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Comparison of the Action of *Escherichia coli* Enterotoxin on the Thymocyte Adenylate Cyclase-Cyclic Adenosine Monophosphate System to That of Cholera Toxin and Prostaglandin E_1

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Mouse thymocytes were used to compare mechanisms by which *Vibrio cholerae* and heat-labile *Escherichia coli* enterotoxins activate the adenylate cyclase-cyclic adenosine monophosphate (AMP) system. Both enterotoxins had their time-delayed increase in cyclic AMP neutralized by antisera to *V. cholerae* or *E. coli* enterotoxin, blocked by low concentrations of ganglioside G_{M1}, and destroyed by prior heating. Enterotoxin activation of adenylate cyclase was similarly affected. By contrast, prostaglandin E_1-mediated increases in cyclic AMP were not affected by specific antitoxins or gangliosides. Combination of maximal stimulatory doses of both enterotoxins did not produce additive increases in cyclic AMP. Wash experiments suggested that both enterotoxins bind rapidly and tightly to thymocytes at 37 C. However, lowering the incubation temperature to 8 C reduced the affinity of *E. coli* enterotoxin but not cholera toxin for thymocytes. Results suggest that heat-labile *E. coli* enterotoxin and cholera enterotoxin may activate the same adenylate cyclase enzyme by similar mechanisms.

Enterotoxins produced by pathogenic strains of *Escherichia coli* are now recognized as a probable cause of acute diarrhea in infants (11), adults (25), and animals (15). These enterotoxins can be either heat-stable or heat labile (8). Heat-labile *E. coli* enterotoxin (ECT) has been shown to be antigenically related to cholera toxin (CT) (14, 26) and may act via the adenylate cyclase-cyclic adenosine monophosphate (AMP) system like CT (7, 13, 19). However, major differences between the action of ECT and CT concerning onset (24) and duration (24) have been shown. In addition, certain experiments have suggested that the receptor sites for ECT and CT may be different (16). Therefore, a considerable amount of uncertainty as to the actual mechanisms of ECT action now exists.

The action of CT has been extensively studied in skin (16), gut (16, 20, 21), fat (6), and lymphatic (3, 4, 17) cells. Although the gut is the natural site of CT action, CT elicited a delayed activation of adenylate cyclase (3, 4, 20, 21) and was specifically inhibited by ganglioside G_{M1} (6, 16, 17) in every tissue or cell type studied. In lymphocytes, the action of CT on the adenylate cyclase-cyclic AMP system is distinct from that of prostaglandin (PG) E_1 (3). Accordingly, we have attempted to compare the action of partially purified heat-labile ECT to the well-studied and diverse actions of PGE_1 and purified CT on thymocytes in the hopes of gaining a better understanding of the cellular and molecular mechanisms of heat-labile ECT action.

MATERIALS AND METHODS

Materials. *E. coli* strain 408-3 (serotype O78:H12) was isolated from the gastrointestinal tract of a patient with severe cholera-like diarrhea in Calcutta (25) and was generously provided by R. Bradley Sack, The Johns Hopkins University. Purified CT (lot 1071) was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by R. A. Finkelstein, Dallas, Tex., as previously described (9), and provided by Carl Miller, National Institutes of Health. Goat cholera antitoxin was a gift from R. Rappaport, Wyeth Laboratories. Antitoxin to ECT was obtained from rabbits immunized with strain 408-3 enterotoxin. Both antitoxins were heated for 30 min at 56 C before use. The ECT preparation was made from an 18-h culture that was incubated in syncase media at 37 C in a 50-liter fermentor. The culture was centrifuged at 16,000 rpm in a continuous flow centrifuge head and the supernatant was concentrated by Amicon XM-50 ultrafiltration. This concentrate was placed on a diethylaminoethyl-cellulose column equilibrated with 0.01 M phosphate buffer, pH 8.0. The fraction eluted with 0.3 M phosphate was

dialyzed against water and lyophilized. This fraction was used throughout the entire study and represents a 10- to 12-fold increase in purity (activity per milligram of protein) over the crude XM-50 concentrate determined by either the rabbit ileal loop or thymocyte cyclic AMP assay methods. The concentrations of ECT and CT employed in our experiments are expressed as milligrams of protein per milliliter. Protein concentrations were estimated by the Lowry method (18) by using bovine serum albumin as a standard.

[^{32}P]α-ATP (8 to 10 Ci/mmol) and cyclic [^{3}H]AMP (24 Ci/mmol) were obtained from New England Nuclear Corp. Adenosine triphosphate (ATP), cyclic AMP, creatine phosphate, and creatine phosphokinase were purchased from Calbiochem. Alumina (neutral, type WN-3) and bovine brain gangliosides (type II) were supplied by Sigma Chemical Co., St. Louis, Mo. Monosialogangliosides (G_{M1} and G_{M2}), disialoganglioside (G_{D1a}), and trisialoganglioside (G_{T1}) were purchased from Supelco. PGE_1 was kindly supplied by John Pike, The Upjohn Co. Male, C57BL/6 mice, 3 to 4 weeks old, were supplied by R. B. Jackson Laboratories, Bar Harbor, Me.

Preparation of thymocytes. Mice were killed by cervical spine dislocation. The thymuses were removed aseptically and cell suspensions were prepared by a mechanical procedure previously described (1). Thymocytes (3×10^7 cells per ml) were suspended in complete tissue culture media, RPMI 1640. Viability of thymocyte preparations ranged from 93 to 97% as assessed by trypan blue exclusion.

Assessment of thymocyte cyclic AMP content. After incubation at 37 C with theophylline and test agents for the appropriate time, cells were centrifuged at 1,000 rpm for 2 min at 4 C, and the cell pellets were immediately homogenized in 400 μliters of hot sodium acetate buffer (pH 4.0, 50 mM). After heating at 100 C for 10 min, the homogenates were cooled and centrifuged at 2,500 rpm for 15 min. Supernatants were removed and kept at −20 C until assay. Portions (50 and 25 μliters) of these supernatants were assayed directly for cyclic AMP content by the protein-binding method of Gilman (10) as modified by Mashiter et al. (22). The lower limit for detection of cyclic AMP was 0.5 pmol. The authenticity of the thymocyte cyclic AMP measured was demonstrated by its destruction by cyclic phosphodiesterase and by linearity of sample dilutions. Three or four determinations of each condition were made. Thymocyte cyclic AMP content is expressed as picomoles of cyclic AMP per 10^7 cells.

Preparation and assay of adenylate cyclase. After incubating thymocytes (20 ml) at 37 C for 60 min with test agents, three 0.5-ml portions were taken from each group for cyclic AMP determination and the remaining cells were pelleted by centrifugation. The cell pellet was resuspended in 10 ml of cold 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.6, 2.0 mM $MgSO_4$, 0.5 mM ethylenediaminetetraacetic acid, and 1.0 mM dithiothreitol. All subsequent tissue processing steps were performed at 4 C. The cells were gently homogenized. After adjusting the volume to 30 ml, the homogenate was passed through four layers of gauze and centrifuged at $3,000 \times g$ for 10 min. The $3,000 \times g$ pellet was resuspended and homogenized by hand in 0.5 ml of buffer.

The incubation mixture for determining adenylate cyclase activity was the same as previously described (29). The reaction was initiated by addition of the $3,000 \times g$ fraction (60 to 100 μg of protein) and was linear for 5 min at 30 C. The cyclic AMP formed was isolated and determined by the method of White and Zenser (28). Adenylate cyclase activity is expressed as picomoles of cyclic AMP formed per milligram of protein per 5 min. Using these assay conditions, cyclic phosphodiesterase activity, determined as described by Thompson and Appleman (27), was not detectable in the washed $3,000 \times g$ pellet. Six determinations of each condition were made.

RESULTS

Effect of enterotoxin concentration and incubation time on cyclic AMP accumulation in thymocytes. A 15- to 20-min delay preceded the increase in cyclic AMP mediated by both enterotoxins, whereas PGE_1 elevated cyclic AMP levels 10-fold higher than basal within 3 min (Fig. 1). Maximal responses were at 10 min for PGE_1 and at 30 min for both enterotoxins. The latter time was therefore chosen as the optimal incubation time in the experiments to follow. Cyclic AMP levels were still elevated 3 h after addition of these agents. As previously shown for CT, the delay period preceding activation by ECT was independent of enterotoxin concentration with either 16 or 32 μg of ECT per ml, requiring approximately 20 min for activa-

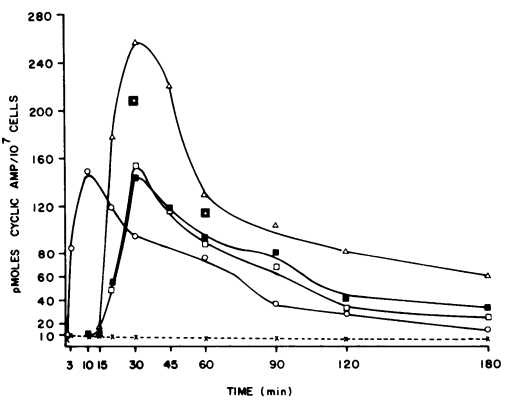

FIG. 1. *Time course of cyclic AMP accumulation in thymocytes. Thymocytes were incubated in the presence of theophylline, 10^{-2} M alone (×); PGE_1, 10^{-6} M (○); E. coli enterotoxin, 32 μg/ml (■) or 16 μg/ml (□); V. cholerae enterotoxin, 0.3 μg/ml (△); or E. coli enterotoxin, 32 μg/ml + V. cholerae enterotoxin, 0.3 μg/ml (◪).*

tion. Combination of maximal stimulatory doses of both enterotoxins incubated for 30 or 60 min failed to produce additive increases in cyclic AMP.

ECT elicited a dose-dependent increase in cyclic AMP (Fig. 2). The lowest effective stimulatory concentration of purified ECT was between 0.5 and 1 μg of enterotoxin protein per ml. By contrast, the lowest effective stimulatory concentration of CT was 3 ng/ml. Maximal ECT stimulation was achieved at approximately 16 μg/ml. Maximal doses of both enterotoxins heated at 95 C for 20 min were nonstimulatory.

Inactivation of enterotoxins by specific antitoxins. Preincubation of CT with *V. cholerae* antitoxin completely blocked the CT-induced accumulation of thymocyte cyclic AMP at a 10^{-4} dilution (Fig. 3). In addition, ECT-induced accumulation of cyclic AMP was completely blocked at a 10^{-3} dilution of antitoxin. *E. coli* antitoxin was much more effective in blocking the ECT- than CT-induced accumulation of cyclic AMP. Although a 10^{-3} dilution completely blocked the ECT response, only at 10^{-1} dilution was effective in blocking CT. Neither antitoxin modified the basal cyclic AMP level or the increase mediated by PGE_1. By using the rabbit ileal loop assay, we were also able to neutralize ECT with either antitoxin.

Inactivation of enterotoxins by specific gangliosides. Using purified gangliosides and submaximal concentrations of CT and ECT of similar potencies, we investigated the ability of gangliosides to inhibit enterotoxin action (Fig. 4). The order and approximate concentrations of ganglioside required to obtain half-maximal inhibition for CT are: G_{M1}, 0.002 μg/ml; G_{D1a}, 0.010 μg/ml; G_{M2}, 3 μg/ml; and G_{T1}, 20 μg/ml. For ECT the values are: G_{M1}, 0.006 μg/ml; G_{D1a}, 0.032 μg/ml; G_{M2}, 3.5 μg/ml; and G_{T1}, 14 μg/ml. Approximately five molecules of G_{M1} were required to completely neutralize one molecule of CT. Gangliosides had no influence on the basal or PGE_1-stimulated cyclic AMP levels.

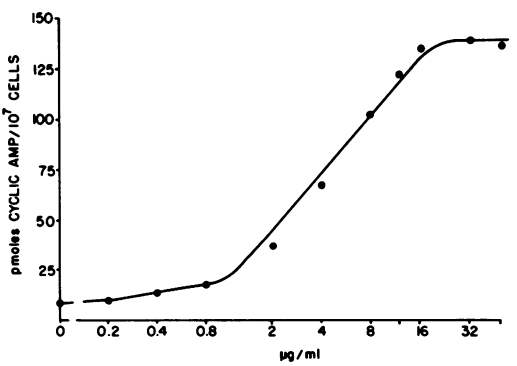

FIG. 2. *Concentration-dependent increase in thymocyte cyclic AMP content mediated by E. coli enterotoxin.*

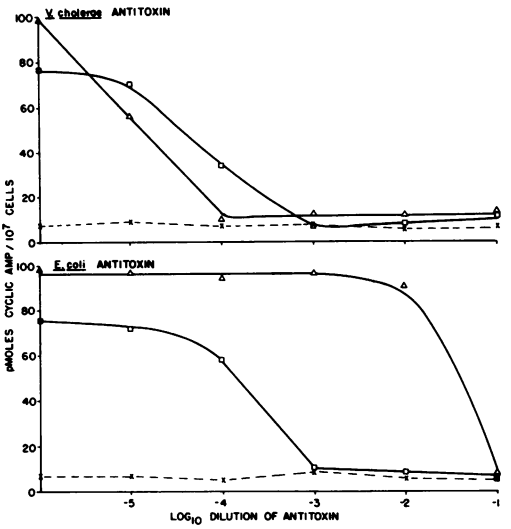

FIG. 3. *Dose response relationship for the inhibitory effect of specific antitoxins on E. coli or V. cholerae enterotoxin-induced accumulation of cyclic AMP in thymocytes: (upper panel) V. cholerae antitoxin and E. coli antitoxin (lower panel). Control (×); E. coli enterotoxin, 8 µg/ml (□); and V. cholerae enterotoxin 0.05 µg/ml (Δ) were investigated with the indicated dilutions of antitoxin at 24 C for 60 min and then were added to an equal volume of thymocytes.*

The effect of gangliosides on thymocyte adenylate cyclase activity was also examined (Fig. 5). Addition of gangliosides to control cells during a 60-min incubation did not effect adenylate cyclase activity or cyclic AMP content. By contrast, gangliosides blocked the increase in adenylate cyclase activity and cyclic AMP content in enterotoxin-treated cells. Addition of CT or ECT to the homogenized 3,000 × *g* thymocyte pellet for periods up to 40 min failed to stimulate adenylate cyclase activity, whereas PGE_1 caused an immediate increase in activity.

Interaction of enterotoxins with thymocytes. As shown above and also in Fig. 6, preincubation of enterotoxins with gangliosides for 20 min completely blocked the toxin-induced increase in cyclic AMP. Although the amount of bovine brain gangliosides used (Fig. 6) was 10-fold greater than the amount re-

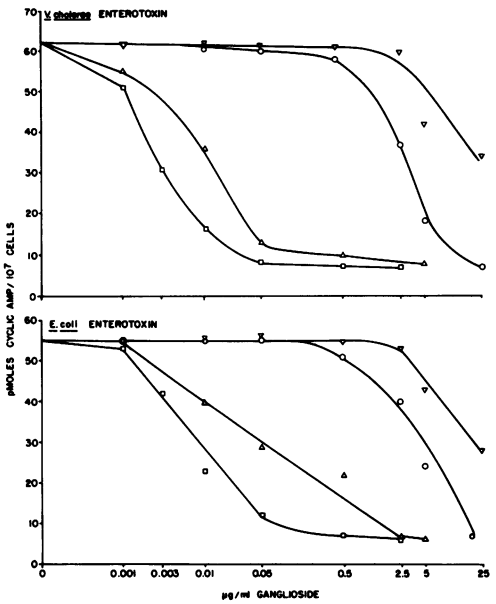

FIG. 4. *Dose response relationship for the inhibitory effect of specific gangliosides on V. cholerae (0.05 μg/ml) or E. coli (4 μg/ml) enterotoxin-induced accumulation of cyclic AMP in thymocytes. Gangliosides* G_{M1} (□), G_{M2} (○), G_{D1a} (△), *and* G_{T1} (▽) *were preincubated at 24 C with enterotoxins for at least 20 min before addition to thymocytes for a 30-min, 37-C incubation. The final concentration of gangliosides in the thymocyte incubation is indicated on the abscissa and represents a 10-fold dilution of the preincubation mixtures.*

quired for maximal inhibition, simultaneous addition of gangliosides and enterotoxin to thymocytes could not completely inhibit the increase in cyclic AMP mediated by either CT or ECT. By contrast, gangliosides added 5 min after either enterotoxin were ineffective in blocking enterotoxin action. As previously shown, gangliosides had no effect on basal levels of cyclic AMP in control cells. Furthermore, preincubation of thymocytes with 100 μg of the bovine brain ganglioside mixture per ml at 24 C for 30 min followed by extensive washing did not appear to change the responsiveness of thymocytes to submaximal doses of CT, ECT, or PGE_1 during a 30-min incubation at 37 C.

To investigate the possibility that rapid, tight binding of enterotoxin is responsible for the time dependency of ganglioside inhibition shown in Fig. 6, a wash experiment was designed (Table 1). Thymocytes were divided into two groups, control and wash. After a 5-min incubation with test agents at either 8 or 37 C, both groups were centrifuged. Thymocytes in the wash group had their supernatants removed and were resuspended in 2 ml of culture media. Both groups of cells were centrifuged again. Supernatants from the wash group were discarded, and 1 ml of medium was added back. Thymocytes were then incubated. The total incubation time was 30 min at 37 C. As shown, washing completely eliminated the PGE_1 response but had little effect on enterotoxin responses. When experiments were performed at 8 C in-

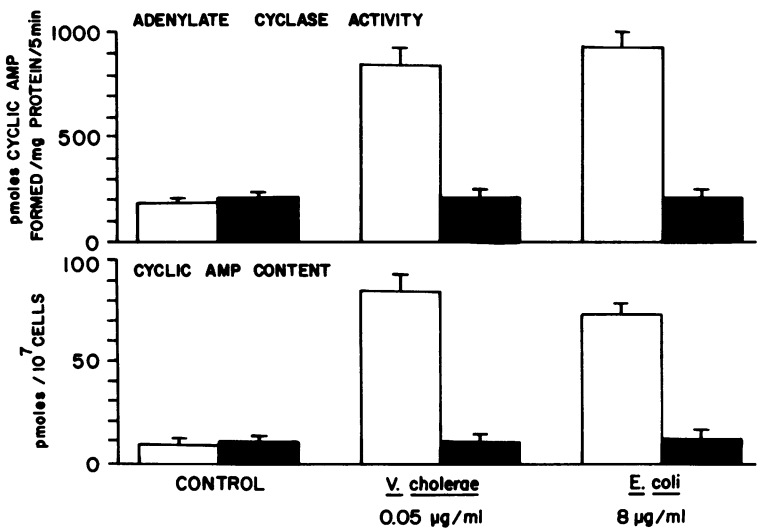

FIG. 5. *Effect of ganglioside blockage on V. cholerae or E. coli enterotoxin activation of thymocyte adenylate cyclase and cyclic AMP accumulation. Thymocytes were incubated with the indicated agents alone (□) or with 5 μg/ml of bovine brain gangliosides, type II (■). See Materials and Methods for details.*

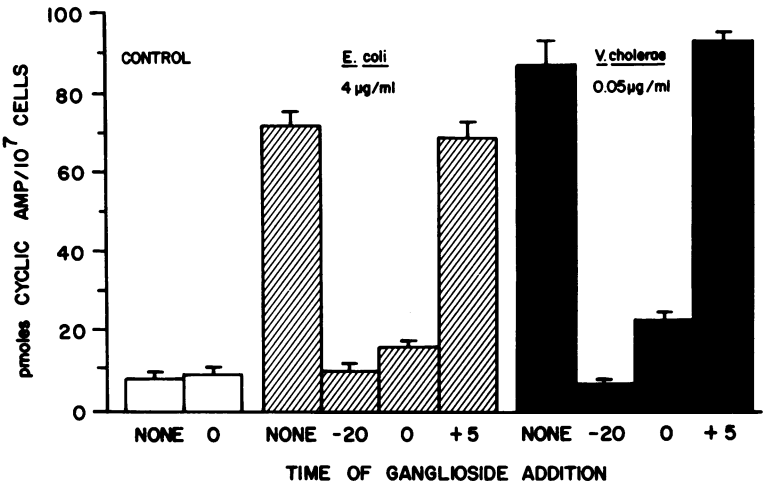

FIG. 6. *Effect of time of ganglioside addition on enterotoxin-induced increase in thymocyte cyclic AMP. When present, bovine brain gangliosides type II were at a final concentration of 5 µg/ml. At the -20-min designation on the abscissa, enterotoxins and gangliosides were mixed for 20 min at 24 C before addition to thymocytes.*

TABLE 1. *Interaction of PGE_1 and V. cholerae and E. coli enterotoxins with thymocytes*

Group	Cyclic AMP per 10^7 cells (pmol)			
	PBS[a]	PGE_1 (2×10^{-7} M)	*V. cholera* enterotoxin (0.1 µg/ml)	*E. coli* enterotoxin (4 µg/ml)
Incubated at 37 C				
Control	9 ± 1	52 ± 3	136 ± 17	60 ± 7
Wash	8 ± 2	6 ± 2	112 ± 21	61 ± 7
Incubated at 8 C				
Control	9 ± 2	36 ± 4	116 ± 8	56 ± 4
Wash	7 ± 2	9 ± 1	102 ± 13	22 ± 3

[a] PBS, Phosphate-buffered saline.

stead of 37 C, differences in the affinity of ECT and CT for thymocytes were observed. Only 30% of the ECT activity remained with the washed thymocytes, compared with 90% or more of the CT.

DISCUSSION

ECT increased thymocyte adenylate cyclase activity and cyclic AMP content. This action of ECT was concentration-dependent and mechanistically similar to that of CT but not PGE_1. Both enterotoxins were inhibited by specific antitoxins and gangliosides. Furthermore, both ECT and CT elicited a delayed activation of adenylate cyclase and exerted their effects on only intact cells. By contrast, PGE_1 produced an immediate increase in adenylate cyclase activity in intact or broken cells. Bourne et al. (3) have previously shown CT and PGE_1 to increase cyclic AMP in this manner in human leukocytes. The delayed onset of the ECT response observed in the present study was not observed by Guerrant et al. (13) in canine intestine but is consistent with a recent report by Kantor et al. (19), where adenylate cyclase activity was assessed in rabbit small intestine. The rapid rise in cyclic AMP levels followed by a sharp decline has also been shown by Boyle and Gardner (4). Essentially the same time course of enterotoxin activation was obtained when the total cyclic AMP content of the incubation mixture was determined. Combining maximal stimulatory doses of both enterotoxins failed to produce additive increases in cyclic AMP, which suggests these enterotoxins activate the same adenylate cyclase enzyme.

Identification of the cell membrane receptor for CT has been extensively investigated. Cuatrecasas working with rat liver membranes (5) and fat cells (6) and Holmgren with intestine (16), skin (16), and thymocytes (17) have demonstrated that ganglioside G_{M1} was the most effective ganglioside in neutralizing CT action. Furthermore, Holmgren (16) showed that G_{M1} was much more effective than other gangliosides in neutralizing ECT. Accordingly, this ganglioside was more effective than G_{D1a}, G_{M2}, or G_{T1} in inhibiting CT- and ECT-induced accumulation of thymocyte cyclic AMP. The inhibition which was seen at only high concentrations of G_{M2} or G_{T1} may have been due to

contaminating amounts of G_{M1} since these gangliosides were not 100% pure. Comparison of half-maximal inhibition data for both toxins shows that the concentrations of the gangliosides required to neutralize concentrations of ECT and CT of similar potency were similar. Gangliosides had no detectable effect on basal cyclic AMP levels or PGE_1 stimulation. These observations imply that G_{M1} may be part of the cellular receptors for ECT as well as for CT.

By contrast, Holmgren (16) suggests that G_{M1} is not the membrane receptor for ECT, since much higher concentrations of gangliosides G_{M1} were required to inhibit the action of ECT than CT. Factors possibly responsible for this discrepancy are differences in the action of porcine and human ECT, in assay, and in the ECT preparation. The amount of incubation time required to monitor enterotoxin activity was also an important variable. In this regard, Cuatrecasas (6) has shown that incubation of CT and gangliosides with fat cells for more than 2 h can result in increased expression rather than inhibition of toxin action. This phenomenon was explained by the ability of gangliosides to become incorporated in fat cell membranes in a manner that permitted them to act as additional binding sites for CT. Since ECT appears to act in a more or less similar manner to CT, biological assays requiring long incubations, as employed by Holmgren, may not be appropriate methods of studying these interactions. In the present study, the 30-min incubation period required for toxin assay was apparently too brief for incorporation of gangliosides into the cell membrane as receptors. In the sorbent assay performed by Holmgren (16), differences in affinity of CT and ECT for G_{M1} were much less than that found in the skin or ileal loop assay and similar to those in this study. However, since results were expressed in dry weight rather than in milligrams of protein, comparison is difficult. The ability of choleragenoid to block the action of CT but not ECT (16, 23) suggests that ECT and CT have different receptor sites with G_{M1}, a functional part of each. An example of different receptors sharing a single enzyme is provided by fat cells where epinephrine, adenocorticotropin (ACTH), and glucagon activate the same adenylate cyclase, but only the epinephrine response is blocked by propranolol and only the ACTH response is blocked by $ACTH_{1\text{-}24}$ analogue (2).

Qualitative characteristics of the ECT and thymocyte interactions were assessed. The effect of time of ganglioside addition on enterotoxin inhibition suggested that ECT, like CT, binds very rapidly to thymocytes and was probably completely bound in the first 5 min of incubation even though activation of adenylate cyclase was not apparent for another 10 to 15 min. To study this interaction more closely, wash experiments were performed. Extensive washing has been shown to displace PGE_1 from its receptors on adipocyte plasma membranes (12) and human leukocytes (3) but has little effect on CT binding even at low temperatures (3, 17). Accordingly, thymocytes treated with PGE_1 for 5 min could be completely separated from this stimulator by washing. By contrast, enterotoxin activity was not removed even after extensive washing. Lowering the temperature has little effect on CT activity. However, considerably less ECT activity remained with thymocytes washed after incubation at 8 rather than 37 C. Qualitatively, it appears that both ECT and CT bind rapidly and tightly to thymocytes, producing a delayed activation of adenylate cyclase, and that the affinity of CT for thymocytes may be greater than ECT. Final verification of these observations await purification of ECT.

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