DIFFERENTIAL INHIBITORY EFFECTS OF CHOLERA TOXOIDS AND GANGLIOSIDE ON THE ENTEROTOXINS OF *VIBRIO CHOLERAE* AND *ESCHERICHIA COLI*

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Diarrhea caused by *Vibrio cholerae* or certain noninvasive strains of *Escherichia coli* appears to be mediated by an enterotoxin(s) released by the organism within the host small bowel (1–3). All strains of *V. cholerae* produce the same protein enterotoxin whereas enterotoxigenic strains of *E. coli* may produce at least two enterotoxins. The predominant one is heat labile and antigenic, giving rise to neutralizing antibodies in serum of convalescent patients.[1] A variety of *E. coli* serotypes associated with acute diarrhea in men and certain domestic animals appear to produce the same heat-labile enterotoxin (4).[2] Some *E. coli* associated with acute diarrhea in men or in piglets produce variable amounts of a heat-stable, nonantigenic enterotoxin (2, 5, 6). Although this heat-stable enterotoxin appears to play a role in the pathogenesis of acute diarrhea in piglets (7), its role in the pathogenesis of human diarrheal disease is uncertain.

The heat-labile enterotoxins of *E. coli* and *V. cholerae* are antigenically related, giving rise to antibodies which show partial cross neutralization (6).[1] Furthermore, there is recent evidence that both enterotoxins induce small bowel secretion by activating the same cyclic 3′,5′-AMP-regulated secretory mechanism in small bowel mucosa (8–10). Despite these similarities there are important differences in the effects of these enterotoxins, the most striking being in the time-course of their effect, which is reflected in the duration of the clinical illness with which they are associated (11, 12). Whereas the secretory response to cholera enterotoxin does not become maximal until 3 h after enterotoxin exposure and is sustained for more than 12 h, the response to heat-labile *E. coli* enterotoxin is maximal within 15 min and is terminated within a similar period after washing the enterotoxin from the mucosa (10, 13). With each enterotoxin the course of the secretory response is closely paralleled by a rise and fall in mucosal adenyl cyclase activity (10, 14).

The differences in the time of onset of action of the two enterotoxins which activate the same membrane-bound enzyme could be due to differences in the rate of

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[1] Sack, R. B. Antitoxin responses to infection with enterotoxigenic *E. coli*. Manuscript in preparation.

[2] Gyles, C. L. Unpublished studies.

enterotoxin binding to the cell, the rate of enterotoxin entry into the cell, or in other cellular events which transpire after enterotoxin exposure but before adenyl cyclase activation. Similarly the differences in duration of enterotoxin effect could be the result of differences in membrane-binding characteristics or in cellular or environmental mechanisms for deactivating the enterotoxin.

The present study attempts to resolve some of these possibilities by comparing the inhibitory effects of three different materials upon the secretory response to *V. cholerae* and *E. coli* enterotoxins. The first is choleragenoid, a natural cholera toxoid isolated during the process of purifying cholera enterotoxin from culture filtrates (15). The second is formalinized cholera toxoid, prepared by formalin treatment of active cholera enterotoxin (16). The third is ganglioside, a normal component of the external membranes of most mammalian cells, minute amounts of which have previously been shown to bind and deactivate cholera enterotoxin (17). The results reveal distinct differences in cellular binding of these enterotoxins and suggest that these binding differences may influence the time-course of the secretory response to these enterotoxins.

Materials and Methods

The secretory response to *V. cholerae* and *E. coli* enterotoxins was determined in 3.5–4.0 cm ligated segments of rabbit small bowel. Albino rabbits weighing 2 kg and provided by a single supplier were used. Rabbits were fasted and ligated segments of small bowel were prepared under local anesthesia as previously described (5). The total volume of enterotoxin-containing or control materials injected into each segment was 1.0 ml, employing a 26-gauge needle. In some instances two separate 0.5 ml injections were made. When these were separated by more than 30 min the abdominal wound was closed for the interval with stainless steel skin clips. 6 or 18 h after enterotoxin injection animals were killed with intravenous pentobarbital and the entire small bowel was excised. The volume contained in each segment was determined by aspiration of the fluid contents into a 10 ml syringe; the length of the empty segment measured, and the secretory response calculated as the volume-to-length ratio in milliliters per centimeter.

Two preparations of cholera enterotoxin were employed. Crude cholera enterotoxin was prepared by Wyeth Laboratories (Philadelphia, Pa., lot no. 001) and supplied by Dr. John Seal, National Institute of Allergy and Infectious Disease, National Institutes of Health (NIH). Highly purified cholera enterotoxin was prepared by Dr. Richard Finkelstein (NIH lot no. 1071) and supplied by Dr. Robert Northrup, National Institute of Allergy and Infectious Disease, NIH. Highly purified natural cholera toxoid was also prepared and kindly supplied as "choleragenoid" by Dr. Richard Finkelstein (15). Formalinized cholera toxoid is a formalinized preparation of purified cholera enterotoxin prepared by Wyeth Laboratories (lot no. 001-01) and supplied by Dr. Robert Northrup. Its preparation has been described elsewhere (18). Purified ganglioside derived from beef brain and containing 20% sialic acid was prepared as previously described (17) and supplied by Dr. W. E. van Heyningen, Oxford University. Two crude preparations of *E. coli* enterotoxin were employed. The first was derived from *E. coli* 334A (015:H11), an organism isolated in pure culture from the jejunum of a man in Calcutta with severe cholera-like diarrhea (3) by a previously described method (2). The resultant lyophilized material contained both heat-labile and heat-stable enterotoxic activity, the major portion being heat labile (5). Each milliliter of original culture filtrate yielded about 140 μg of dry material after dialysis and lyophilization. The second preparation of *E. coli*

enterotoxin was prepared and supplied by Doctors Harley Moon and Shannon Whipp as previously described (19). The organism employed was *E. coli* 263 (08:K87, K88a,b:H19), originally isolated from piglets with acute diarrhea. The method of preparation yielded a liquid containing enterotoxin that was completely destroyed by boiling (19). The enterotoxin preparation represented a 65.5-fold concentration of the original culture filtrate.

Injection mixtures were injected in random distribution throughout the segments of small bowel to eliminate the influence of any difference in secretory response between anterior and posterior portions. In each rabbit two segments were injected with diluent controls, at least two with any nonenterotoxic materials employed (i.e. ganglioside, formalinized cholera toxoid, natural cholera toxoid), and at least two with a combination of the enterotoxin and diluent employed. Results from a rabbit were accepted only if both diluent controls were negative and all enterotoxin controls contained measurable fluid.

Lyophilized preparations of purified cholera enterotoxin, formalinized cholera toxoid, natural cholera toxid, and *E. coli* enterotoxin from strain 334A were reconstituted in pH 7.4, 0.01 M phosphate-buffered saline. Samples of the former three were shell frozen and stored at −40°C until used. *E. coli* 334A enterotoxin was reconstituted at the time of each study. Two diluents were employed, normal saline and pH 7.5 borate-gelatin buffer (H_3BO_3 3.09 g/liter, NaCl 7.01 g/liter, gelatin [Difco Laboratories, Detroit, Mich.] 0.02%). Borate-gelatin buffer was used in all studies in which purified cholera enterotoxin or ganglioside were used, normal saline was used in the remainder. All solutions injected into rabbit bowel were isosmotic with plasma. Statistical analysis of results employed the t test applied to the difference between pairwise comparisons.

RESULTS

Interference with the Secretory Response to Cholera-Enterotoxin by Natural Cholera Toxoid and Formalinized Cholera Toxoid.—

Natural cholera toxoid: Preliminary studies demonstrated that when crude cholera enterotoxin (Wyeth lot no. 001) and natural cholera toxoid were premixed, but not incubated, and 1 ml of the mixture (containing 2 mg cholera enterotoxin and 0.78 μg natural toxoid) was injected into segments of rabbit small bowel, the near-maximal secretory response normally produced by this amount of enterotoxin after 18 h was almost completely inhibited. Further studies were performed to clarify the nature of this inhibition of the secretory response to cholera enterotoxin.

Segments of rabbit small bowel were injected with 1 ml volumes containing 2 mg of crude cholera enterotoxin and serial twofold dilutions of natural cholera toxoid. The mixtures were either incubated for 1 h at 37°C or placed in an ice bath before injection. In other studies 0.5 ml volumes of serial twofold dilutions of natural toxoid were injected 30–480 min before injection of 0.5 ml containing 2 mg of crude cholera enterotoxin into the same segments. Control segments were injected with 0.5 ml of diluent at the time of natural toxoid injection.

Results are summarized in Fig. 1. The mean secretory response in control segments injected only with 2 mg crude cholera enterotoxin varied somewhat among groups of rabbits used for different portions of the study. It was 2.1 ± 0.2 (mean ± SE in milliliters per centimeter) in rabbits in which natural toxoid was premixed with or injected 30 min before cholera enterotoxin, 1.5 ± 0.1 in rabbits in which natural toxoid was injected 120 min before cholera
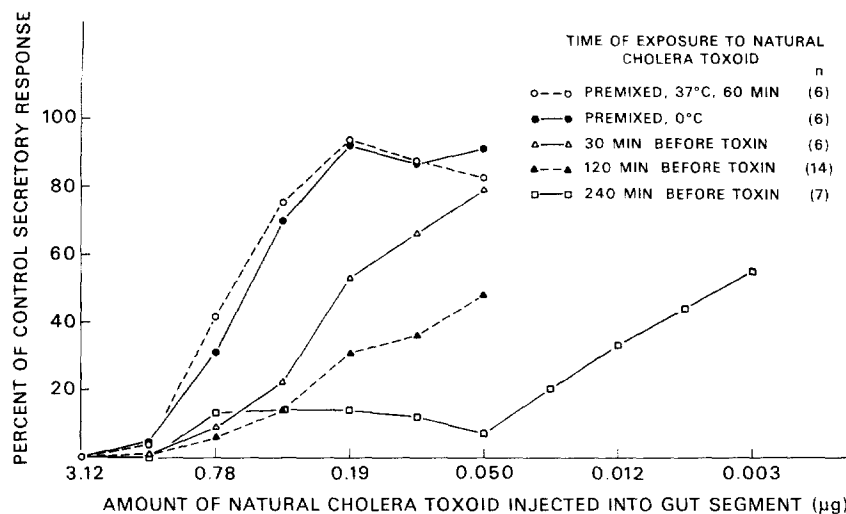
FIG. 1. Effect of natural cholera toxoid on secretory response to crude cholera enterotoxin. All segments were injected with 2 mg crude cholera enterotoxin premixed with, or at indicated interval after injection of, natural cholera toxoid.

enterotoxin, and 1.3 ± 0.1 in rabbits in which natural toxoid was injected 240 min before cholera enterotoxin. Because of these differences results in Fig. 1 are expressed as percent of control response in the same group of rabbits. There was no appreciable difference in the pattern of inhibition of cholera enterotoxin by natural toxoid which was premixed with enterotoxin with or without subsequent incubation at 37°C before injection. When natural toxoid was injected before cholera enterotoxin the amount required to achieve 50% inhibition of the secretory response decreased markedly as the period separating the injection of natural toxoid and cholera enterotoxin increased. This amount (in nanograms $\pm$ SE) was: 670 ± 170 (when premixed with cholera enterotoxin at 0°C), 650 ± 200 (when premixed and incubated for 60 min at 37°C), 220 ± 70 (when injected 30 min before cholera enterotoxin), 50 ± 30 (when injected 120 min before cholera enterotoxin), and 4.2 ± 3.8 (when injected 240 min before cholera enterotoxin). Extending the period between injection of the toxoid and cholera enterotoxin to 8 h did not further enhance the inhibitory effect of the toxoid (not shown). In no instance did control segments injected with natural toxoid alone (3.12 μg) contain measurable fluid at the time of sacrifice.

Further studies were conducted to determine if natural cholera toxoid could inhibit the effect of cholera enterotoxin when placed in the bowel after the enterotoxin. Because preliminary studies showed that as much as 25 μg of natural toxoid placed in the bowel 30 min after 2 mg of crude cholera enterotoxin failed to inhibit the secretory response to enterotoxin, the study design was altered to permit detection of inhibition of the secretory response to smaller

amounts of enterotoxin. A constant amount of natural toxoid was injected into gut segments at varying intervals before or after injection of serial twofold dilutions of cholera enterotoxin. The amount of natural toxoid used was the approximate molar equivalent of the 2 mg of crude cholera enterotoxin employed above. This amount, 129 ng or 2.3 pmol, was calculated by comparing the relative enterotoxin potency of crude and highly purified cholera enterotoxin in rabbit small bowel (which revealed a potency ratio of 1:10,280) and adjusting for the reported molecular weights of cholera enterotoxin (84,000) and natural toxoid (56,000) (20). The results, determined 18 h after enterotoxin injection, are summarized in Fig. 2. Inhibition of the secretory response to cholera enterotoxin was clearly related to the interval separating enterotoxin and natural toxoid injection. The equivalent amounts of pure enterotoxin required to produce a 0.50 ml/cm secretory response in the presence of 2.3 pmol of natural toxoid are shown in Table I. When injection of natural cholera toxoid followed that of cholera enterotoxin by as little as 30 min there was little, if any, inhibition of the subsequent secretory response.

Formalinized cholera toxoid: Preliminary studies with formalinized cholera toxoid indicated that injection of as much as 50 µg (based on Lowry protein content) into a segment of gut 30 min before injection of 2 mg crude cholera enterotoxin gave no inhibition of the secretory response measured 18 h later. In further efforts to detect any inhibitory capacity of formalinized cholera toxoid 193 ng (2.3 pmol) of the toxoid in 0.5 ml volume was injected into segments of rabbit small bowel 120 min before injection of graded amounts of
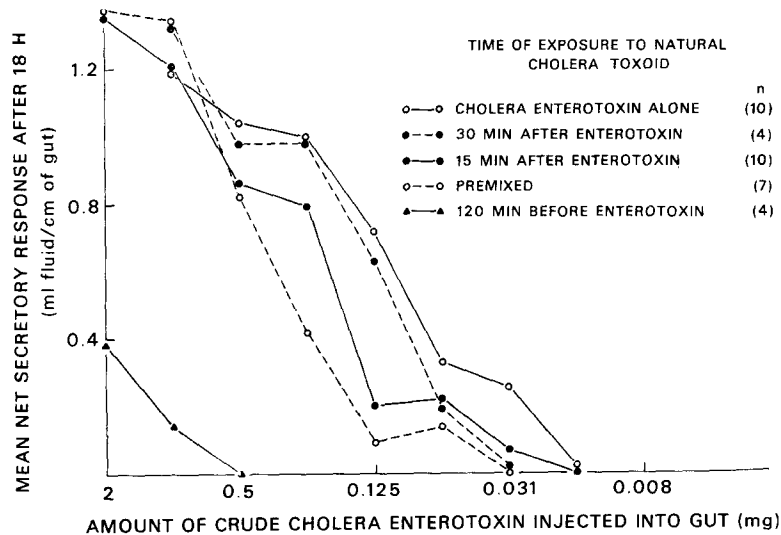

FIG. 2. Influence of injection sequence on inhibitory effect of natural cholera toxoid on cholera enterotoxin. All segments were injected with 129 ng natural cholera toxoid premixed with, or at indicated interval before or after injection of, crude cholera enterotoxin.

crude cholera enterotoxin. The results, determined 18 h after enterotoxin injection, are shown in Fig. 3. Formalinized cholera toxoid had no inhibitory effect on the secretory response to cholera enterotoxin. By contrast, when natural cholera toxoid was studied under similar conditions marked inhibition was observed (Fig. 2).

Effect of Natural Cholera Toxoid on the Secretory Response to E. coli Enterotoxin.—

To determine the effect of natural cholera toxoid on the secretory response to *E. coli* enterotoxin segments of bowel were injected with 0.5 ml containing 12.5 µg natural toxoid or normal

TABLE I

Effect of Time of Exposure upon Inhibition of the Secretory Response to Cholera Enterotoxin by Natural Cholera Toxoid

Time of natural toxoid injection relative to that of enterotoxin*	Equivalent amount of pure enterotoxin producing 0.50 ml/cm secretory response‡ (geometric mean)	No. of studies
	pmol ± SE	
120 min before	>2.3	4
Premixed	0.33 ± 0.08§	7
15 min after	0.20 ± 0.04	10
30 min after	0.13 ± 0.03	4
Control (enterotoxin alone)	0.10 ± 0.03	10

* 2.3 pmol of natural cholera toxoid used throughout.
‡ Secretory response measured 18 h after enterotoxin injection.
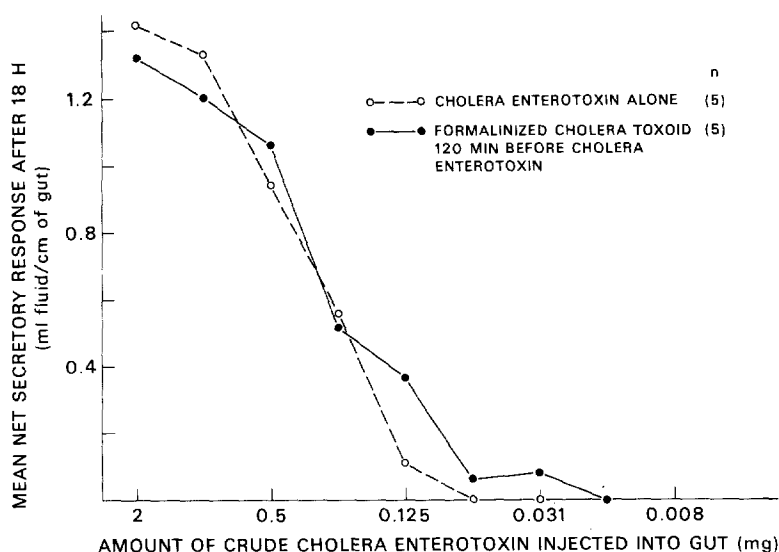§ Significantly different from response to enterotoxin alone, $P < 0.05$.

FIG. 3. Effect of formalinized cholera toxoid on secretory response to cholera enterotoxin. Formalinized toxoid (193 ng) was injected 120 min before indicated amount of crude cholera enterotoxin.

saline. 1 h later 0.5 ml containing one of three concentrations of *E. coli* enterotoxin from human strain 334 or porcine strain 263 was injected into each gut segment. The secretory response was determined at 6 h in one series and at 18 h in another. Results are summarized in Table II.

TABLE II

Effect of Natural Cholera Toxoid on Secretion Induced by E. coli Enterotoxin

(A) Strain 334	Enterotoxin challenge, μg/gut segment		
	500	150	50
(1) 6 h			
Natural toxoid*	0.56‡	0.28	0.13
± SE	0.05	0.05	0.04
Control§	0.51	0.19	0.10
± SE	0.06	0.05	0.03
P‖ (n = 15)	NS¶	NS	NS
(2) 18 h			
Natural toxoid*	0.68	0.38	0.13
± SE	0.10	0.09	0.07
Control§	0.30	0.05	0.00
± SE	0.07	0.04	—
P‖ (n = 12)	<0.02	<0.01	NS

(B) Strain 263	Enterotoxin challenge, μl/gut segment**		
	167	42	10
(1) 6 h			
Natural toxoid*	0.68	0.57	0.17
± SE	0.09	0.07	0.05
Control§	0.74	0.43	0.13
± SE	0.05	0.06	0.06
P‖ (n = 15)	NS	<0.05	NS
(2) 18 h			
Natural toxoid*	0.67	0.54	0.36
± SE	0.10	0.09	0.09
Control§	0.60	0.32	0.12
± SE	0.10	0.08	0.07
P‖ (n = 15)	NS	<0.05	<0.02

* 12.5 μg, 1 h before *E. coli* enterotoxin. In each rabbit two segments receiving 12.5 μg natural toxoid alone contained no fluid at time of sacrifice.

‡ Mean milliliters per centimeter, at indicated time after enterotoxin injection.

§ 0.85% NaCl.

‖ By pairwise comparison using *t* test.

¶ NS, not significant.

** Represents volume of concentrated liquid enterotoxin described in text.

Natural cholera toxoid failed to inhibit the secretory response to either preparation of *E. coli* enterotoxin. Rather, in most instances, the mean secretory response to *E. coli* enterotoxin was greater in the presence of natural toxoid and in several instances the difference was statistically significant. Control segments in each rabbit injected with 12.5 μg of natural cholera toxoid alone showed no secretory response at either 6 or 18 h.

Effect of Ganglioside on the Secretory Response to Enterotoxins of V. cholerae and E. coli.

The inhibitory effect of ganglioside upon the secretory response to these enterotoxins was studied by premixing serial fourfold dilutions of ganglioside with equal volumes containing the enterotoxin. All dilutions were in borate-gelatin buffer. The enterotoxin content of the 1 ml injected into small bowel was 1 mg of crude cholera enterotoxin, 1 mg of crude *E. coli* enterotoxin from strain 334, or the equivalent of a 1:6 dilution of the concentrated crude *E. coli* enterotoxin from strain 263. Mixtures of enterotoxin and ganglioside were placed on ice after mixing until injected unless otherwise specified. The secretory response was determined after both 6 and 18 h in studies of *E. coli* enterotoxin and only after 18 h in studies of *V. cholerae* enterotoxin. Results are summarized in Fig. 4.

The mean secretory responses in segments injected only with enterotoxin were: 0.94 ± 0.04 and 0.96 ± 0.07 (6 and 18 h after 1 mg *E. coli* 334 enterotoxin), 0.60 ± 0.04 and 0.98 ± 0.11 (6 and 18 h after 167 μl *E. coli* 263 enterotoxin), and 1.63 ± 0.10 ml/cm ± SE (18 h after 1 mg crude *V. cholerae* en-
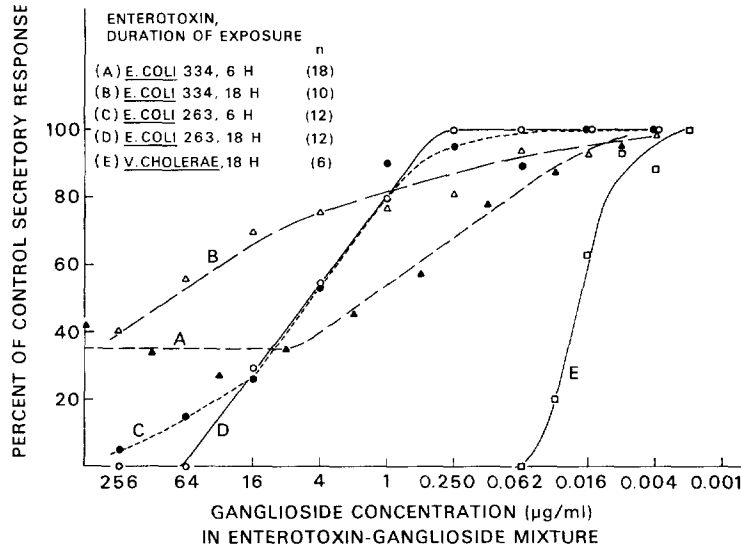

FIG. 4. Comparison of inhibitory effect of ganglioside on secretory response to *V. cholerae* and *E. coli* enterotoxins. The amounts of enterotoxin used are indicated in the text. The secretory response was measured at indicated interval after injection of enterotoxin-ganglioside mixture.

terotoxin). Ganglioside inhibited the secretory responses of both *V. cholerae* and *E. coli* enterotoxins. In neither case was the inhibition increased by pre-incubation of the mixture for 60 min at 37°C. There were, however, striking differences between the nature of this inhibition of *V. cholerae* enterotoxin on the one hand and of the *E. coli* enterotoxins on the other. There were also differences between the different preparations of *E. coli* enterotoxin. All control segments containing 256 μg of ganglioside in buffer were negative. The mean amount of ganglioside producing a 50% decrease in secretory response to the dose of cholera enterotoxin employed was 18.9 ng, whereas comparable reduction in the secretory response to the *E. coli* enterotoxins required 1,500–94,000 ng depending upon the enterotoxin and the time of reading. Furthermore the slopes of the curves of ganglioside inhibition of the secretory effect of the *E. coli* enterotoxins, though variable, were consistently more shallow than that of *V. cholerae* enterotoxin. Finally, it is evident that a portion of the secretory response to *E. coli* enterotoxin from the 334 strain was uninhibited at the highest concentrations of ganglioside used, whereas complete, or nearly complete, inhibition of the response to *E. coli* enterotoxin from the 263 strain was eventually observed.

The possiblity that the residual secretory activity of *E. coli* enterotoxin from strain 334 which was uninhibited by ganglioside was due to the heat-stable component of the enterotoxin was further studied. Segments of gut were injected with: (*a*) 1 mg of the *E. coli* enterotoxin, (*b*) 1 mg of the *E. coli* enterotoxin previously heated to 100°C for 15 min, or (*c*) 1 mg heated *E. coli* enterotoxin premixed with 2 mg of ganglioside. The secretory response was determined 6 h later. Results are summarized in Table III. Heating to 100°C reduced the mean secretory response at 6 h by 36% ($P < 0.001$) but there was no further reduction in secretory response when ganglioside was added to previously heated enterotoxin before injection. Control segments injected with 2 mg of ganglioside alone contained no measurable fluid.

TABLE III

Effect of Ganglioside on Heat-Stable E. coli Enterotoxin

	Secretory response*	
	Amount	*n*
	ml/cm ± SE	
E. coli enterotoxin‡	0.73 ± 0.10	12
E. coli enterotoxin‡ (heated 100°C, 15 min)	0.47 ± 0.03§	24
E. coli enterotoxin‡ (premixed with ganglioside)‖	0.48 ± 0.06	6

* 6 h after enterotoxin injection.

‡ 1 mg from strain 334.

§ Significantly less than response to unheated enterotoxin, $P < 0.001$.

‖ 2 mg ganglioside.

Observations on the Binding of V. cholerae and E. coli Enterotoxins to Gut Mucosa.—Several observations suggest that natural cholera toxoid acts as a competitive inhibitor of cholera enterotoxin: (a) natural cholera toxoid bears considerable antigenic and structural similarity to cholera enterotoxin (15, 20) and thus might be expected to bind to a mucosal receptor for cholera enterotoxin; (b) with prolonged preexposure to natural toxoid the response to cholera enterotoxin is inhibited by approximately equimolar amounts of natural toxoid; and (c) natural toxoid does not block the mucosal secretory mechanism since it fails to inhibit the secretory response to *E. coli* enterotoxin, which appears to be mediated by the same adenyl cyclase-controlled system (8–10). The availability of an agent antigenically related to cholera enterotoxin but which acts as a competitive inhibitor of the enterotoxin provides a very useful tool for study of the interaction of cholera enterotoxin with cell membranes.

Cholera enterotoxin and its natural toxoid appear to differ appreciably in the rate at which they achieve essentially irreversible binding to bowel mucosa. The effect of cholera enterotoxin is unmodified if given 30 min before natural toxoid, whereas the inhibitory effect of natural toxoid is maximal only when it is exposed to gut mucosa 4 h before cholera enterotoxin. Previous studies have also indicated that cholera enterotoxin binds very rapidly to gut mucosa (17, 21). Nevertheless, once binding of natural toxoid occurs it appears to be prolonged. This is suggested by the demonstration of a marked reduction in net fluid secretion 18 and 22 h after injection of cholera enterotoxin and natural toxoid, respectively, in essentially equimolar amounts. If this long-lasting inhibitory effect is caused by prolonged occupation of the cellular binding site by natural toxoid it suggests that active cholera enterotoxin may also exhibit prolonged binding to the cellular receptor.

Formalinized cholera toxoid is antigenically similar to natural toxoid and active cholera enterotoxin (22). Nevertheless, it shows little, if any, ability to inhibit the action of cholera enterotoxin, suggesting that it does not compete for the mucosal binding site of cholera enterotoxin. This conclusion is consistent with the observation by others that formalinized toxoid could not be demonstrated to bind to gut mucosa by techniques in which antibody labeled with fluorescein or horseradish peroxidase were used and which readily demonstrated mucosal binding of natural toxoid and active cholera enterotoxin (23). These observations suggest that the properties of antigenicity, mucosal binding, and enterotoxicity (manifested by induction of gut secretion) belong to separate portions or characteristics of the active enterotoxin molecule. Formalinized toxoid is antigenic, giving rise to antibodies which neutralize cholera enterotoxin (22), but it does not exhibit mucosal binding or enterotoxicity; natural toxoid is antigenic and appears to compete for the mucosal binding site of cholera enterotoxin but it is not enterotoxic; while active cholera enterotoxin has all three properties (Table IV). These observations also indicate that

TABLE IV

Interrelation of V. cholerae Enterotoxin, Cholera Toxoids, and Heat-Labile E. coli Enterotoxin

	Cholera enterotoxin	Natural cholera toxoid	Formalinized cholera toxoid	Heat-labile E. coli enterotoxin
(1) Neutralized or bound by cholera antitoxin	Yes	Yes	Yes	Yes*
(2) Binds to cellular receptor of cholera enterotoxin	Yes	Yes	No	No
(3) Induces gut secretion by adenyl cyclase activation	Yes	No	No	Yes

* Cholera antitoxin is more effective in neutralizing heat-labile *E. coli* enterotoxin than is antibody to heat-labile *E. coli* enterotoxin in neutralizing cholera enterotoxin.[1,2]

simple binding of an agent (i.e., natural toxoid) to the cellular receptor of cholera enterotoxin is not sufficient to activate the mucosal secretory process.

Failure of natural cholera toxoid to inhibit the secretory response to *E. coli* enterotoxin indicates that the mucosal binding sites for *E. coli* enterotoxin and natural cholera toxoid (and presumably cholera enterotoxin) are different. This difference was apparent with *E. coli* enterotoxins prepared from bacterial strains of diverse origins associated with both human and porcine diarrheal disease. The demonstration of antigenic similarity between heat-labile *E. coli* enterotoxin and cholera enterotoxin[1,2] again suggests that the molecular determinants of cell binding and antigenicity are largely unrelated to those of enterotoxicity (Table IV). These observations suggest that an agent in the gut lumen need not bind to the mucosal receptor for cholera enterotoxin in order to activate adenyl cyclase and induce gut secretion. They also do not exclude the possibility that the molecules of heat-labile *E. coli* enterotoxin and cholera enterotoxin share common or similar enterotoxic radicals which act upon the same secretory mechanism, but which differ in their interaction with the cell membrane because of differences in the structure of the large molecule of which they are a part.

Previous studies have indicated that ganglioside may be the mucosal receptor of cholera enterotoxin (17). Ganglioside, in minute amounts, binds and deactivates cholera enterotoxin in vitro and ganglioside-like substances isolated from gut mucosal membranes also deactivate cholera enterotoxin (17). The highly efficient deactivation of cholera enterotoxin by ganglioside is contrasted in this study with its much less efficient deactivation of heat-labile *E. coli* enterotoxin from strains pathogenic for both men and pigs and its lack of effect upon heat-stable *E. coli* enterotoxin. The latter observations suggest that ganglioside would function ineffectively as a cellular binder of *E. coli* enterotoxins and, if ganglioside is the cellular binding site of cholera enterotoxin, they are consistent with the previous conclusion that *E. coli* and *V. cholerae* enterotoxins have different cellular binding sites. The demonstration

that ganglioside fails to inhibit the heat-stable component of E. coli entero-
toxin suggests that ganglioside binding might be used as a means of separating
and partially purifying the heat-labile E. coli enterotoxin from crude culture
filtrates which frequently contain both heat-labile and heat-stable enterotoxins.
To date attempts to purify the heat-labile E. coli enterotoxin have met with
failure.[3]

The observation that preexposure to natural cholera toxoid produces a
small but significant enhancement of the secretory response to E. coli entero-
toxin has at least two possible explanations. It is possible that natural cholera
toxoid has a low level of adenyl cyclase-stimulating activity which, in the
amounts used, is insufficient to produce fluid accumulation in small bowel
segments but which could act in concert with adenyl cyclase activation by
E. coli enterotoxin resulting in an enhanced secretory response. This possi-
bility is also suggested by previous observations that natural cholera toxoid
produces some of the effects ascribed to active cholera enterotoxin, including
hyponatremia and, occasionally, death when given parenterally to dogs[4] and
increased induration of skin after intracutaneous injection in rabbits[5] and
monkeys.[6] These effects could be due either to a very low level of contamina-
tion of the natural toxoid with cholera enterotoxin, or to direct effects of
the toxoid molecule. Alternatively, it is possible that a portion of E. coli entero-
toxin is normally bound to the receptor for cholera enterotoxin but that such
binding actually prevents E. coli enterotoxin from activating adenyl cyclase.
Occupation of the cholera enterotoxin-binding site by natural toxoid could,
then, increase the effective dose of E. coli enterotoxin available for adenyl
cyclase activation.

*Possible Influence of Binding Characteristics upon Time-Course of Secretory
Response to V. cholerae and E. coli Enterotoxins.*—Despite rapid binding to gut
mucosa the secretory response to cholera enterotoxin does not become maximal
until at least 3 h after exposure (13). The metabolic effect of cholera entero-
toxin on the function of other intact cell systems shows a similar delay in
onset after exposure (24, 25). It has been proposed that this delay may be due
to the time required for cellular synthesis of a protein regulator of the secretory
process (26, 27). This seems unlikely in view of the demonstration that the
antigenically similar heat-labile E. coli enterotoxin stimulates the same secre-
tory mechanism with little, if any, delay in onset of peak secretory rate (10).
Recent studies have indicated that the adenyl cyclase enzyme activated by
cholera enterotoxin lies in the lateral or basal membranes of the mucosal cell
(28). It is possible that slow release of all or a portion of the cholera entero-

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[3] Jacks, T. M., B. J. Wu, and A. C. Braemer. Properties of the enterotoxic component in an
Escherichia coli enteropathogenic for swine. Manuscript submitted for publication.
[4] Pierce, N. F. Unpublished studies.
[5] Finkelstein, R. A. Unpublished studies.
[6] Northrup, R. S. Unpublished studies.

toxin molecule or slow changes in membrane configuration which follow binding of cholera enterotoxin result in its slow delivery to the adenyl cyclase regulator mechanism. If so, access to the adenyl cyclase regulator might be enhanced by rupturing cells before exposing them to cholera enterotoxin. This has been reported to occur by Zieve and his co-workers who showed that cholera enterotoxin fails to induce glycogenolysis in intact platelets but induces glycogenolysis reaching a maximum rate within 15 min when platelets are ruptured before enterotoxin exposure (29). In contrast to cholera enterotoxin the secretory response to E. coli enterotoxin is rapid in onset, being fully developed within 15 min after exposure (10). Since both enterotoxins act upon the same secretory mechanism it is likely that the demonstrated differences in cell binding, and possibly in molecular structure, are responsible for the rapid effect of E. coli enterotoxin.

The prolonged duration of the secretory response to cholera enterotoxin has not been explained. The present study suggests that its prolonged action may be related to its very long occupation of the cellular binding site. The possibility that cholera enterotoxin produces long-lasting "damage" to the cell which could persist after disappearance of the enterotoxin, while not excluded by this study, is not a necessary consideration in explaining the duration of its secretory effect. Similarly, the brief duration of effect of E. coli enterotoxin may also be due to the nature of its cellular binding which other studies suggest to be readily reversible (5, 10). Previous studies have shown that the brief duration of its effect is not due to tachyphylaxis of the secretory response (5). Evidence has also been presented that E. coli enterotoxin is slowly inactivated after exposure to gut mucosa (10), which may also serve to limit its duration of action.

SUMMARY

Natural cholera toxoid appears to act as a competitive inhibitor of cholera enterotoxin and is thus a useful tool for studying the interaction of cholera enterotoxin with cell membranes. Cholera enterotoxin binds to gut mucosa more rapidly than does its natural toxoid. Once binding occurs, however, it appears to be prolonged for both materials. Formalinized cholera toxoid has no inhibitory effect upon cholera enterotoxin. Enterotoxic activity, ability to bind to gut mucosa, and antitoxigenicity appear to be independent properties of cholera enterotoxin.

Natural cholera toxoid does not inhibit Escherichia coli enterotoxin, indicating that although the two enterotoxins activate the same mucosal secretory mechanism they occupy different binding sites in the mucosa. Ganglioside, which may be the mucosal receptor of cholera enterotoxin, is highly efficient in deactivating cholera enterotoxin. By contrast, ganglioside is relatively inefficient in deactivating heat-labile E. coli enterotoxin and is without effect upon the heat-stable component of E. coli enterotoxin. These findings suggest

that ganglioside is not likely to be the mucosal receptor for *E. coli* enterotoxin. Differences in cellular binding of *E. coli* and cholera enterotoxins may explain, at least in part, the marked differences in the time of onset and duration of their effects upon gut secretion.

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