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Mobility of cholera toxin receptors on rat lymphocyte membranes

(immunofluorescence/cap formation/adenylate cyclase/gangliosides/ganglioside-agarose)

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ABSTRACT **Fluorescein-labeled cholera toxin binds detectably to 40–60% of rat mesenteric lymph node cells and induces a temperature-dependent redistribution (patch and cap formation) of cell surface toxin receptors. The redistribution is inhibited by several "metabolic," "microtubule," and "microfilament" inhibitors, by concanavalin A, and by anti-cholera toxin IgG. Various studies indicate that cholera toxin is at least bivalent, and that this property may be related to both the induction of receptor redistribution and to the activation of adenylate cyclase. Membrane components which are probably identical to the sialo-glycolipid, G_{M1} ganglioside, appear to be mobile in the plane of the membrane. The possible role of toxin multivalency and receptor mobility in the mechanism of toxin action is considered.**

Cholera toxin (choleragen), a protein produced by *Vibrio cholerae*, activates adenylate cyclase [EC 9.6.1.1; ATP pyrophosphate-lyase (cyclizing)] in all cell types studied (1). Choleragen binds selectively and with high affinity to cell surface ganglioside G_{M1} (2–7). Binding is followed by a lag of 20–60 min before activation of adenylate cyclase (8–11) or of other biological activities (6, 12, 13) is detectable. To obtain more information on the manner by which the toxin–receptor complex, once formed, might produce biological effects, we have studied the interaction of fluorescein-labeled cholera toxin (F-CT) with rat lymphocytes.

MATERIALS AND METHODS

Choleragen was obtained from Dr. C. E. Miller, choleragenoid from Dr. R. A. Finkelstein, gangliosides G_{M1} and G_{M2} from Supelco, and G_{D1b} and G_{A1} from Dr. Peter Fishman. Rabbit anti-cholera toxin was obtained 3 weeks after subcutaneous immunization with 100 μg of toxin emulsified in complete Freund's adjuvant (Difco). [α-^{32}P]ATP was synthesized (11, 14) and purified on DEAE-cellulose. ^{125}I-Labeled toxin, 30–50 μCi/μg (6), and ganglioside-Sepharose derivatives (15) have been described. Fluoresceinated toxin and choleragenoid were prepared by the method of Cebra and Goldstein (16). Membrane staining of viable lymphocytes with F-CT and examination of stained cells by fluorescence microscopy were done by standard methods (17).

RESULTS

Binding Properties of F-CT. F-CT retains 80% of its biological activity (lipolysis in isolated fat cells, ref. 6). F-CT (10 nM), incubated with viable lymphocytes (10^7 in 1 ml) at 0° for 30 min, binds to the surface of some of the cells. The binding can be blocked by preincubating the cells with unlabeled choleragen or choleragenoid or by preincubating F-CT with anti-toxin or ganglioside G_{M1} (Table 1). Other gangliosides structurally related to G_{M1} (i.e., G_{M2}, GA_1, Gd1b, and GT_1) are ineffective. Thus, F-CT retains the binding and biological properties of native toxin (6).

Mobility of Lymphocyte Toxin–Receptor Complexes. If lymphocytes incubated at 0° with F-CT are warmed (37°) for 20–30 min, the surface fluorescence, which is initially diffuse and patchy (Fig. 1a), redistributes and becomes concentrated preferentially at one pole of the cell (Fig. 1b and c; Table 2), indicating mobility in the plane of the membrane. Binding and redistribution are detectable with 10^{-10} M F-CT (Fig. 2). Since this concentration is well within the linear dose response range for toxin activation of adenylate cyclase in these cells, the binding and redistribution events described most likely reflect the properties of the functionally relevant toxin receptors.

Multivalency of Cholera Toxin. Redistribution of cell surface glycoproteins by antibodies (17, 18) and lectins (19) depends on the multivalent nature of the capping agents. Since lymphocyte-bound cholera toxin can crosslink the cells

Table 1. Specificity of F-CT binding to lymphocyte membranes

Pretreatment of cells	Stain	Pretreatment of F-CT	% Stained cells Exp. 1	% Stained cells Exp. 2
None	10 nM F-CT	—	59	47
0.1 μM CT	10 nM F-CT	—	7	
1 μM CT	10 nM F-CT	—	0	
0.1 μM CG	10 nM F-CT	—	3	
1 μM CG	10 nM F-CT	—	0	
None	—	Anti-CT	0	0
None	—	G_{M1}		0
None	—	G_{d1b} (or G_{A1}, G_{M2}, G_{T1})		47–50

In Exp. 1, 1×10^7 lymphocytes were incubated (0°, 30 min) in 1 ml of Hanks' solution containing native cholera toxin (CT) or choleragenoid (CG); 10 nM F-CT (10 μl) was added and the incubation continued for another 30 min at 0°. In Exp. 2, 2 μg of F-CT (20 μl) was incubated with 20 μl of the indicated ganglioside (500 μg/ml) for 60 min, 24° before addition (20 μl) to lymphocytes (as in Exp. 1). 200 to 500 cells were scored per slide in all experiments involving cell counts.

Abbreviation: F-CT, fluorescein-labeled cholera toxin.

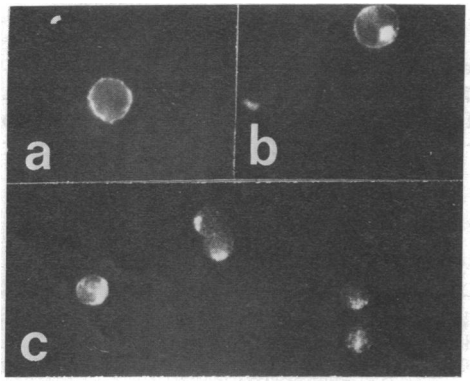

FIG. 1. Patterns of fluorescence on rat lymphocytes incubated in F-CT. (a) Lymphocytes (10^7) were incubated (0°, 30 min) in 1 ml of Hanks' solution containing 1 μg of F-CT. The cells were washed in cold Hanks' solution before fixation and mounting. (b and c) Lymphocytes stained at 0° were washed and incubated at 37° for 20–30 min before fixing. All 37° incubations were carried out in a humidified incubator equilibrated with 95% air–5% CO_2.

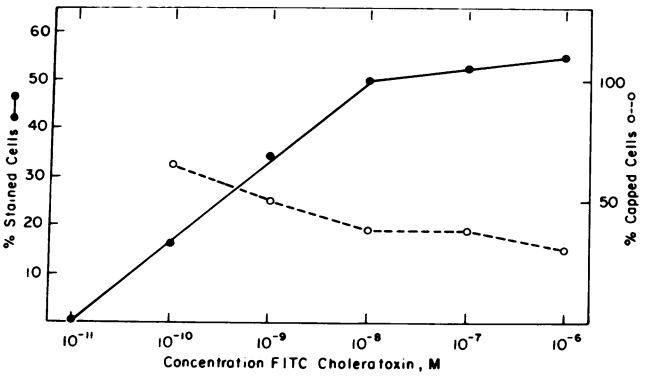

FIG. 2. Concentration dependence of binding and redistribution of F-CT. Binding and redistribution of F-CT were as described in the text and in Fig. 1. ●, % of stained cells; ○, % of cells with caps.

to agarose beads derivatized covalently with gangliosides (Fig. 3a and b), toxin too, is at least bivalent. The binding of toxin-treated lymphocytes to ganglioside-Sepharose is specific since untreated lymphocytes do not bind and ganglioside G_{M1} blocks the binding (Table 3).

Effects of Anti-Cholera Toxin IgG. Anti-cholera toxin IgG added at 0° to cells treated with F-CT at 0° inhibits redistribution and patching seen on warming to 37° (Fig. 4). This inhibition is selective for toxin receptors since capping of surface immunoglobulin, detected by rhodamine-labeled anti-rat IgG, is not affected by toxin and anti-toxin IgG (data not shown). The inhibition is dependent on the dose of anti-toxin IgG used (Fig. 4). The fact that anti-toxin inhibits capping of toxin–receptor complexes may indicate that these complexes are too far apart to be crosslinked by the antibody (20). This is supported by the observation that goat anti-rabbit Fc can partially reverse the inhibitory effect of anti-toxin IgG (Table 4). Anti-toxin blocks the ability of toxin-treated cells to crosslink to ganglioside-Sepharose (Table 3), suggesting that anti-toxin can reduce the effective valence of the cell-bound toxin. Thus, it may be proposed that crosslinking of toxin receptors is required for their redistribution and that toxin multivalency is essential for such reorganizations.

Effect of Agents Which Restrict Capping. Metabolic poisons which inhibit capping of membrane glycoproteins (17, 21, 22) also inhibit redistribution of toxin receptors (Table 5). Depending on the system, microtubule and microfilament inhibitors have been reported to inhibit capping, or to have no effect (23, 24–27). The mobility of toxin receptors is inhibited substantially by all the microtubule and microfilament inhibitors tested (Table 5) and by concanavalin A, which inhibits mobility of various lymphocyte surface antigens (19, 22).

Table 2. Effect of temperature on the distribution of F-CT on rat lymphocytes

	0°		37°	
Exp. no.	% Stained cells	% Stained cells that capped	% Stained cells	% Stained cells that capped
1	26	4	36	67
2	47	0	53	73
3	13	0.4	31	60
4	19	2	34	53
5	37	0	36	52

The experiments were performed as stated in the legend of Fig. 1. Different preparations of F-CT were used; the A 280/515 ranged from 0.66 to 1.8. Cells were counted as capped if the bulk of the fluorescence was concentrated over one-half, or less, of the cell surface.

Relationship Between Multivalent Binding of Toxin and Activation of Adenylate Cyclase. Various studies have suggested that crosslinking of cell surface receptors by acti-

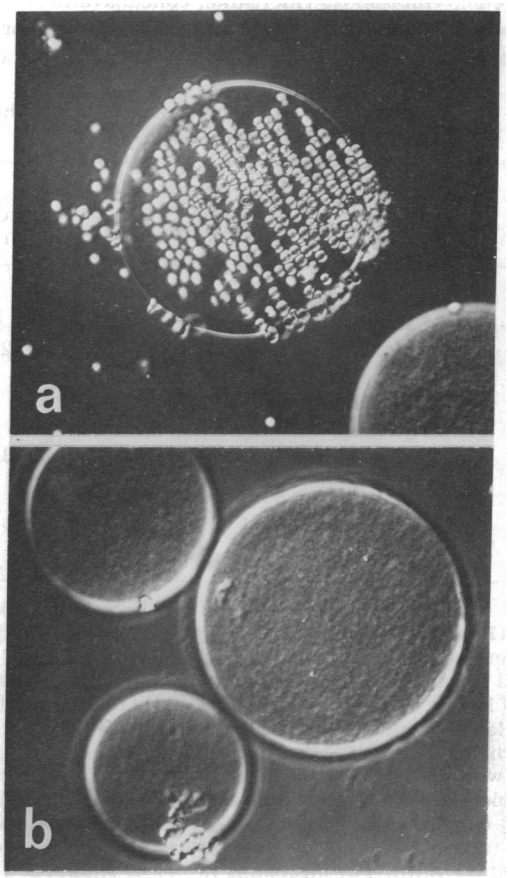

FIG. 3. Binding of toxin-treated (a) or untreated (b) lymphocytes to ganglioside-Sepharose beads. For details see Table 3.

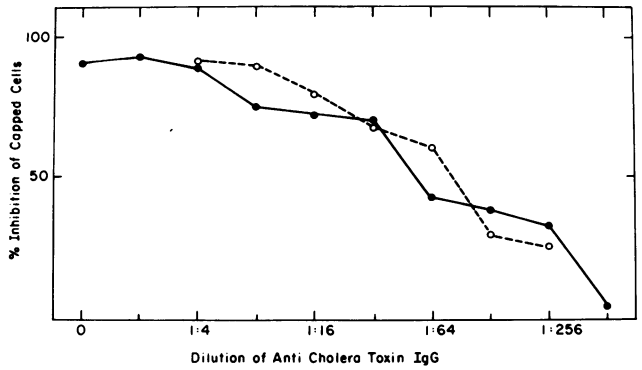

FIG. 4. Inhibition of F-CT capping by rabbit anti-toxin IgG. Lymphocytes stained with F-CT at 0° were washed and incubated (30 min, 0°) with rabbit anti-CT IgG (28 mg/ml, undiluted). After incubation for 20 to 30 min at 37°, the cells were washed and assessed for F-CT cap formation. Two independent experiments are presented.

vating molecules, such as mitogens (19, 31, 32), antigens (33, 34), and anti-immunoglobulins (17, 35), might be integral to activation or repression of particular cell functions. The relationship between the time courses of toxin receptor redistribution and activation of adenylate cyclase (Fig. 5) shows that there is substantial reorganization of the toxin receptors during the lag period. The possible relationship between toxin capping and activation of cyclase was examined by testing the effect of inhibitors of capping on toxin activation of cyclase (Table 6). The effects of these inhibitors can be grouped into three categories: those which (*i*) have little or no effect on either basal or toxin-stimulated cyclase activity (colchicine, vinblastine, vincristine, concanavalin A), (*ii*) affect both activities (NaF, Dnp), and (*iii*) have little or no effect on basal but markedly reduce toxin-stimulated cyclase

Table 3. Binding of cholera toxin-treated rat lymphocytes to ganglioside-Sepharose beads

Exp.	Addition to G-beads	Cells/bead ≤5	Cells/bead >20	Beads with >20 cells*
		no. of beads		%
1	RMLN	526	5	0.8
	CT-RMLN	342	150	28
	G_{M1} blocked CT-RMLN	430	31	6
2	RMLN	474	1	0.2
	CT-RMLN	679	135	13
	G_{M1} blocked CT-RMLN	429	32	6
	Anti-toxin CT-RMLN	578	12	2

One × 10^7 lymphocytes in 1 ml of Hanks' solution were incubated (15 min, 37°) with (CT-RMLN) or without (RMLN) 1 μg of toxin and washed in cold Hanks' solution. One milliliter of packed, washed ganglioside-Sepharose beads (G-beads) were suspended in 2 ml of Hanks' solution and 200 μl was added to 1 × 10^7 cells in 100 μl of Hanks' solution. After incubating at 37° for 40 to 60 min followed by chilling to 0°, the suspensions were diluted (0°) and the beads were allowed to settle out several times. Toxin-treated cells were blocked with G_{M1} (G_{M1}-blocked CT-RMLN) by incubating (0°, 40–60 min) with 10 μg/ml of G_{M1} before adding to the beads. Toxin-treated cells were blocked with anti-toxin IgG (anti-toxin blocked CT-RMLN) by incubating (0°, 40–60 min) in 50 μl of anti-toxin IgG (14 mg/ml) before exposure to the beads.
* 500–600 beads were counted.

Table 4. Reversal of anti-toxin inhibition by anti-Fc (IgG)

Treatment of CT stained cells: Anti-CT	Anti-Fc	Time at 37°	% Capped cells
–	–	20	69
+	–	20	5
+	+	20	25
+	+	40	30
+	+	60	36

Lymphocytes were stained with F-CT and treated with anti-CT IgG as described in Fig. 4. After washing, cells were treated with the IgG fraction of goat anti-rabbit Fc (IgG), 0°, 30 min before being warmed at 37° for various times.

(NaN_3 and cytochalasin B). The first inhibitors are not informative since they only partially depress toxin mobility (Table 5); sufficient receptor rearrangement or multipoint attachment may still occur to permit enzyme activation. Group 2 inhibitors are not useful since their alteration of basal cyclase activity indicates unrelated effects on membrane properties. Group 3 inhibitors, however, may *preferentially* affect membrane events involved in toxin activation of cyclase. Although both cytochalasin B and NaN_3 partially reduce toxin mobility and rearrangement and partially inhibit toxin activation, lack of knowledge concerning their site(s) of action frustrates interpretations of the interdependence of these two effects.

The effect of multivalent binding and receptor redistribution on cyclase activation was better analyzed with anti-toxin IgG, since it is known that the suppression of patching and capping results from specific interaction of the antibody with cell-bound toxin. When anti-toxin IgG is used to restrict redistribution of toxin receptors, enzyme activation is mark-

Table 5. Effect of agents that restrict capping of membrane proteins on the redistribution of cholera toxin receptors

Reagent	% Inhibition of capped cells
Me_2SO, 0.05%	2
Vinblastine, 25 μM	60
Vincristine, 2.5 μM	56
Colchicine, 5 μM	58
Cytochalasin B, 20 μg/ml	23
Concanavalin A, 10 μg/ml	80
WGA, 10 μg/ml	0
Dnp, 10 mM	98
NaN_3, 10 mM	96
NaF, 10mM	78
None	0 = 60% capped cells

Lymphocytes (1 × 10^7 cells per ml) were incubated (30 min, 37°) in 1 ml of Hanks' solution containing the indicated inhibitors. The cells were chilled (0–4°) and 1 μg of F-CT (10 μl) was added. After incubating for 30 min at 0°, the cells were washed, resuspended in 1 ml of Hanks' solution, and incubated for 20–30 min at 37°. All wash and incubation media contained the appropriate inhibitor. The plant alkaloids were dissolved in neat dimethylsulfoxide (Me_2SO) which was diluted to a concentration of 0.05% in the incubation. The percentage of viable cells ranged from 61 to 70% under all conditions. Treated cells bound from 94–108% of the amount of ^{125}I-labeled toxin bound by control cells. Similar results were obtained in two other experiments.
Con A, concanavalin A; WGA, wheat germ agglutinin.

Table 6. Effect of inhibitors of capping on activation of adenylate cyclase

	Adenylate cyclase activity*					
	Exp. 1		Exp. 2		Exp. 3	
Addition	Basal	Toxin	Basal	Toxin	Basal	Toxin
None	5.5	67.2	3.4	38.6	11.8	103.0
NaN_3, 10 mM	9.2	31.7	3.4	16.9	13.6	45.7
NaF, 10 mM	34.6	39.1	31.0	27.0	24.9	28.4
Dnp, 10 mM	0.15	3.1	0.4	1.6	4.6	4.8
Colchicine, 5 μM	13.8	75.5	4.5	39.8	17.8	77.6
Con A, 25 μg/ml	12.7	52.0			17.2	89.6
0.05% Me_2SO	8.0	78.0	3.0	25.4	9.6	67.6
Vinblastine, 2.5 μM (0.05% Me_2SO)	14.9	75.8	4.2	26.3	15.7	101.0
Vincristine, 25 μM (0.05% Me_2SO)	7.1	85.0	5.4	37.0	6.4	65.2
Cytochalasin B, 20 μg/ml (0.05% Me_2SO)	8.2	42.6†	3.9	16.5	13.4	33.4

Lymphocytes (1 to 4 × 10^8) were incubated (30 min, 37°) in 2 ml of Hanks' solution with 0.1% albumin and the indicated inhibitor. After 30 min, 3 μg of toxin was added. Incubation was continued at 37° for 1.5 hr, and the cells were washed with cold Hanks' solution. For adenylate cyclase assays the cells were suspended in 0.5 ml of Hanks' solution containing 10 μg of micrococcal nuclease, diluted with 2 ml of cold 25 mM Tris, pH 7.5, and homogenized (Polytron, Brinkmann). The homogenate was diluted with 7 ml of Tris buffer, and centrifuged. The pellets were resuspended in 100–300 μl of cold 25 mM Tris, pH 7.5; 20-μl samples were used. The assay medium contained (volume, 0.1 ml) 40 μM ATP, 100 μM GTP, 5 mM $MgCl_2$, 10 mM aminophylline, 2.5 mM phosphoenolpyruvate, 25 μg/ml of pyruvate kinase, 0.1% albumin, 25 mM Tris·HCl, pH 7.6, and 2–4 million cpm of [α-^{32}P]ATP (20–60 Ci/mmol). After 15 min at 37° the assay was terminated by boiling. Cyclic [^{3}H]AMP was added for analysis of recovery, and cyclic AMP was isolated on neutral alumina columns (29). Protein was estimated by the method of Lowry *et al.* (30).

* pmol of cAMP/15 min per mg of protein; mean of duplicate determinations, 600–2000 cpm/pmol.

† Cytochalasin B at 1 μg/ml.

edly inhibited (Table 7). However, if anti-toxin is added after considerable toxin–receptor reorganization has occurred, the inhibitory effect is diminished. The effect of anti-toxin IgG is abolished by preabsorption with choleragenoid, and nonimmune rabbit IgG has no effect on cyclase activity. Anti-toxin IgG does not inhibit by removing toxin from the cells in excess of the amount that normally dissociates at 37°. F-CT stained cells treated with anti-toxin remain brightly fluorescent.

DISCUSSION

The effect of anti-cholera toxin IgG demonstrates that the interaction of anti-toxin with cell-bound toxin can partially

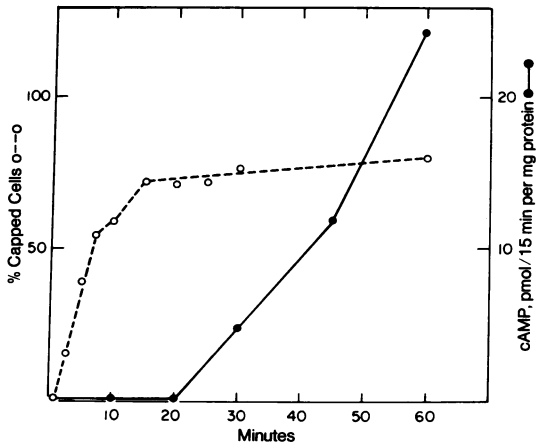

FIG. 5. Relationship between the time courses of cholera toxin redistribution and activation of adenylate cyclase. Adenylate cyclase activity was assayed on particulate fractions prepared from 1 to 4 × 10^8 cells at various times after incubation (37°) with 2 μg/ml of toxin (see Table 1). Capping experiments were as described in Fig. 1. ●, Toxin-stimulated cyclic AMP production minus basal activity, pmol of cyclic AMP/15 min per mg of protein; O, % of cells with caps.

inhibit toxin activation of adenylate cyclase. Since partial inhibition still occurs after incubation with toxin for 30 min (37°) (i.e., after the lag phase), all the cell-bound toxin does not form irreversible active complexes with cyclase (36) at the same time. Apparently, transformation from an inactive, neutralizable state to an active, nonneutralizable form occurs during, and extends somewhat beyond, the lag phase. In toxin-induced erythema and induration, some neutralization was observed even when anti-toxin was added as long as 6 hr later (37). The seemingly contradictory reports (reviewed in ref. 1) that the *biological* effects (e.g., fluid loss in gut) are

Table 7. Effect of anti-cholera toxin on activation of adenylate cyclase

		Adenylate cyclase activity*	
Exp.	Addition	Toxin stimulated	Basal
1	None	11.1	2.2
	Anti-CT IgG (30 min, 0°)†	1.8	1.8
	Anti-CT (after 30 min, 37° C)	6.8	3.1
	N-R IgG (0°)	11.3	N.D.
	N-RIgG (after 30 min, 37°)	9.6	N.D.
2	None	99.1	13.2
	Anti-CT IgG (30 min, 0°)†	62.6	22.8
	Anti-CT (after 30 min, 37°)‡	71.1	21.4
	Toxoid adsorbed anti-CT (0°)	96.9	N.D.
3	None	33.8	18.7
	Anti-CT IgG (30 min, 0°)†	18.6	24.9
	Anti-CT (after 30 min, 37°)‡	28.1	20.3

* pmol/15 min per mg of protein; mean of duplicate determinations, 600–2000 cpm/pmol; as described in Table 5.

† Cells (2 × 10^8) previously incubated with toxin (1.5 μg/ml, 30 min, 0°, in 1.8 ml) were washed and incubated (30 min, 0°) with 200 μl of anti-CT IgG (28 mg/ml) and then transferred to 37° for 1.5 hr.

‡ Cells previously incubated with toxin (1.5 μg/ml, 30 min, 0°) and washed were transferred to 37°. After 30 min, 200 μl of anti-toxin IgG was added, the incubation continued at 37° for 1 hr, and the cells were washed.

not reversible if anti-toxin is added as late as 5 min after toxin might be explained in quantitative terms if the residual, unneutralized toxin still generates enough cyclic AMP to produce the biological response.

The kinetics of toxin activation of cellular processes (6) and of adenylate cyclase (11) suggest that the toxin binds to the membrane in a biologically inactive form, and that during the lag phase it is converted to an active form (6, 11). It was speculated (6, 11, 36) that this transformation might involve lateral mobility of toxin–receptor complexes in the plane of the membrane. This report demonstrates that toxin–receptor complexes are indeed mobile in the plane of the membrane, that toxin is at least bivalent, and that the latter property may be related to the observed toxin–receptor redistribution. Anti-toxin reduces the effective valence of cell-bound toxin and it inhibits CT patching, capping, and activation of adenylate cyclase. It is suggested that anti-toxin inhibits activation of cyclase by interfering with a rearrangement normally required to convert the inactive toxin–receptor complex to the active form.

Since choleragenoid, a biologically inactive competitive antagonist of toxin action which lacks the 36,000 molecular weight subunit (6), can induce receptor redistribution as well as toxin (data not shown), receptor crosslinking and rearrangement are not sufficient conditions for stimulation of cyclase. It is possible that multivalent binding places a strain on the toxin molecule, causing it to dissociate into its "active" (36,000 molecular weight) and "binding" subunits. Since the free "active" subunit is quite hydrophobic, it might be readily incorporated into the substance of the membrane, where it may interact with and activate adenylate cyclase (26, 28).

The lateral mobility of toxin receptors might reflect the properties of membrane ganglioside molecules (2–6). Since it is unlikely that the polar head group of a single ganglioside provides more than one site of attachment for choleragen, it is difficult to see how toxin could induce capping by crosslinking gangliosides into a network, a mechanism thought to operate in analogous redistributions of surface glycoproteins. It is equally difficult to see how gangliosides might be connected to submembrane colchicine-binding proteins thought to be involved (22) in surface protein translocations since the hydrophobic portion of gangliosides is too short (30 Å) to span the membrane. It is possible that gangliosides are tightly associated with membrane proteins, and bridging of any two glycolipids on separate proteins may automatically cause all the glycolipids on each of the two proteins to behave as a single unit. In fact, recent evidence (38, 39) suggests that in erythrocyte ghosts glycolipids may be specifically associated with membrane proteins. Whether such proteins are associated with a submembrane protein network is not known.

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1. Finkelstein, R. A. (1973) *CRC Crit. Rev. Microbiol.* **2,** 533–623.
2. Holmgren, J., Lonnroth, I. & Svennerholm, L. (1973) *Infect. Immun.* **8,** 208–214.
3. Holmgren, J., Lonnroth, I., & Svennerholm, L. (1973) *Scand. J. Infect. Dis.* **5,** 77–78.
4. King, C. A. & van Heyningen, W. E. (1973) *J. Infect. Dis.* **127,** 639–647.
5. Pierce, N. F. (1973) *J. Exp. Med.* **137,** 1009–1023.
6. Cuatrecasas, P. (1973) *Biochemistry* **12,** 3547–3558; 3558–3566; 3567–3577; 3577–3581.
7. Hollenberg, M. D., Fishman, P. H., Bennett, V. & Cuatrecasas, P. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 4224–4228.
8. O'Keefe, E. & Cuatrecasas, P. (1974) *Proc. Nat. Acad. Sci. USA* **71,** 2500–2504.
9. Kimberg, D. V., Field, M., Johnson, J., Henderson, A. & Gershon, E. (1971) *J. Clin. Invest.* **50,** 1218–1230.
10. Guerrant, R. L., Chen, L. C. & Sharp, G. W. G. (1972) *J. Infect. Dis.* **125,** 377–384.
11. Bennett, V. & Cuatrecasas, P. (1975) *J. Memb. Biol.* **22,** 1–28; 29–52
12. Carpenter, C. C. J. & Greenough, W. B., III (1968) *J. Clin. Invest.* **47,** 2600–2607.
13. Wolff, J., Temple, R. & Cooke, G. H. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 2741–2744.
14. Symons, R. H. (1968) *Biochim. Biophys. Acta* **155,** 609–610.
15. Cuatrecasas, P., Parikh, I. & Hollenberg, M. D. (1973) *Biochemistry* **12,** 4253–4263.
16. Cebra, J. J. & Goldstein, G. (1965) *J. Immunol.* **95,** 230–245.
17. Taylor, R. B., Duffus, W. P. H., Raff, M. C. & de Petris, S. (1971) *Nature New Biol.* **233,** 225–229.
18. de Petris, S. & Raff, M. C. (1974) *Eur. J. Immunol.* **4,** 130–137.
19. Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A. & Edelman, G. M. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1012–1016.
20. Unanue, E. R. & Karnovsky, M. J. (1973) *Transplant. Rev.* **1973,** 184–210.
21. Edidin, M. & Weiss, A. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 2456–2459.
22. Yahara, I. & Edelman, G. M. (1972) *Proc. Nat. Acad. Sci. USA* **69,** 608–612.
23. Edelman, G. M., Yahara, I. & Wang, J. L. (1973) *Proc. Nat. Acad. Sci. USA* **70,** 1442–1446.
24. de Petris, S. (1974) *Nature* **250,** 54–55.
25. Stackpole, C. W., Jacobson, J. B. & Lardis, M. P. (1974) *Nature* **248,** 232–234.
26. Cohen, H. J. & Gilbertson, B. B. (1975) *J. Clin. Invest.* **55,** 84–93.
27. Unanue, E. R., Karnovsky, M. J. & Engers, H. D. (1973) *J. Exp. Med.* **137,** 675–689.
28. Sayhoun, N. & Cuatrecasas, P. (1975) *Proc. Nat. Acad. Sci. USA* **72,** in press.
29. Ramachandran, J. (1971) *Anal. Biochem.* **43,** 227–239.
30. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
31. Greaves, M. F. & Bauminger, S. (1972) *Nature New Biol.* **235,** 67–70.
32. Lotan, R., Lis, H., Rosenwasser, A., Novogrodsky, A. & Sharon, N. (1973) *Biochem. Biophys. Res. Commun.* **13,** 1347–1355.
33. Feldman, M. & Basten, A. (1971) *J. Exp. Med.* **134,** 103–119.
34. Mitchison, N. A. (1972) *In Vitro.* **7,** 88–94.
35. Fanger, M. W., Hart, D. A., Wells, J. V. & Nisonoff, A. (1970) *J. Immunol.* **105,** 1484–1492.
36. Bennett, V. B., O'Keefe, E. & Cuatrecasas, P. (1975) *Proc. Nat. Acad. Sci. USA* **72,** 33–37.
37. Craig, J. P. (1970) *J. Infect. Dis.* **121,** Suppl.: S100–S110.
38. Ji, T. H. (1974) *J. Biol. Chem.* **249,** 7841–7847.
39. Springer, G. F., Adye, J. C., Bezkorovainy, A. & Jirgensons, B. (1974) *Biochemistry* **13,** 1379–1389.