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Comparison of the Tissue Receptors for *Vibrio cholerae* and *Escherichia coli* Enterotoxins by Means of Gangliosides and Natural Cholera Toxoid

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The in vitro binding properties of enterotoxins of *Vibrio cholerae* and *Escherichia coli* to different pure gangliosides and related neutral glycosphingolipids were analyzed with a sorbent assay utilizing plastic tubes to which the glycolipid substances had been coupled. It was found that the cholera toxin bound to G_{M1} ganglioside better than to the other tested substances G_{M3}, G_{M3}-NGN, G_{M2}, G_{D1a}, G_{D1b}, G_T, G_{A1}, tetrahexoside-GlcNac and globoside. With this assay using G_{M1}-coated tubes it is possible to measure cholera toxin even at concentrations below 1 ng/ml. Also enterotoxin of various *E. coli* strains bound to G_{M1}, but the affinity was much less than for cholera toxin. The G_{M1} ganglioside, in contrast to the other glycosphingolipids, effectively inactivated cholera toxin as determined with the intradermal and the ileal loop assays; approximately equimolar concentrations of the ganglioside in relation to toxin sufficed. Also, the skin and ileal loop activities of *E. coli* enterotoxins could be inhibited by G_{M1}; however, several orders more of the ganglioside were required for such inhibition than for inactivation of the cholera toxin, and the differences between G_{M1} and the other substances were less pronounced for *E. coli* toxins. Preincubation of rabbit ileal loops with choleragenoid, a natural toxoid of *V. cholerae* which has binding properties to the G_{M1} ganglioside similar to cholera toxin, made the loops resistant to subsequently added enterotoxin of *V. cholerae*. The responsiveness to enterotoxin of *E. coli* was not reduced by this toxoid. A likely interpretation of these data is that the G_{M1} ganglioside constitutes or at least contains the structure of functional tissue receptors for the cholera toxin, whereas the weak binding to G_{M1} by *E. coli* enterotoxins is probably a pathogenetically insignificant reflection of structural similarities between these toxins and cholera toxin. Consequently, the cholera toxoid by occupying functional intestinal G_{M1} receptors for the cholera toxin could inhibit the ileal response to this toxin, but not the response to *E. coli* enterotoxin since the intestinal receptors for the latter toxin are not affected by the cholera toxoid.

Enterotoxin production has been recognized in *Vibrio cholerae* and in certain strains of *Escherichia coli*, and is probably responsible for the acute diarrhea caused by these organisms (3, 6). The enterotoxins of the two principal serotypes of *V. cholerae* (Inaba and Ogawa) are immunologically identical (9), whereas it is possible that more than one immunological type of enterotoxin from *E. coli* is produced (17). The heat-labile enterotoxins of *V. cholerae* and *E. coli* are antigenically related although not identical as judged from cross-neutralization studies (17; J. Holmgren, O. Söderlind, and T. Wadström, Acta Pathol. Microbiol. Scand., in press). The toxins also seem to affect the same transport process in the intestine, probably by activating intestinal adenyl cyclase (3).

It is important to learn whether the hypersecretion in the intestine resulting from the action of both *V. cholerae* and *E. coli* enterotoxins is triggered from the binding of the toxins to the same or to different tissue receptors. A better understanding, along with the theoretical implications, might facilitate the development of means to prevent the toxic effects of these enterotoxins through competition at the recep-

tor level. Such competition could be obtained by blocking the active site(s) of the toxin molecules with free receptor substance or a receptor-analogue. Alternatively, the tissue receptors could be covered with some agent devoid of toxin activity.

The observation by van Heyningen et al. (20) that a ganglioside mixture could inactivate cholera toxin led to the identification by Holmgren et al. (10, 11) of the possible tissue receptor structure for the cholera enterotoxin. Analyses of the reactivity of the toxin with various pure gangliosides and neutral glycosphingolipids (10, 11) revealed that the sialidase-resistant ganglioside G_{M1} fixed and inactivated the cholera toxin with an affinity and specificity that indicated that this ganglioside constitutes or at least contains the tissue receptor structure (11). The G_{M1} ganglioside can therefore be used as "receptor substance" in studies of cholera toxin blocking. A natural cholera toxoid which can bind to intestinal mucosal cells (14) showed a similar affinity for G_{M1} ganglioside as the active toxin (11). This suggested that this toxoid could possibly interfere with the activity of enterotoxins by competing for the same intestinal receptors.

The aim of the present study was to elucidate whether the cellular binding sites for the enterotoxin of *V. cholerae* also function as tissue receptors for *E. coli* heat-labile enterotoxin. One approach was to compare the binding properties in vitro to various pure glycosphingolipids including the G_{M1} ganglioside and the inactivation of the toxins that may be caused by the contact with such substances. A second approach was to test whether the natural cholera toxoid could interfere with the intestinal secretory response to enterotoxins of the two bacterial species.

MATERIALS AND METHODS

V. cholerae enterotoxin and enterotoxoid. Isolated exo-enterotoxin (choleragen) of *V. cholerae* (Inaba) was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by R. A. Finkelstein, Dallas, Texas, as described (5). The lot number of the preparation was 1071 and the material was provided by NIAID via R. Northrup. R. A. Finkelstein provided isolated natural cholera toxoid (choleragenoid) prepared as described (5). These freeze-dried materials were dissolved to a 0.2% (wt/vol) solution in sterile tris(hydroxymethyl)aminomethane(Tris) - ethylenediaminetetraacetic acid (EDTA) buffer, pH 7.5 (0.05 M Tris, 0.001 M Na_2EDTA, 0.003 M NaN_3, and 0.2 M NaCl) and dispensed into portions which were kept in stoppered glass tubes at −20 C until use. Culture filtrates from *V. cholerae* Inaba and Ogawa bacteria were used as crude cholera enterotoxins. The preparations, lots 4493G and 001, respectively, were provided as lyophilized samples from NIAID via J. R. Seal, and stored at +4 C until use.

A diluent consisting of 0.1 M Tris-hydrochloride buffer, pH 7.5, and containing 0.2% (wt/vol) of gelatin (TG buffer) was used in experiments with these toxin and toxoid preparations.

E. coli enterotoxin. Crude enterotoxins from *E. coli* were prepared by O. Söderlind, Stockholm, from six strains isolated in Sweden from pigs in herds with acute enteric disease and from a porcine enteropathogenic strain, P5 (O 141), isolated in England. The strain designations of the Swedish strains (the somatic O antigens within parentheses) were: 915/66 (O 8), 163/67 (O 138), 887/64 (O 141), B 2577/67 (O 147), 537/67 (O 149), and 853/67 (O 149).

Roux bottles containing 200 ml of tryptose agar (tryptose phosphate broth, Oxoid, 29.5 g; agar, Difco, 15 g; deionized water, 1,000 ml) supplemented with 0.2% (wt/vol) glucose, were inoculated with 5 ml of an 18-h-old culture of the bacterial strain in nutrient broth. After incubation at 37 C for 18 h the organisms were harvested by shaking and washing the cells from the agar with 20 ml of distilled water containing 12 g of glass beads per bottle. The bacterial suspension (approximately 3×10^{10} viable cells per ml) was subjected to ultrasonic treatment (MSE, 60-W ultrasonic disintegrator, Measuring Scientific Equipment Ltd., London) at 1.3 A, 10 μm amplitude, in 12-ml batches for 5 min. After 3 h centrifugation at 4,000 × g, the clear supernatant fluid was filtered through 0.65 μm and 0.45-μm (pore size) cellulose acetate filters (Millipore Corp.) and neomycin was added to a final concentration of 100 μg/ml. The dry weight of this material was approximately 30 mg/ml.

The enterotoxins from P5 and 853/67 were stored in portions at −20 C until use within 3 months, whereas the other *E. coli* enterotoxin preparations were lyophilized and kept at 4 C until use up to a year later, when they were dissolved in TG buffer for each test occasion. Two toxin preparations from different cultures were used from strain B2577/67, I and II.

Antisera. Antiserum to cholera toxin was obtained from a rabbit given three subcutaneous injections of 30 μg of the purified toxin without adjuvant 3 weeks apart. Bleeding was performed 2 weeks after the second injection.

Antiserum against enterotoxin from *E. coli* strain P5 was provided by O. Söderlind. It was produced in a rabbit given three subcutaneous injections of partially purified enterotoxin 3 weeks apart. Bleeding was performed 10 days after the last injection.

Glycosphingolipids. L. Svennerholm, Göteborg, Sweden, provided a series of gangliosides and allied neutral glycosphingolipids isolated from human brains with the chromatographic methods recently described (19). The substances had been analyzed for total neutral sugars, hexosamine, sialic acid, and fatty acids by gas-liquid chromatography (GLC). The positions of glycosidic bonds had been determined by permethylation (6), and the partially methylated sugars had been converted into alditol acetates and analyzed by GLC and mass spectrometry (1, 2). The anomeric configuration of the glycolipids had been determined by the sequential hydrolysis of the oligo-

saccharide chain by specific glycosidases (7, 12). Dissolved in TG buffer the following neutral glycosphingolipids and gangliosides were tested for their capacity to react with enterotoxins: globoside, GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer; G_{A1}, Galβ1-3GalNAcβ1-4Galβ1-4Glc-Cer; tetrahexoside-GlcNAc, Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer; G_{M3}, NANα2-3Galβ1-4Glc-Cer and NGNα2-3Galβ1-4Glc-Cer; G_{M2}, GalNAcβ1-4(NANα2-3)Galβ1-4Glc-Cer; G_{M1}, Galβ1-3GalNAcβ1 - 4(NA2 - 3)Galβ1 - 4Glc - Cer; G_{D1a} NANα2-3Galβ1-3GalNAcβ1-4 (NANα2-3)Galβ1-4Glc-Cer; G_{D1b}, Galβ1-3GalNAcβ1-4(NANα2-8NANα2-3) Galβ1-4Glc-Cer; G_{T1}, NANα2-3Galβ1-3GalNAcβ1-4(NANα2-8NANα2-3)Galβ1-4Glc-Cer. (Abbreviations used are: NAN, N-acetylneuraminic acid; NGN, N-glycolylneuraminic acid; Gal, galactose; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcNAc, N-acetylglucosamine; Cer, ceramide. The ganglioside nomenclature is according to Svennerholm [18]).

The purity of the various gangliosides and neutral glycolipids was in general better than 99%, and in the case of G_{M1} better than 99.9%.

Control reagents. For control purposes purified *V. cholerae* Inaba lipopolysaccharide (9), rabbit antiserum to this antigen, and rabbit antiserum to a derivate from Inaba culture filtrate (approximately 10% lipopolysaccharide and 0.1% exo-enterotoxin) were used. Tritiated G_{M1} ganglioside provided by L. Svennerholm and rabbit antisera reactive with the glycosphingolipids G_{A1}, tetrahexoside-GlcNAc, and globoside were also employed.

Double diffusion in gel. A sensitive microplate double diffusion-in-gel method was used for analyses of the capacity of the glycosphingolipids to fix enterotoxins in vitro (11). In volumes of 25 μliters, 1.2 μg of isolated cholera toxin was tested against glycosphingolipid amounts ranging from 2.5 ng to 10 μg. Similar tests were done with the crude enterotoxins of *V. cholerae* and *E. coli* at concentrations of up to 100 mg/ml or, in the case of *E. coli* P5 and 853/67, with the undiluted lysates. Registration of precipitation lines was done after diffusion for 4 days at room temperature, as well as after staining with Coomassie blue.

ELISA. The enzyme-linked immunosorbent assay (ELISA) technique of Engvall and Perlmann (4), as adapted to cholera serology by Holmgren and Svennerholm (12), was used with minor modifications. Isolated *V. cholerae* enterotoxin at a concentration of 2 μg/ml or crude *E. coli* enterotoxins at one-fifth concentrations of the original lysates was incubated in volumes of 0.5 ml in polystyrene test tubes at 37 C for 3 h to bind the antigens to the inner surface of the tubes. After removal of nonattached antigen by washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20, the tubes were incubated for 6 h at room temperature in a roller drum (60 rpm) with 0.5 ml of dilutions of antiserum in PBS-Tween, whereupon non-antigen-bound material was washed off with PBS-Tween. A 0.5-ml amount of anti-rabbit immunoglobulin (Ig)G conjugated by glutaraldehyde to alkaline phosphatase and diluted to an enzyme concentration of 0.2 ng/ml was then allowed to react with antigen-bound IgG antibodies, the tubes were thoroughly washed, and 1 ml of nitrophenylphosphate (1 mg/ml) was added as substrate. After suitable incubation time, the enzyme-substrate reaction was stopped by addition of 1 N NaOH, and the yellow color obtained was registered spectrophotometrically at 400 nm. Details about the reagents and procedures are given in a previous paper (12).

Glycosphingolipid sorbent assay (GSA). In 0.5-ml volumes the individual gangliosides and the neutral glycosphingolipids dissolved in TG buffer were coupled to the inner surface of disposable polystyrene tubes (11 by 70 mm) Nunc, Roskilde, Denmark) by mere incubation at 37 C for 4 to 5 h. Nonattached material was then removed by washing three times with PBS-Tween. Enterotoxin diluted in TG buffer was added in a volume of 0.5 ml, and the tubes were incubated at room temperature for 2 h in a roller drum (60 rpm) to permit binding to the glycosphingolipid. After washing three times with PBS-Tween, bound enterotoxin was demonstrated with a dilution of the anti-cholera toxin antiserum followed by anti-IgG conjugated with alkaline phosphatase, and nitrophenylphosphate as described for the ELISA technique (12). The extinction values recorded were transformed to extinction change per 100 min ($E_{100\ min}$).

Toxicity tests. The ileal loop technique and the intradermal test in rabbits were used to study the capacity of the gangliosides and the related neutral glycosphingolipids to inactivate the gut and the skin effects of enterotoxins.

In the loop tests 8- to 12-week-old partially inbred rabbits were used, and five or six loops were arranged in each animal as previously described (8) with the modification that the terminal 70 cm of ileum was rinsed with 20 ml of PBS before the loops were prepared. Isolated cholera toxin, 500 ng or 3 μg (approximately 1 × mean effective dose [ED_{50}] and 6 × ED_{50}, respectively) of this toxin, or crude *E. coli* enterotoxin diluted to approximately 2 × ED_{50} was mixed with various amounts of glycosphingolipid, incubated at 20 C for about 15 min in a volume of 2 ml, and then injected into a ligated loop. Each combination of materials was tested in randomized positions in two to six animals; positive controls (enterotoxin incubated in TG buffer) were tested in adjacent loops and each test also included a negative control (only the TG buffer).

In the skin assays, the rabbits were selected to weigh approximately 2 kg. In a volume of 100 μliters, 500 pg (3 to 5 blueing doses [BD]) of isolated cholera toxin or diluted *E. coli* enterotoxin (attempted concentration 3 to 4 BD/0.1 ml) which had been incubated with different amounts of glycolipid at 20 C for 15 min was tested, each combination in at least two positions in two animals. Positive and negative controls analogous to those described for the ileal loop tests were included in each animal.

Ligated small bowel loops were also used to study the possible interference by natural cholera toxoid with the secretory response to enterotoxins. The loops were preincubated with 1 ml of toxoid in various concentrations for 10 to 15 min, and then 1 ml containing 500 ng of isolated cholera toxin or 1 ml of a dilution of *E. coli* 853/67 enterotoxin containing approximately 2 × ED_{50} was injected. Positive (TG

followed by enterotoxin) and negative (toxoid followed by TG) controls were tested in adjacent loops.

Only data from rabbits responding properly to the controls are included. The rabbits varied in responsiveness to the lower dose of isolated cholera toxin used in the ileal loops experiments, and to the doses of *E. coli* enterotoxins used in the skin assays, which led to exclusion of results in about one-third of the tested animals.

RESULTS

Binding of enterotoxins to glycosphingolipids. Fixation studies of cholera toxin to glycosphingolipid by means of double diffusion-in-gel techniques (11) were extended to the crude *V. cholerae* toxins in addition to isolated toxin. The G_{M1} ganglioside was the only glycosphingolipid which caused precipitation; with this substance both the crude and the isolated toxin formed a single line which fused, *i.e.*, gave a "reaction of identity" on comparative analysis. Approximately 10 μg of the isolated toxin per ml and 100 mg of the crude toxin per ml was required for visible precipitation. Tested in the latter concentration or, in the case of the materials from strain P5 and 853/67, as undiluted preparations, none of the *E. coli* enterotoxins precipitated with any of the glycosphingolipids including G_{M1}. This could of course be due to an insufficient concentration of enterotoxin in these preparations, and therefore a sorbent technique, GSA, which is approximately 10,000-fold more sensitive than the gel precipitation test was elaborated to measure in vitro binding of enterotoxins to glycosphingolipids.

The GSA is based on the principle that, by mere incubation in polystyrene tubes, gangliosides and their related neutral glycosphingolipids attach to the plastic surface, probably with their hydrophobic ceramide portion. Good attachment was demonstrated for tritiated G_{M1}, and with ELISA for G_{A1}, tetrahexoside-GlcNAc, and globoside against which rabbit antisera were available. It is therefore assumed that, in addition, the other ceramide-containing glycosphingolipids attach to polystyrene in a similar manner. Incubation of glycosphingolipid-coated tubes with enterotoxin permits binding of the toxin to the glycosphingolipid, and the bound enterotoxin is immunospecifically detected by means of antibodies and the ELISA principle. The technique was elaborated with the G_{M1} ganglioside, isolated cholera toxin, and rabbit antiserum to this toxin. This antiserum had an ELISA IgG antibody titer of $10^{-4.9}$; for the GSA it was, when not otherwise specified, used diluted $10^{-4.0}$. For coating of the tubes in the GSA it was found that a G_{M1} concentration of 2 μg/ml gave maximal fixation of the cholera

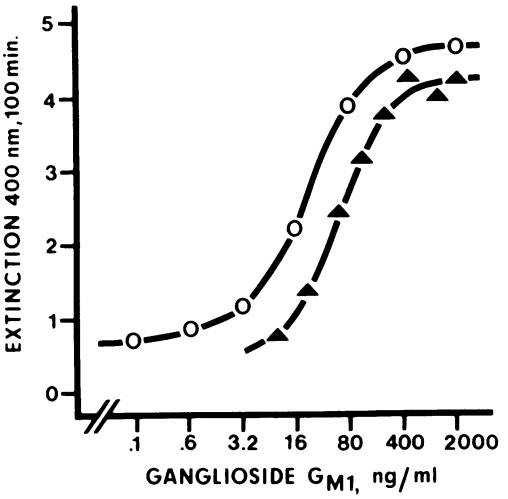

FIG. 1. *Binding of isolated cholera toxin (2 μg/ml) to polystyrene tubes coated with different concentrations of G_{M1} ganglioside. The test procedure was that of the GSA (see text), and the circles and triangles represent values obtained in experiments on different occasions.*

toxin, but 200 ng/ml was almost as efficient when tested with 2 μg/ml of isolated toxin (Fig. 1). However, the higher G_{M1} concentration permitted detection of lower concentrations of cholera toxin, 630 pg/ml compared to 3.5 ng/ml for an extinction of 1.0 per 100 min ($E_{100\ min}$) as shown in Fig. 2.

The enterotoxin of the *V. cholerae* culture filtrates bound to G_{M1} with a slope similar to that observed with pure toxin (Fig. 2). No difference was noted between the filtrates of Inaba and Ogawa bacteria; for $E_{100\ min} = 1.0$, approximately 65 μg/ml was required, i.e., approximately 20,000 times more than of the isolated toxin. Control experiments showed that lipopolysaccharide (LPS) of *V. cholerae* (serotype Inaba) did not bind to G_{M1} in GSA. This was assessed with purified LPS and antisera to LPS and to crude cholera enterotoxin, as well as with crude enterotoxin from *V. cholerae* (Inaba) and the anti-LPS antiserum; in no instance did the $E_{100\ min}$ exceed 0.10.

Immunological cross-reactivity between the enterotoxins of *V. cholerae* and *E. coli* was demonstrated with ELISA using tubes coated with isolated cholera toxin and anti-*E. coli* enterotoxin antiserum, as well as in tubes coated with crude *E. coli* enterotoxins and tested with antiserum to isolated cholera toxin. The ELISA IgG titer of the latter antiserum against the enterotoxin of *E. coli* 853/67 was $10^{-3.8}$, and the titer was similar against the other tested *E. coli* enterotoxins. This antiserum against isolated cholera toxin was

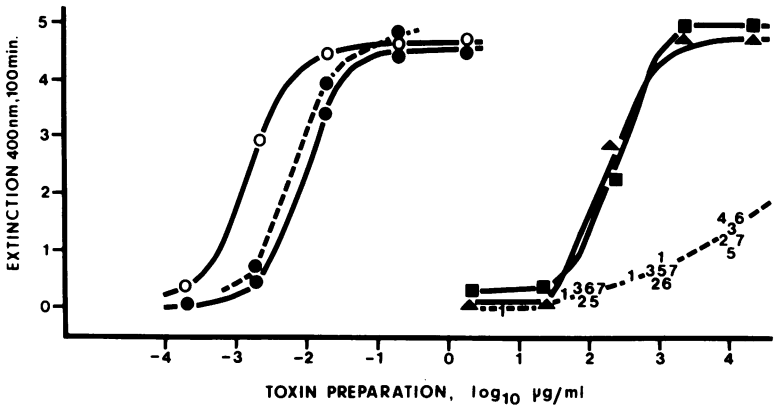

FIG. 2. *Comparison of the G_{M1}-binding properties in the GSA of isolated and crude V. cholerae enterotoxin and crude E. coli enterotoxin. The circles show results with isolated cholera toxin, and the squares and triangles show results with crude toxin of Inaba and Ogawa vibrios, respectively. The Arabic numerals show the values obtained with crude toxins of different E. coli strains (1 = 853/67, 2 = 887/64, 3 = B2577/67 I, 4 = 915/66, 5 = 163/67, 6 = B2577/67 II, 7 = 537/67). Broken and solid lines distinguish tests using anti-cholera toxin antiserum diluted 10^{-3} and 10^{-4}, respectively. For coating, 200 ng of G_{M1} per ml was used, except in the test represented with open circles when it was 2 µg/ml.*

therefore used in GSA to detect glycosphingolipid-bound *E. coli* enterotoxin; a serum dilution of $10^{-3.0}$ was chosen. It was found that *E. coli* enterotoxins also showed some binding to G_{M1} ganglioside (Fig. 2). However, the binding curves differed much from those observed with *V. cholerae* enterotoxin by being flatter and requiring more material for demonstrable binding (Fig. 2).

The binding properties of the isolated enterotoxin of *V. cholerae* and of the enterotoxin of *E. coli* 853/67 to a series of pure gangliosides and neutral glycosphingolipids was studied by the GSA and compared with the binding to G_{M1} (Table 1). Both enterotoxins bound to G_{M1} better than to any other substance, but at much different levels. Both toxins also showed binding to G_{D1a} but differed in their reactivity with G_{D1b}-G_T and G_{A1}. In general, the binding of the cholera toxin to certain substances but not to others was more distinct than was the case for the *E. coli* enterotoxin.

Inactivation of enterotoxins by glycosphingolipids. The capacity of G_{M1} and the other glycosphingolipids to inactivate the toxicity of *V. cholerae* enterotoxin in the skin and in the gut (11) was reinvestigated and compared with the effects of these compounds on the *E. coli* enterotoxins. Figure 3 shows the effect of pure G_{M1} on isolated cholera toxin and on enterotoxin from *E. coli* 853/67. The skin manifestations and the ileal loop activity of both toxins were inhibited by this ganglioside. However, much more G_{M1} was required for a corresponding degree of inactivation of the *E. coli* toxin than of the cholera toxin, for which approximately 1:1 molar ratios of G_{M1} to toxin were almost completely inactivating. The inactivation curve was also flatter for the *E. coli* toxin. Similar inactivation curves by G_{M1} were found for enterotoxins of other *E. coli* strains.

The inactivating capacity of a series of other glycosphingolipids on *E. coli* and *V. cholerae*

TABLE 1. *Binding of V. cholerae and E. coli enterotoxins to gangliosides and related neutral glycosphingolipids tested with a sorbent assay (GSA)*

Sorbent[a]	Enterotoxin[b]			
	V. cholerae	Buffer control	*E. coli*	Buffer control
G_{M3}	0.04[c]	0.11[d]	0.22[c]	0.12[d]
G_{M3}-NGN	0.03	0.07	0.15	0.06
G_{M2}	0.08	0.11	0.23	0.12
G_{M1}	4.68	0.12	0.90	0.13
G_{D1a}	2.06	0.07	0.35	0.09
G_{D1b}, G_T	0.16	0.13	0.45	0.18
G_{A1}	0.91	0.10	0.21	0.14
Tetrahexoside-GlcNac	0.16	0.12	0.18	0.12
Globoside	0.03	0.11	0.18	0.11
None	0.05	0.06	0.06	0.07

[a] A concentration of 2 µg/ml was used for coating of polystyrene tubes.

[b] Isolated cholera toxin, 2 µg/ml, or *E. coli* 853/67 enterotoxin, fivefold diluted.

[c] Extinction change per 100 min at 400 nm; anti-cholera toxin antiserum 10^{-4} with *V. cholerae* toxin and 10^{-3} with *E. coli* toxin.

[d] As in footnote c but incubation with TG buffer instead of enterotoxin.

enterotoxins was compared with that of G_{M1}. The glycosphingolipid amount required to give half-maximal inhibition of approximately three skin BD and 2 × ED_{50} ileal loop doses was calculated from curves of the type shown in Fig. 3. The values are given in Table 2. Enterotoxins from different strains of *E. coli* were inactivated by very similar amounts of G_{M1} ganglioside; in the skin test approximately 10,000-fold and in the loop assay 300-fold those required to inactivate the cholera toxin. The G_{M1} ganglioside was approximately 400 times more effective than any other substance in the inactivation of cholera toxin, whereas the G_{M1} specificity seemed less pronounced for inactivation of *E. coli* enterotoxin, although the various toxin preparations differed in this respect (Table 2).

Interference by natural cholera toxoid on the ileal secretory response to enterotoxins. Choleragenoid, a natural cholera toxoid, is almost entirely devoid of toxicity in spite of marked in vitro affinity for the G_{M1} ganglioside (11) and good binding to intestinal mucosal cells (14). The effect on the ileal loop response to cholera toxin and to enterotoxin from *E. coli* 853/67 by preincubating the loops with this toxoid for 10 to 15 min was analyzed (Table 3). It was found that the response to cholera toxin was reduced by equimolar and higher amounts of the toxoid. By contrast, the toxoid caused no

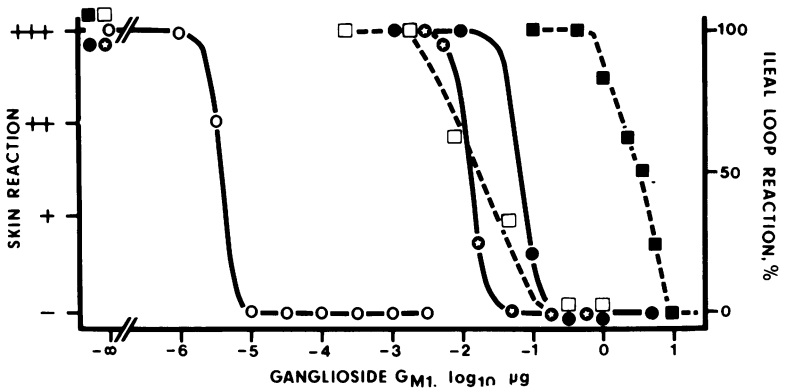

FIG. 3. *Tests of the inactivating capacity of G_{M1} ganglioside on the skin response to 500 pg of isolated cholera toxin (○) and approximately 3 BD of E. coli 853/67 enterotoxin (□), and on the ileal loop response to the cholera toxin (◉, 500 ng; ●, 3 µg) and the E. coli toxin (■, approximately 2 × ED_{50} which means a toxin amount 150-fold that used in the skin test).*

TABLE 2. *Inactivating capacity of various gangliosides and neutral glycosphingolipids on the ileal loop, and skin toxicity of enterotoxins of V. cholerae and E. coli*

Substance	*V. cholerae*		*E. coli* 853/67		*E. coli* B2577/67				*E. coli* 915/66	
					I		II			
	Gut	Skin	Gut	Skin	Gut	Skin	Gut	Skin	Gut	Skin
G_{M3}	>5[a]	>0.005	>5	>2	NT[b]	~2	NT	~1	NT	>2
G_{M3}-NGN	>5	>0.005	>5	>2	NT	>2	NT	>2	NT	>2
G_{M2}	>5	>0.005	>5	>2	NT	~1	NT	~1	NT	>2
G_{M1}	0.01	0.000004	3	0.02	1-5	0.03	1-5	0.04	1-5	0.05
G_{D1a}	1-5	0.0016	5	>2	NT	~1	NT	~1	NT	>2
G_{D1b}, G_T	>5	>0.005	>10	>2	NT	~1	NT	~ 1	NT	>2
G_{A1}	1-5	0.0024	>5	>2	NT	~1	NT	~1	NT	>2
Tetrahexoside-GlcNAc	>5	>0.005	>5	>2	NT	2	NT	~2	NT	>2
Globoside	>5	>0.005	>5	>2	NT	NT	NT	~2	NT	>2

[a] Micrograms required for half-maximal inhibition of approximately 2 × ED_{50} in the loop assays and of 3 BD in the skin tests.

[b] Not tested.

TABLE 3. *Effect on ileal loop responsiveness to V. cholerae and E. coli enterotoxins by preincubation of loops with choleragenoid*

Choleragenoid[a] (μg)	Intestinal response to enterotoxins of	
	V. cholerae[b]	*E. coli*[c]
10	0.00 (n = 2)[d]	1.50 (n = 2)
5.6		1.93 (n = 4)
3.3	0.00 (n = 2)	2.00 (n = 2)
1.7		1.83 (n = 6)
1.0	0.27 (n = 7)	
0.56		1.88 (n = 6)
0.33	0.58 (n = 4)	
0.19		1.55 (n = 2)
0.10	1.43 (n = 4)	
0.06		1.40 (n = 2)
0	1.48 (n = 23)	1.55 (n = 12)

[a] A 1-ml amount injected 10 to 15 min before injection of enterotoxin.

[b] Isolated toxin injected, 500 ng in 1 ml.

[c] Crude toxin of strain 853/67 injected, approximately 2 × ED_{50} in 1 ml.

[d] Arithmetic mean of fluid accumulation in loops expressed as milliliters of fluid per centimeter of gut; number of tested loops is within parentheses.

reduction of the response to the *E. coli* enterotoxin.

DISCUSSION

Interference with the binding of bacterial toxins to their tissue receptors leading to inhibition of the toxic manifestations can be effected by blocking the binding site of the toxin with a substance identical to or closely resembling the receptor structure, or by occupying the cellular receptors with an inactive toxin analogue. Previous studies have shown that the monosialoganglioside G_{M1} probably corresponds to the receptor structure for the cholera toxin, and that a natural cholera toxoid binds to this ganglioside with similar affinity as the active toxin and therefore may be regarded as an inactive cholera toxin analogue (10, 11). The G_{M1} ganglioside and the natural cholera toxoid were therefore used in the present study as probes to elucidate whether the intestinal hypersecretion which is caused both by enterotoxin of *V. cholerae* and by enterotoxin of *E. coli* is initiated through the binding of these two types of toxins to the same or to different cellular binding sites.

A whole series of pure and structurally defined gangliosides and neutral glycosphingolipids was tested in addition to pure G_{M1}, and not only was inactivation of toxicity measured but also the primary binding of enterotoxins to the glycosphingolipids. These precautions are important to permit evaluation of the specificity and the nature of observed interactions, since tissue lipid extracts devoid of gangliosides can cause a nonspecific inactivation of cholera enterotoxin (J. Holmgren and L. Svennerholm, unpublished results). In this study it was shown that the cholera enterotoxin binds much better than *E. coli* enterotoxins to the G_{M1} ganglioside, and also that this ganglioside inactivates the cholera toxin much better than the *E. coli* enterotoxins. The other glycosphingolipids were less effective than G_{M1} both to bind and to inactivate enterotoxins, but the difference between G_{M1} and other substances was more distinct for the cholera toxin than for *E. coli* enterotoxins. From these studies it cannot be stated with certainty whether the binding properties of the *E. coli* enterotoxins to G_{M1} ganglioside are significant with regard to diarrheogenic action. However, it is noteworthy that G_{D1a} and G_{A1} bound cholera toxin to a higher extinction value than reached with the G_{M1}-bound *E. coli* enterotoxins. These substances were approximately 400 and 600 time less efficient than G_{M1} to inactivate the cholera toxin and are not likely to function as receptors for this toxin, which might suggest that the binding of *E. coli* enterotoxin to G_{M1} is probably pathogenetically insignificant. It is also unlikely that any of the other glycosphingolipids tested would correspond to the functional receptor for *E. coli* enterotoxin.

Despite affinity similar to that of the cholera toxin for the G_{M1} ganglioside (11) and binding properties similar to those of the toxin to mammalian cell membranes (J. Holmgren, L. Lindholm, and I. Lönnroth, unpublished results), the natural cholera toxoid, choleragenoid, is almost entirely devoid of toxicity. In this study, brief preincubation of ileal loops with the natural toxoid made the loops resistant to the cholera toxin; an inhibitory effect was seen with equimolar and higher amounts of toxoid compared to toxin. These findings suggest that the cholera toxoid, through binding to the same intestinal cell membrane receptors as the cholera toxin, can occupy these sites so that later added toxin cannot bind to the intestinal cells and therefore is incapable to initiate fluid accumulation in the loops. The fact that the toxoid has no toxic activity shows that only the capacity to bind to the receptors for the cholera toxin is not by itself sufficient to activate intestinal cells to hypersecretory processes.

In contrast to the cholera toxin, the *E. coli* enterotoxin tested had full effect in ileal loops preincubated with natural cholera toxoid. This indicates that the functional receptors for the *E.*

coli enterotoxin are different from the intestinal binding sites for the *V. cholerae* enterotoxin and its natural toxoid. Since the latter proteins probably bind to cell membrane-located G_{M1} molecules, this is another indication that the observed low-avidity in vitro binding of the *E. coli* enterotoxins to G_{M1} is probably not an important in vivo event for cellular activation resulting in intestinal hypersecretion.

Until reproduced with pure toxins, as yet unavailable, results obtained with *E. coli* enterotoxin preparations should be interpreted with some caution. The relative inefficiency of the ganglioside G_{M1} in inactivating the *E. coli* preparations and the failure of the natural cholera toxoid to block the ileal loop effects of *E. coli* enterotoxin could possibly be ascribed to the presence of heat-stable enterotoxin acting independent of the heat-labile toxin. However, this possibility seems ruled out by the observations that the *E. coli* enterotoxin preparations used lost their skin and ileal loop toxicity by heating at 65 C for 10 to 15 min, and by incubation with the antisera to *E. coli* P5 enterotoxin or to isolated cholera toxin (J. Holmgren, O. Söderlind, and T. Wadström, Acta Pathol. Microbiol. Scand. B, in press, and O. Söderlind, personal communication), since these procedures should not affect the heat-stable, nonantigenic type of enterotoxin (3, 16). Another possibility which is difficult to exclude with certainty is that potent sialidase production by the enterotoxigenic *E. coli* could have caused the failure of cholera toxoid to inhibit the fluid accumulation. This enzyme, if present in the crude enterotoxin used, could have converted di- and trisialogangliosides on the cell surface to G_{M1} ganglioside (11, 18), thereby providing new receptors uncovered by the toxoid and available for *E. coli* enterotoxin (although still in competition with remaining unbound toxoid). This alternative is less likely, since ganglioside-converting sialidase activity was not demonstrable in the *E. coli* enterotoxin preparation used in this experiment (J. Holmgren, J.-E. Månsson and L. Svennerholm, unpublished results).

Some of the results obtained are of methodological interest. The use of G_{M1}-coated plastic tubes in a "receptor-specific" sorbent assay permitted exceptionally sensitive in vitro measurement of cholera enterotoxin. With the immunological detection used, toxin concentrations below 1 ng/ml were measured. It is also possible to quantitate cholera toxin in G_{M1}-coated tubes by means of a radiosorbent modification with iodine-labeled cholera toxin, and a further use of polystyrene coated with G_{M1} is for affinity chromatography with cholera toxin (unpublished data). It is possible that the latter approach could be useful for purification of *E. coli* enterotoxins, since these toxins as demonstrated bind to the G_{M1} ganglioside, although with low affinity. An alternative way suggested by this investigation to purify these toxins could be through immunosorbent affinity chromatography using antibodies to the cholera toxin, since such antibodies showed binding to *E. coli* enterotoxins.

During the preparation of this manuscript independent work by N. F. Pierce was published which also made use of cholera toxoid and of ganglioside to elucidate the intestinal receptors for enterotoxins of *V. cholerae* and *E. coli* (15). The results of the two studies complement each other and provide mutual support for the conclusions reached.

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ADDENDUM IN PROOF

After submission of this manuscript, reports by P. Cuatrecasas (Biochemistry **12:** 3547–3588, 3559–3566) and C. A. King and W. E. van Heyningen (J. Infect. Dis **127:** 639–647) were published which further document that G_{M1} is the receptor for cholera toxin.

LITERATURE CITED

1. Björndahl, H., C. G. Hellerqvist, B. Lindberg, and S. Svensson. 1970. Gas-liquid chromatography and mass spectrometry in methylation analysis of polysaccharides. Angew. Chem. Int. Ed. Engl. **9:**610–619.
2. Björndahl, H., B. Lindberg, and S. Svensson. 1970. Gas-liquid chromatography of partially methylated alditols as their acetates. Acta Chem. Scand. **21:**1801–1804.
3. Craig, J. P. 1972. The enterotoxic enteropathies, p. 129–155. *In* H. Smith and J. H. Pearce (ed.), Microbial pathogenicity in man and animals. Cambridge University Press, Cambridge.
4. Engvall, E., and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109:**129–135.
5. Finkelstein, R. A., and J. J. LoSpalluto. 1970. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. **121:**S63–S72.
6. Hakomori, S. 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. J. Biochem. Tokyo **55:**205–208.
7. Hakomori, S., B. Siddiqui, Y.-T. Li, S.-C. Li, and C. G. Hellerqvist. 1971. Anomeric structures of globoside and ceramide trihexoside of human erythrocytes and hamster fibroblasts. J. Biol. Chem. **246:**2271–2277.

8. Holmgren, J., Å. Andersson, G. Wallerström, and Ö. Ouchterlony. 1972. Experimental studies on cholera immunization. II. Evidence for protective antitoxic immunity mediated by serum antibodies as well as local antibodies. Infect. Immunity **5**:662-667.
9. Holmgren, J., I. Lönnroth, and Ö. Ouchterlony. 1971. Immunochemical studies of two cholera toxin-containing preparations of *Vibrio cholerae*. Infect. Immunity **3**:747-755.
10. Holmgren, J., I. Lönnroth, and L. Svennerholm. 1973. Fixation and inactivation of cholera toxin by G_{M1}, ganglioside. Scand. J. Infect. Dis. **5**:77-78.
11. Holmgren, J., I. Lönnroth, and L. Svennerholm. 1973. Tissue receptor for cholera exotoxin: postulated structure from studies with G_{M1} ganglioside and related glycolipids. Infect. Immunity **8**:208-214.
12. Holmgren, J., and A.-M. Svennerholm. 1973. Enzyme-linked immunosorbent assays for cholera serology. Infect. Immunity **7**:759-763.
13. Li, Y.-T., and S.-C. Li. 1971. Anomeric configuration of galactose residues in ceramide trihexoside. J. Biol. Chem. **246**:3769-3771.
14. Peterson, J. W., J. J. LoSpalluto, and R. A. Finkelstein. 1972. Localization of cholera toxin in vivo. J. Infect. Dis. **126**:617-628.
15. Pierce, N. F. 1973. Differential inhibitory effects of cholera toxoids and ganglioside on the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. J. Exp. Med. **137**:1009-1023.
16. Pierce, N. F., W. B. Greenough III, and C. C. J. Carpenter. 1971. *Vibrio cholerae* enterotoxin and its mode of action. Bacteriol. Rev. **35**:1-13.
17. Smith, N. W., and R. B. Sack. 1973. Immunologic cross-reactions of enterotoxins from *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. **127**:164-170.
18. Svennerholm, L. 1963. Chromatographic separation of human brain gangliosides. J. Neurochem. **10**:613-623.
19. Svennerholm, L. 1972. Gangliosides, isolation p. 464-474. *In* R. L. Whistler and J. N. BeMiller (ed.), Methods in carbohydrate chemistry, vol. 6. Academic Press Inc., New York.
20. van Heyningen, W. E., C. C. J. Carpenter, N. F. Pierce, and W. B. Greenough III. 1971. Deactivation of cholera toxin by ganglioside. J. Infect. Dis. **124**:415-418.