Fig. 2*A*). Significant activation occurred by 3 min and within 25 min the oligosaccharide no longer inhibited. When the cells were preincubated at different temperatures for 15 min with choleragen, the inhibitory effect of the oligosaccharide decreased with increasing temperature (Fig. 2*B*). Below 15 °C, there was little activation in the presence of oligosaccharide, whereas above 18 °C activation of adenylate cyclase rapidly increased with the preincubation temperature. When the effect of time and temperature on displacement of bound ^{125}I-choleragen by G_{M1}-oligosaccharide was examined, comparable results were obtained (Fig. 3). The longer the cells were exposed to the labeled toxin at 37 °C before addition of the oligosaccharide, the more toxin remained bound to the cells (Fig. 3*A*). When the cells were preincubated for 15 min at different temperatures with ^{125}I-choleragen, the amount of toxin not displaced by the oligosaccharide increased sharply at around 18 °C (Fig. 3*B*). These effects were not simply a consequence of the rate of choleragen binding during the preincubation period; HeLa cells incubated with ^{125}I-choleragen at 4 °C bound more toxin than cells incubated at 37 °C (Fig. 4). Similar results were obtained with C6 cells but not N18 or G_{M1}-treated C6 cells (Table 6).

Effect of Time and Temperature on the Stability of the Toxin-Receptor Complex

These results indicated that at elevated temperatures, choleragen irreversibly binds to cells with low receptor densities in a time-dependent manner. Since internalization of the toxin may occur at temperatures above 18 °C (Hansson, Holmgren & Svennerholm, 1977; Joseph, Stieber & Gonatas, 1979), we repeated some of the above experiments with membranes pre-

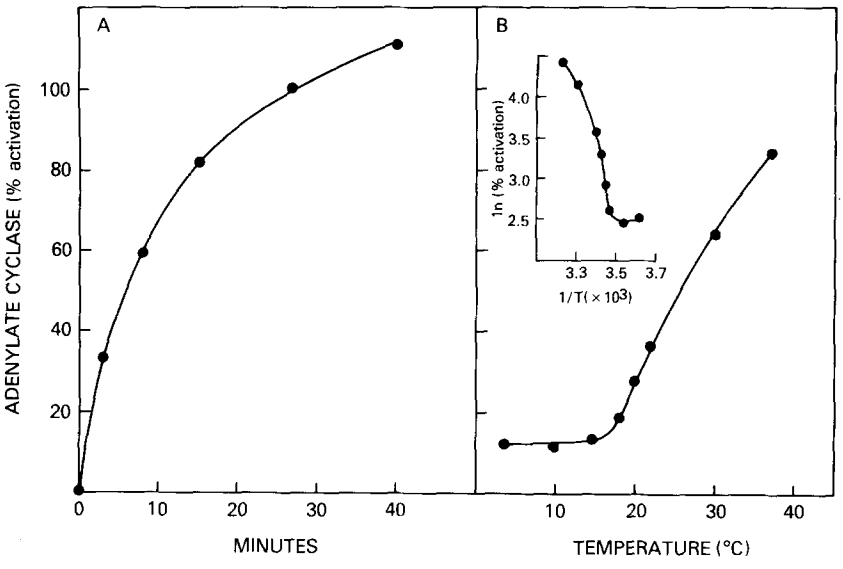

Fig. 2. Effect of time and temperature on inhibition of choleragen-activation of adenylate cyclase by G_{M1}-oligosaccharide. (*A*) HeLa were incubated at 37 °C with 2×10^{-10} M choleragen; at the indicated times, 50 μM G_{M1}-oligosaccharide was added and the incubations were continued for 2 hr. Adenylate cyclase activities were assayed as described in Materials and Methods and corrected for basal activity. (*B*) Same as in (*A*) except G_{M1}-oligosaccharide was added after the cells were incubated with choleragen for 15 min at the indicated temperatures; then the cells were incubated 2 hr at 37 °C. Results are expressed as % of activation observed in cells not exposed to G_{M1}-oligosaccharide

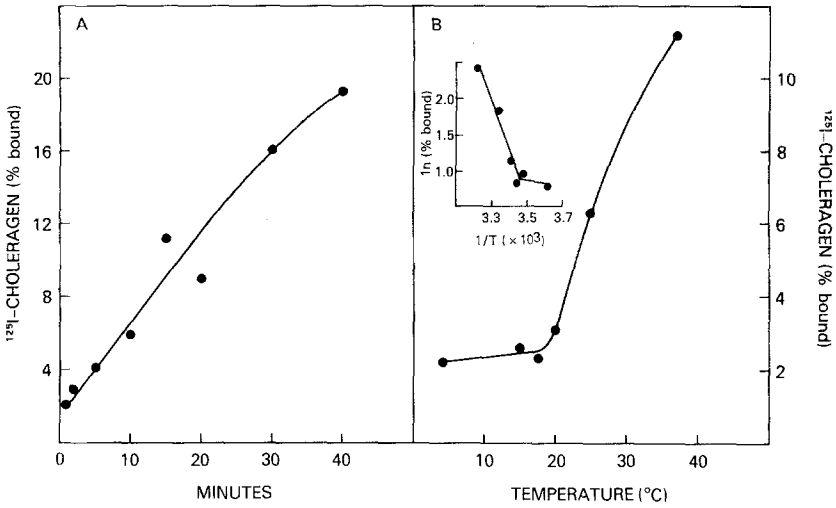

Fig. 3. Effect of time and temperature on displacement of bound ^{125}I-choleragen from HeLa cells by G_{M1}-oligosaccharide. Details are the same as described in the legend to Fig. 2 except the cells were incubated with 2×10^{-10} M ^{125}I-choleragen. After addition of G_{M1}-oligosaccharide, the cells were incubated 1 hr at 37 °C. Binding of labelled toxin was determined as described in Materials and Methods and corrected for nonspecific binding in the presence of 2×10^{-7} M unlabeled choleragen. Results are expressed as % of toxin bound to cells not exposed to G_{M1}-oligosaccharide.

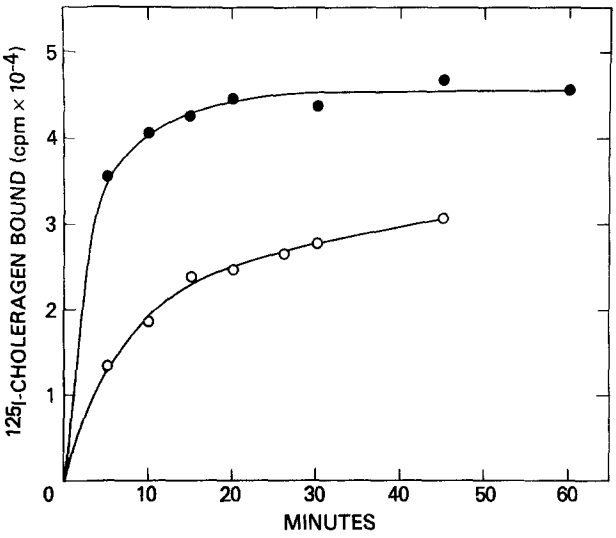

Fig. 4. Effect of temperature on rate of binding of ^{125}I-choleragen to HeLa cells. HeLa cells were incubated with ^{125}I-choleragen at 4 °C (●) or 37 °C (○) in the presence and absence of excess unlabeled toxin and assayed for bound iodotoxin at the indicated times as described in Materials and Methods. Values have been corrected for nonspecific binding

Table 6. Effect of temperature on binding of ^{125}I-choleragen to cells[a]

Cell line	^{125}I-choleragen bound (cpm)	
	4 °C	37 °C
HeLa	47,310	27,840
Rat glial C6	37,270	23,460
G_{M1}-treated C6[b]	37,010	56,580
Mouse neuroblastoma N18	32,210	49,740

[a] Cells were incubated with 4×10^{-10} M ^{125}I-choleragen for 30 min at 4 or 37 °C and assayed for bound iodotoxin as described in Materials and Methods. The number of cells was varied to ensure that less than 25% of the total toxin bound. Values have been corrected for nonspecific binding.

[b] C6 cells were incubated for 1 hr with 1 μM G_{M1} and washed extensively.

pared from the cells. The membranes from HeLa and C6 cells were incubated with ^{125}I-choleragen at 37 °C, and 50 μM G_{M1}-oligosaccharide was added at different times. The amount of iodotoxin remaining bound increased with time and the results were identical to those shown in Fig. 3*A*.

In addition, we examined the spontaneous dissociation of toxin-receptor complexes formed at different temperatures. Cells were incubated with ^{125}I-choleragen at 4 and 37 °C for 1 hr, centrifuged, and suspended in fresh medium at 37 °C. There was much more extensive dissociation of iodotoxin from C6 cells incubated at 4 °C than at 37 °C, whereas there was less dissociation from N18 cells and the extent of dissociation was similar, irrespective of the initial temperature.

We also observed that unlabeled choleragen could displace bound ^{125}I-toxin from HeLa cells at 37 °C, and the degree of displacement depended on the temperature at which the radioligand-receptor complexes were initially formed. Cells incubated with ^{125}I-choleragen at 4 °C displaced 2.5 times more bound toxin than cells incubated at 37 °C. Similar effects of temperature on the displacement of labeled toxin from rat adipocytes and liver membranes (Cuatrecasas, 1973*b*) and mouse thymocytes have been reported (Holmgren & Lonnroth, 1976).

Reversibility of the Inhibition by G_{M1}-Oligosaccharide

Since even high concentrations of G_{M1}-oligosaccharide were unable to displace all of the bound toxin

from cells with low numbers of toxin receptors (*see* Table 5), we tested the possibility that the remaining bound toxin might activate adenylate cyclase once the oligosaccharide was removed. The cells were incubated at 4 °C with a saturating concentration of toxin, then at 37 °C with G_{M1}-oligosaccharide, and finally at 37 °C after the oligosaccharide had been removed. A significant activation of adenylate cyclase occurred in both HeLa and C6 cells (Table 7). Since only a small percentage of the toxin remained bound under these conditions, only a partial activation was expected. Less activation was observed with C6 than with HeLa cells as the K_a for choleragen activation of adenylate cyclase is similar to the K_d for toxin binding in C6 cells, whereas in HeLa cells the K_a is an order of magnitude lower than the K_d.

Specificity of G_{M1} in Reversing G_{M1}-oligosaccharide Action

As indicated above, G_{M1}-oligosaccharide was unable to displace bound cholergen from HeLa and rat glial C6 cells treated with G_{M1} nor was it able to inhibit activation of adenylate cyclase by the toxin in the treated cells. To ensure that this reversal of the action of the oligosaccharide was due to an increase in the number of choleragen receptors and not to some other effect of ganglioside treatment on the cell membrane,

Table 7. Reversibility of inhibition of choleragen activation of adenylate cyclase by G_{M1}-oligosaccharide[a]

Incubation conditions	HeLa cells	C6 cells
	adenylate cyclase (%)	choleragen-activated
1 hr at 37 °C with toxin	100	100
10 min at 4 °C with toxin, then 2 hr at 37 °C with G_{M1}-oligosaccharide	25	4
10 min at 4 °C with toxin, then 1 hr at 37 °C with G_{M1}-oligosaccharide then 1 hr at 37 °C without	43	15[b]

[a] Cells were incubated with and without 10 nM choleragen for 10 min at 4 °C at which time G_{M1}-oligosaccharide was added to some of the samples (20 μM for HeLa and 100 μM for C6 cells) and all were incubated at 37 °C. After 1 hr, 10 nM toxin was added to some of the samples not yet exposed to the toxin. Ten volumes of ice cold saline were added to some of the samples containing G_{M1}-oligosaccharide, and these were centrifuged at $600 \times g$ for 3 min. The cells were suspended in fresh medium and incubated at 37 °C. After 2 hr, all of the samples were assayed for adenylate cyclase activity as described in Materials and Methods. Activities have been corrected for basal activities.

[b] Activation in C6 cells exposed to 20 pM choleragen was 16%.

Table 8. Effect of ganglioside treatment of rat glial C6 cells on ability of G_{M1}-oligosaccharide to inhibit choleragen binding and action[a]

Ganglioside treatment[b]	Choleragen receptors[c]	Effect of G_{M1}-oligosaccharide on	
		Inhibition of choleragen activated cyclase (%)	Displacement of bound ^{125}I-Choleragen (%)
None	19.0	92.3	96.3
G_{M3}	18.4	97.5	94.9
G_{M2}	22.1	91.6	91.3
G_{D1a}	37.9	95.4	86.5
G_{M1}	672	0	15.1

[a] C6 cells were incubated with 0.5 μM of the indicated gangliosides for 1 hr at 37 °C, washed extensively, and harvested. The effect of G_{M1}-oligosaccharide (50 μM) on activation of adenylate cyclase by choleragen and displacement of ^{125}I-choleragen was determined as described in Tables 4 and 5. All values are the mean of triplicate determinations.

[b] *See* Fishman (1980) for ganglioside structures.

[c] fmol of ^{125}I-choleragen specifically bound per mg cell protein.

C6 cells were treated with several other gangliosides which do not increase choleragen binding (*see* Fishman, 1980). Activation of adenylate cyclase by choleragen in cells treated with G_{M3}, G_{M2} and G_{D1a} was effectively blocked by G_{M1}-oligosaccharide (Table 8). In addition, the oligosaccharide displaced bound choleragen from cells treated with these gangliosides and the extent of displacement was similar to that observed in cells not treated with gangliosides. In contrast, G_{M1}-treated cells, which bound increased amounts of toxin, were resistant to the effects of G_{M1}-oligosaccharide (Table 8). We also examined the specificity of G_{M1}-oligosaccharide. When neuramin lactose was substituted for G_{M1}-oligosaccharide, no inhibition of cyclase activation or displacement of bound choleragen was observed (data not shown).

Discussion

Although choleragen had a similar affinity for the various cell lines that we examined, we found large differences in the amounts of toxin that could bind to these cells. Presumably this reflects the different amounts of G_{M1} present in these cells. Previous studies indicated that no G_{M1} could be detected chemically in HeLa (Simmons, et al., 1975; Fishman & Atikkan, 1979) and rat glial C6 cells (Dawson et al., 1971; Robert, Rebel & Mandel, 1977); but N18 neuroblastoma cells contained large amounts of this ganglioside (Dawson & Stoolmiller, 1976; Duffard et al., 1977). Small amounts of G_{M1} were detected in Friend

erythroleukemic cells (Fishman & Atikkan, 1979) but not in human lymphocytes (P.H. Fishman, *unpublished observations*). Although some investigators have argued that the absence of chemically detectable amounts of G_{M1} in choleragen-sensitive cells indicates that this ganglioside is not the toxin receptor (Kanfer, Carter & Katzen, 1976), the use of sensitive techniques has in fact demonstrated that G_{M1} is present in such cells (Pacuszka, Moss & Fishman, 1978). We also were able to create new toxin binding sites on HeLa and C6 cells by incubating them with G_{M1}.

The ability of G_{M1}-oligosaccharide to block the activation of adenylate cyclase in cells preincubated with choleragen varied among the different cell lines but correlated with their densities of toxin receptors. Thus HeLa, C6 glial, and human lymphocytes with few receptors were affected by the oligosaccharide, and Friend erythroleukemic and N18 neuroblastoma cells with many receptors were not affected. By incubating HeLa and C6 cells in medium containing G_{M1} and thus increasing the number of toxin receptors per cell, we could prevent the inhibitory effect of the oligosaccharide. The inhibition of choleragen activation of adenylate cyclase by G_{M1}-oligosaccharide also correlated with its ability to displace bound toxin from the cells. When additional toxin binding sites were created on HeLa cells, the diplacement of bound toxin from the cells by the oligosaccharide decreased as the receptor density increased.

Both inhibition of adenylate cyclase activation and displacement of bound choleragen by G_{M1}-oligosaccharide dependend on the time and temperature of the preincubation phase with the toxin. The oligosaccharide was an effective inhibitor even when cells were preincubated for up to 1 hr with the toxin at 4° C. As the preincubation temperature was increased, there was a sharp transition in these two effects of the oligosaccharide at 18 °C. Below this temperature, inhibition and displacement were maximum; above 18 °C, activation of adenylate cyclase increased and displacement of bound toxin decreased as the temperature rose. When the cells were incubated with toxin at 37 °C, inhibition of cyclase activation and displacement of bound toxin by the oligosaccharide decreased rapidly with time.

It would appear that a large part of the inhibitory effect of G_{M1}-oligosaccharide on activation of cyclase by choleragen is due to its ability to displace bound toxin. We observed, however, that in the presence of even 100 μM oligosaccharide, 5% of the toxin remained bound to HeLa and C6 cells and less than 10% of the cyclase was activated. With human lymphocytes, there was no activation even when 20% of the toxin remained bound. This suggested that the bound toxin was unable to activate adenylate cyclase in the presence of the oligosaccharide. We were able to demonstrate this by diluting out the oligosaccharide and obtaining the expected degree of activation. Thus the choleragen remaining bound to the cells in the presence of G_{M1}-oligosaccharide is in a form incapable of activating adenylate cyclase until the oligosaccharide is removed.

Our results are consistent with the following model for the mechanism of action of choleragen. The toxin initially binds to one G_{M1} molecule on the cell surface. If the density of membrane receptors is low, then at temperatures where the membrane is sufficiently fluid, the toxin-receptor complex moves laterally in the plane of the membrane or other G_{M1} molecules move into its vicinity[3]. Both types of movement have been observed by photobleaching recovery techniques (Schlessinger et al., 1977; Reidler, et al., 1978). With increasing time, the toxin binds to several receptors and becomes stably attached to the membrane in a form that can activate adenylate cyclase. At this point, our model is similar to that proposed by Bennett et al. (1976)[4]. If the receptor density, however, is high, multivalent binding occurs rapidly, even at temperatures where the membrane is rigid and no lateral diffusion of receptors or toxin-receptor complexes is necessary to obtain a stably bound form of choleragen, which can activate adenylate cyclase.

Multivalent binding of choleragen to several membrane receptors may orient the toxin, which appears to be asymmetric (van Heyningen, 1977; Gill, 1977), in proper relationship to the plane of the membrane. In addition, binding of the B protomer to the oligosaccharide chain of G_{M1} induces a perturbation in the toxin molecule which is maximum when 5 molecules of the oligosaccharide are bound to each choleragen molecule (Fishman et al., 1978). Such a change in the toxin structure may permit the A protomer to come in contact with the membrane and separate from the B protomer. If choleragen binds to only

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[3] The increased amount of choleragen bound to HeLa and C6 cells at 4 °C compared to that at 37 °C supports this model. At the lower temperature, each toxin molecule may be binding to fewer receptors than at the higher temperature and thus the same number of receptors could accommodate more toxin molecules.

[4] The model of Bennett et al. (1976) that lateral mobility of toxin-receptor complexes are involved in activation of cyclase is based mainly on observations of Craig & Cuatrecasas (1975). They found that capping of choleragen in lymphocytes paralleled activation of adenylate cyclase and both processes were inhibited by NaN_3. Revesz & Greaves (1975), however, observed that lymphocytes pretreated with G_{M1} did not cap. Both NaN_3 and G_{M1}-oligosaccharide inhibited activation of adenylate cyclase by choleragen in control but not in G_{M1}-treated lymphocytes (P.H. Fishman, *unpublished observations*).

two G_{M1} receptors, it may remain stably attached to the membrane in the presence of G_{M1}-oligosaccharide but not be properly oriented to allow the A protomer to interact with the membrane. It is important to note that the choleragen protomers do not dissociate in the presence of the oligosaccharide (Fishman et al., 1978). Studies on the interaction of choleragen with artificial lipid membranes containing G_{M1} indicated that toxin binding caused a perturbation in the lipid membrane (Moss et al., 1976; Moss et al., 1977); this may be a consequence of the clustering of G_{M1} molecules in a small region of the membrane. Such an alteration in membrane structure may facilitate the penetration of the A protomer into the lipid bilayer where it can come in contact with the adenylate cyclase complex.

Thus, multivalent binding of choleragen to G_{M1} molecules on the cell surface appears to be an essential step in the mechanism of action of the toxin. In contrast, lateral movement of toxin-receptor complexes appears not to be essential for choleragen action and may be necessary only to achieve multivalent binding in cells with a low density of toxin receptors[5]. Finally, the technique developed in the present study may be useful for examining the effects of temperature and membrane fluidity on both lateral and transmembrane mobility of toxin and receptor components and the subsequent activation of adenylate cyclase.

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