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Rabbit Intestinal Glycoprotein Receptor for *Escherichia coli* Heat-Labile Enterotoxin Lacking Affinity for Cholera Toxin

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The receptors for cholera toxin and *Escherichia coli* heat-labile toxin (LT) in rabbit small intestinal epithelium were characterized and compared. (i) In vivo studies in ligated intestinal loops showed that whereas LT B subunits could block the fluid secretogenic action of purified LT as well as cholera toxin, cholera toxin B subunits did not inhibit the LT response even when tested in a concentration 100-fold higher than one which gave complete blocking of cholera toxin action. (ii) In vitro studies indicated that isolated intestinal epithelial cells or brush-border membranes could bind about 10-fold more of *E. coli* LT than of cholera toxin. (iii) All binding sites for cholera toxin in duodenal, jejunal, or ileal mucosal cells or brush-border membranes were extracted by chloroform-methanol-water (4:8:3), which removed lipids quantitatively but did not extract glycoproteins. The extracted cholera toxin binding sites were to >95% recovered in a monosialoganglioside fraction; quantitatively these sites closely corresponded to the concentration of chromatographically identified mucosal GM1 ganglioside (1 nmol of cholera toxin was bound per 1 to 2 nmol of GM1). In contrast, a substantial fraction of mucosal binding sites for *E. coli* LT remained in the delipidized tissue residue, and these sites had properties consistent with a glycoprotein nature. Thus, whereas cholera toxin appeared to bind highly selectively to GM1 ganglioside receptor sites of rabbit small intestine, *E. coli* LT bound both to GM1 ganglioside and to a main glycoprotein receptor for which cholera toxin lacks affinity.

The heat-labile enterotoxins of *Escherichia coli* (LT) and *Vibrio cholerae* (cholera toxin) have many properties in common (for recent reviews see references 11, 15, 19, 22, 31, and 35). Both toxins, after binding to specific receptors on the luminal surface of the intestinal mucosal cells, stimulate the adenylate cyclase-cyclic AMP system of the exposed cells by enzymatically modifying a regulatory component of adenylate cyclase. Structurally, cholera toxin and *E. coli* LT are similar, too, both being composed of five B subunits which mediate binding to cell surface receptors, and an A subunit which contains the enzymic toxic activity (11, 14, 15, 19, 22, 31, 35). The cholera and *E. coli* A subunits cross-react immunologically, as do the B subunits (4, 43); of the 103 amino acid residues of the B subunits only 20 have been found to differ between the two toxins (9).

The ganglioside GM1 has been identified as receptor for cholera toxin in various cell types (for recent reviews, see references 12, 19, 20, and 22). GM1 specifically binds and inactivates cholera toxin (8, 24, 26), and studies of various cells, including small intestinal mucosal cells of different species, have demonstrated a direct relationship between the cell content of GM1 and the number of binding sites for cholera toxin (16, 23). Furthermore, exogenous GM1 has been found to be incorporated into the membrane of exposed cells and thus sensitize the cells to cholera toxin, supporting the hypothesis that GM1 functions as a biological cholera toxin receptor (7, 23, 29). Morita et al. (28) reported the additional presence of cholera toxin-binding glycoproteins in rat intestinal microvillus membranes, but this could not be confirmed by Critchley et al. (6), who found all of the cholera toxin-binding activity of such membranes to be associated with a glycolipid which chromatographically behaved as GM1 ganglioside.

The nature of the receptor(s) for *E. coli* LT is less well established. Similar to cholera toxin LT is known to bind avidly to GM1 in vitro and to be inactivated by this ganglioside (18). A diagnostic assay of LT in diarrheal stools or bacterial culture filtrates is based upon the receptor-like binding of the toxin to plastic-adsorbed GM1 ganglioside (1, 34, 38). Moreover, the incorporation of GM1 ganglioside into a ganglioside-

deficient cell line of mouse fibroblasts was found to sensitize these normally toxin-resistant cells to form cyclic AMP in response to both cholera toxin and *E. coli* LT (30). However, choleragenoid, a protein composed of the B subunits of cholera toxin, was unable to inhibit the biological activity of LT in rabbit intestine even though it effectively blocked the action of cholera toxin (18, 33); thus, it was unclear whether *E. coli* LT and cholera toxin actually shared the same receptors in the intestine, which is the natural target tissue for the toxins.

MATERIALS AND METHODS

Cholera toxin, CT-B, and antiserum. Highly purified cholera toxin was purchased from Sigma Chemical Co. (St. Louis, Mo.). After the lyophilized toxin was dissolved, it was kept in a concentration of 1 mg/ml at −70°C, and a fresh sample was used and further diluted for each experiment. Highly purified cholera B subunit (CT-B) was prepared from affinity-purified cholera toxin (40) as described previously (25). Specific, high-titer antiserum to cholera toxin was prepared by repeated subcutaneous immunization of a rabbit with purified toxin; the serum was prepared from a bleeding taken after three injections with 30 μg of toxin per injection.

***E. coli* LT, B subunits (LT-B), and antiserum.** Highly purified *E. coli* LT was prepared from the LT-only strain 286C2 (isolated from a patient with diarrhea), kindly provided by D. Robertson (University of Kansas, Lawrence). Concentrated crude LT was prepared from this strain as described earlier (43), and the LT was subsequently purified essentially by the procedure of Clements and Finkelstein (5) by a sequence of precipitation with ammonium sulfate, affinity chromatography on a Bio-Rad agarose column (Bio-Rad Laboratories, Richmond, Calif.), and gel filtration on Sephacryl S-200 (Pharmacia, Uppsala, Sweden). The protein concentration of the purified LT was determined with Bio-Rad protein assay based on the method of Bradford (2) with purified cholera toxin as a reference. The LT protein was shown to give a single-band precipitate in Ouchterlony immunodiffusion tests against anti-crude LT antiserum and to have biological activity (fluid accumulation in ligated intestinal loops of rabbits) and GM1-binding activity (GM1-enzyme-linked immunosorbent assay [ELISA] tests, see below) similar to those of highly purified LT prepared and kindly provided by D. Robertson or of purified cholera toxin. Crude LT, when used, was examined for its specific content of LT protein by determination of its GM1-ELISA titer in comparison with purified LT as well as by single radial immunodiffusion tests of concentrated crude toxin against anti-purified LT serum with purified LT as a reference.

Culture filtrate from an *E. coli* strain carrying a hybrid DNA B-only plasmid providing synthesis of the LT B protomer (LT-B), but not any A subunit or holotoxin, was kindly provided by N. Zygraich (Smith, Kline & French Laboratories, Belgium). It was concentrated against polyethylene glycol to give a concentration of 50 μg of LT-B protein per ml as determined by GM1-ELISA tests. The LT-B preparation was shown to lack any detectable A subunit as tested with a specific anti-A subunit antiserum as well as any toxic activity as assayed in rabbit ligated intestinal loops.

Specific antiserum to *E. coli* LT was prepared by giving a rabbit three subcutaneous injections with the purified LT (20 μg of LT protein) at 3-week intervals; the first two injections were given together with Freund complete adjuvant, whereas the last injection was without adjuvant. Serum was prepared from a bleeding 10 days later, and the antiserum was shown to have a high anti-LT titer and to give a single-line precipitate with crude 286 C2 culture filtrate in immunodiffusion tests which fused (reaction of identity) with the precipitation line obtained with the purified LT preparation.

Receptor blocking tests in rabbit intestine. Rabbits (1.7 to 2.0 kg) who had been given no food but water ad libitum for the past 24 h were used. Animals were anesthetized, and the jejunum and ileum were ligated into 22 to 28 5-cm loops (37). Into each loop was then injected 1.0 ml of either CT-B or LT-B in various concentrations, or for comparison 1.0 ml of buffer (phosphate-buffered saline [PBS]: 0.15 M NaCl–0.01 M sodium phosphate, pH 7.2, with 0.1% bovine serum albumin [BSA]); these solutions were placed in randomly positioned loops, each dilution of CT-B or LT-B or the buffer being tested in at least two loops in each animal. After fifteen min each loop was injected with 0.5 ml of either purified cholera toxin (0.1 μg per 0.5 ml of PBS with 0.1% BSA) or purified *E. coli* LT (0.05 μg per 0.5 ml of PBS with 0.1% BSA) in alternating animals. "Negative" controls were also tested in each animal; they were the PBS-BSA buffer without toxin and with the highest concentration of the blocking agent (500 μg of CT-B per ml or 50 μg of LT-B protein per ml). The ligated intestine was then put back into the abdominal cavity of the animal, and the abdominal cavity was then closed with sutures. Results were registered after 18 h by determining the volume of accumulated fluid per centimeter of intestine.

Preparation of epithelial cells and brush-border membranes. Rabbits who had fasted for 24 h but had received water ad libitum were killed by rapid intravenous injection of sodium barbiturate (Mebumal). The small intestine was excised surgically and rinsed with ice-cold PBS. The intestine was opened longitudinally and then cut into pieces which were thoroughly rinsed with ice-cold PBS. The tissue was placed in phosphate-buffered citrate (0.096 M NaCl, 0.008 M KH_2PO_4, 0.027 M sodium citrate, 0.056 M Na_2HPO_4, 0.0015 M KCl) at pH 7.0 for 30 min at 37°C with intermittent stirring (3). It was then transferred to calcium- and magnesium-free Hanks balanced salt solution (0.14 M NaCl, 0.005 M KCl, 0.005 M D-glucose, 0.0005 M Na_2HPO_4, 0.0005 M KH_2PO_4), containing EDTA (1 mM) and incubated for 30 min at 4°C. The epithelial cells were scraped off the mucosa and incubated with stirring for 15 min at 4°C in Hanks balanced salt solution-EDTA. The cell suspension was then filtered through a nylon mesh and centrifuged (600 × *g* for 5 min). The cells were washed three times with ice-cold Hanks balanced salt solution-EDTA and suspended in PBS–0.2% BSA for use in the studies.

Brush-border membranes of rabbit jejunum were prepared essentially by the procedure of Miller and Crane (27). The small intestine was removed as for the preparation of epithelial cells. About 30 cm of the jejunum was thoroughly washed with ice-cold PBS.

The mucosa was scraped into 100 ml of cold 0.005 M EDTA (pH 8.0) and homogenized in an MSE Ato mix blender at 12,000 rpm for 30 s. The homogenate was filtered through a nylon net and then centrifuged at 1,500 × *g* for 10 min at 4°C. The pellet was suspended in 0.005 M EDTA (pH 8.0) and recentrifuged as above. This washing and centrifugation was repeated until the supernatant was clear. The final pellet was then washed twice with ice-cold PBS and used for the experiments.

Extraction and separation of lipids. The mucosa of duodenum, jejunum, and ileum of rabbits were isolated by scraping, and the epithelial cells were washed twice by centrifugation. These cells or brush-border membranes from the jejunum were homogenized in a Potter-Elvehjem homogenizer at 4°C and extracted twice, each time with 20 volumes of chloroform-methanol-water (4:8:3, by volume) (39). The lipid extracts were centrifuged at 1,000 × *g* for 10 min, and the resulting tissue pellets—the delipidized tissue residue—were collected.

The two lipid extracts were combined, evaporated to dryness, and redissolved in chloroform-methanol-water (60:30:4.5, by volume). Salt and low-molecular-weight contaminants were removed by gel chromatography on Sephadex G-25 (42). Total lipids were separated into neutral and acidic fractions by anion exchange chromatography on Spherosil-DEAE-Dextran (13). The neutral lipids were eluted from the resin with 10 volumes of chloroform-methanol-water (60:30:4.5, by volume). The gangliosides were subsequently eluted by potassium acetate in methanol in a monosialoganglioside fraction and a second fraction containing two or more sialic acids, termed the di- plus polysialoganglioside fraction (13).

Neutral glycosphingolipids were separated from other neutral lipids by silica gel chromatography (P. Fredman, G. W. Klinghardt, O. Nilsson, and L. Svennerholm, Biochem. J., in press) followed by peracetylation and subsequent chromatography on Florisil (36). The isolated neutral glycosphingolipids were separated into two fractions by silica gel chromatography. The first fraction, eluted from the gel with chloroform-methanol (4:1, by volume), contained mono- and diglycosylceramides; the second fraction, eluted with chloroform-methanol-water (60:35:8, by volume), contained all higher oligoglycosylceramides, here termed tri- plus polyglycosylceramides.

Monosialogangliosides were eluted from the resin together with acidic phospholipids and sulfatides. After desalting on Sephadex G-25 (42) the contaminating lipids were removed by saponification and subsequent silica gel chromatography as described elsewhere (Fredman et al., in press). The fraction, containing the di- plus polysialogangliosides, was desalted by dialysis and then purified by silica gel chromatography, which reduced any possible contamination of glycoproteins or glycopeptides in the fraction. The neutral glycolipids and gangliosides isolated were rechromatographed on the anion-exchange resin Spherosil-DEAE-Dextran.

The total amount of ganglioside was measured as sialic acid by the resorcinol method, and the ganglioside pattern was determined by thin-layer chromatography and densitometric scanning (39). Neutral glycosphingolipids were determined by measuring the sphingosine by a modification (17) of the methyl orange method of Trams and Lauter (41). None of the glycolipid fractions was detectably contaminated with protein (<10 μg/g [wet weight] of the fraction).

Delipidized tissue residues. Epithelial cells and brush-border membranes were extracted with chloroform-methanol-water and centrifuged as described above. The delipidized pellet was homogenized in PBS with a glass pestle and then placed in an ultrasonic bath for 10 min, a procedure repeated three times. The resulting suspension was used for the experiments. The pellet was free from lipid as assayed by determination of fatty acids after hydrolysis with 1 M HCl.

Periodate oxidation and proteolytic digestion of brush-border membranes. Sodium periodate to a final concentration of 0.05 M was added to a suspension of the delipidized brush-border membrane fraction (5 mg/ml). The mixture was kept in the dark for 48 h at 4°C and then centrifuged at 3,000 × *g* for 5 min. The pellet was suspended in PBS and recentrifuged. This washing and centrifugation was repeated four times to get rid of the periodate. Suspensions of the last pellet were used in the experiments.

A mixture of the delipidized brush-border membrane material (100 mg), pronase (Sigma; 10 mg), and urea (360 mg) in 2 ml of 0.1 M Tris buffer (pH 8.3) was incubated at 37°C for 72 h. After 48 h another portion of pronase (4 mg) was added. The incubation was terminated by boiling for 10 min and then centrifuged at 10,000 × *g* for 2 min. The supernatant was desalted on a Sephadex G-10 column (0.9 by 12.5 cm) and then used.

Assay of toxin binding. Binding of cholera toxin or *E. coli* LT to intestinal cells, brush-border membranes, or tissue extracts was assayed indirectly by determining the amount of residual, unbound toxin after incubations were completed. The tests were performed by preparing two rows containing serial dilutions of 200 μl of mucosal cells (or brush-border membranes or tissue extract fractions) in cold PBS-BSA. To the first row was added an equal volume of purified cholera toxin, and to the second row was added the same volume of crude or purified *E. coli* LT; the concentration of the toxins in the final mixture was in all cases the same: 0.025 μg/ml in PBS–0.2% BSA buffer. The mixtures were incubated for 1 h at 21°C, the cells were sedimented by centrifugation at 3,000 × *g* for 10 min, and the supernatant fluid was transferred to new tubes. One hundred microliters of the final supernatant fluid was then tested for its concentration of residual toxin with the GM1-ELISA procedure (see below).

GM1-ELISA. Concentrations of cholera toxin or *E. coli* LT were determined by the GM1-ELISA method, a procedure described in detail previously (21, 38). In short, the wells of polyvinyl microtiter plates (ELISA plates; Dynatech, Plochingen, West Germany) were coated with GM1 ganglioside (0.1 ml of 1.5 μM concentration at 21°C for 18 to 20 h), unoccupied plastic sites were blocked with 1% BSA, and then the toxin-containing test solution was added. Cholera toxin or *E. coli* LT bound to the wells was then assayed immunologically by sequential incubations with rabbit antitoxin, goat anti-rabbit immunoglobulin conjugated with alkaline phosphatase, and alkaline phosphatase substrate. Absorbance readings at 405 nm for unknown test samples were compared with readings for known concentrations of cholera toxins or *E. coli* LT tested concurrently.

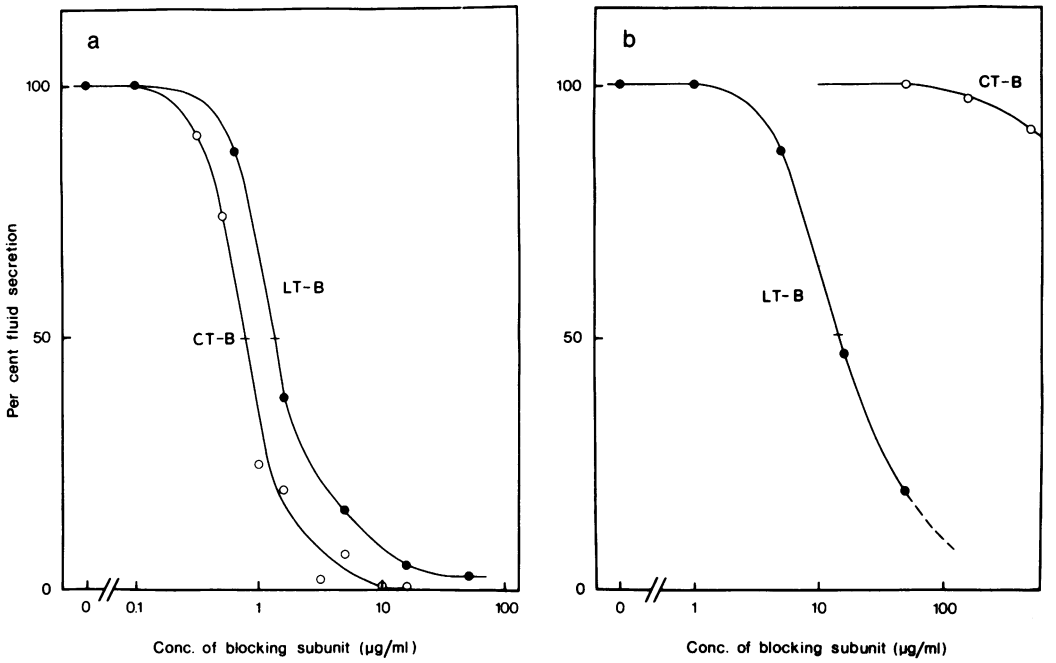

FIG. 1. Blocking of cholera toxin (a) or *E. coli* LT action (b) in rabbit intestine by cholera (CT-B) or *E. coli* (LT-B) binding subunits. Mean values of three to six experiments in different animals are shown. The uninhibited cholera toxin-induced fluid accumulation observed in the absence of blocking subunits was 1.51 ± 0.14 ml of fluid per cm of intestine (=100%), and uninhibited *E. coli* LT secretion was 0.94 ± 0.21 ml/cm.

RESULTS

In vivo blocking studies with B subunits. The B subunits of *E. coli* LT (LT-B) and cholera toxin (CT-B) were tested for their ability to inhibit the biological activity of LT and cholera toxin in rabbit intestine by blocking the mucosal receptors for the respective toxins.

The results are summarized in Fig. 1. Whereas cholera toxin activity was completely blocked by either CT-B or LT-B, *E. coli* LT activity was only blocked by LT-B and not by CT-B. Half-maximal inhibition of cholera toxin activity was attained by about 1 μg of CT-B per ml and by 1.5 to 2 μg of LT-B per ml (Fig. 1a). *E. coli* LT was inhibited 50% by 10 to 15 μg of LT-B per ml, but less than 10% by as much as 500 μg of CT-B per ml (Fig. 1b). The different blocking effect of LT-B as compared with CT-B on *E. coli* LT intestinal action (Fig. 1b) was statistically significant; at either 15 or 50 μg of LT-B per ml the reduction of LT-induced fluid output was greater ($P < 0.05$) than that observed with 50, 150, or 500 μg of CT-B per ml, and when loop responses of these two LT-B concentrations were combined and compared with the combined results for the three above CT-B concentrations the difference was highly significant ($P < 0.001$).

Control experiments showed that cholera toxin and *E. coli* LT activities in rabbit intestine were completely inhibited by preheating the toxins at 80°C for 15 min or by preincubation with 1 μM GM1 ganglioside or with either rabbit anti-cholera toxin or anti-LT (data not shown).

Toxin-binding capacity of isolated mucosal cells. The capacity of isolated epithelial cells from rabbit small intestine to bind cholera toxin and *E. coli* LT, respectively, was determined.

Whereas the slope of the binding curves did not differ much for the two toxins, the intestinal cells could be diluted manyfold more for the same degree of LT binding as compared with cholera toxin binding (Fig. 2a). Table 1 summarizes results of five separate experiments on cells from different animals. As a mean half-maximal binding of LT occurred at about a cell concentration 13 times lower than that giving similar binding of cholera toxin, consistent with a correspondingly higher number of binding sites for LT than for cholera toxin on these cells.

Studies with isolated brush-border membranes showed a toxin-binding pattern very similar to that described above (Fig. 2b). The membranes consistently bound larger quantities of *E. coli* LT than of cholera toxin. Periodate treatment of brush-border membranes was found to completely inactivate the binding sites for cholera toxin and *E. coli* LT (data not shown).

Interaction of toxins with glycolipids. The different ganglioside and neutral glycolipid frac-

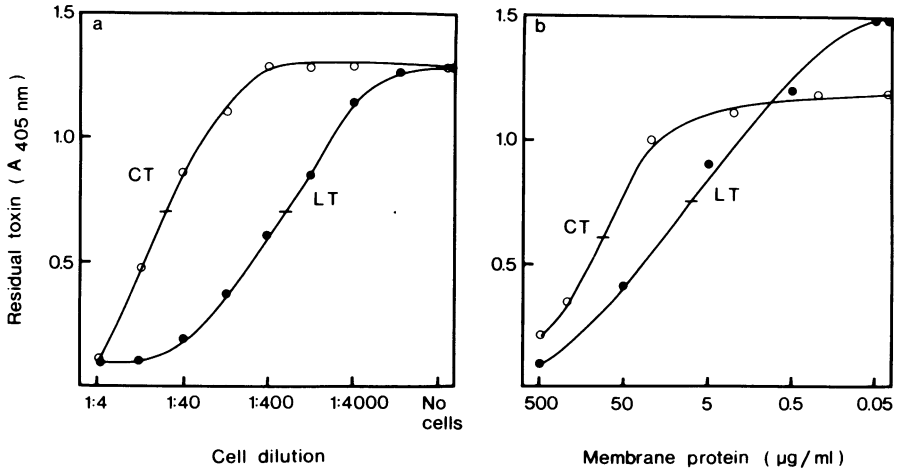

FIG. 2. Binding of cholera toxin (CT) and *E. coli* LT by rabbit small intestinal epithelial cells (a) or brush-border membranes (b). Binding was calculated indirectly by determination with the GM1-ELISA method of the amount of residual, unbound toxin after incubation and centrifugation of a mixture of the toxin (0.025 µg/ml) and serial dilutions of the cells or membranes.

tions of duodenal, jejunal, and ileal mucosal cells were tested by the GM1-ELISA method for their ability to inhibit *E. coli* LT and cholera toxin from binding to GM1-coated tubes. Each fraction was tested in serial dilutions against a fixed concentration of either toxin (Fig. 3). The results show that for cholera toxin more than 95% of the total extracted inhibitory activity was found in the monosialoganglioside fraction; only minor activity was found in the di- plus polysialoganglioside and neutral tri- plus polyglycosylceramide fractions.

The *E. coli* LT inhibitory activity of the monosialoganglioside and other glycolipid fractions did not differ much from that observed with cholera toxin (Fig. 3 and Table 2); this is consistent with the finding that purified reference brain GM1 ganglioside had only twofold stronger inhibitory activity for cholera toxin than for LT (Table 2). The fractions from different parts of the intestine gave in general very similar results, although ganglioside fractions of the ileum were slightly more inhibitory than those from duodenum and jejunum for both cholera toxin and LT (Table 2). These results were in agreement with results of chemical analyses of epithelium of the different parts of intestine (Table 3). Generally, both phospholipids and glycolipids, acid as well as neutral ones, differed only little between duodenal, jejunal, and ileal mucosa, although gangliosides including GM1 increased about twofold from duodenum to ileum (Table 3).

TABLE 1. Different capacity of epithelial cells from rabbit small intestine to bind cholera toxin as compared to *E. coli* LT (five animals)

Toxin or ratio	Cell dilution giving 50% binding of 0.025 µg of toxin per ml[a]	µg of toxin bound per g of packed cells[b]
Cholera toxin	32 (17–67)[c]	0.40 (0.21–0.83)
E. coli LT	440 (260–910)	5.43 (3.21–11.4)
Ratio *E. coli*/cholera	13 (7.6–23)	

[a] Determined as illustrated in Fig. 2.
[b] Calculated at the cell dilution binding 50% of 0.025 µg of toxin per ml.
[c] Data are given as geometric means with the mean ± one SEM within parentheses.

It was of particular interest to compare toxin inhibitory titers of the intestinal monosialoganglioside fractions with on one hand the contents of chemically identifiable GM1 ganglioside in these fractions and on the other hand the inhibitory activity of highly purified reference GM1 from human brain. The results showed a close agreement between the content of chemically determined GM1 (Table 3) and GM1 determined functionally (50% inhibitory titer multiplied by the inhibitory concentration of reference GM1 preparation; Table 2).

Toxin-binding activity of delipidized tissue glycoprotein. The delipidized tissue fraction of intestinal cells (from duodenum, jejunum, or ileum) or of isolated brush-border membranes completely lacked any cholera toxin-binding activity (Fig. 4). This contrasted with the findings for *E. coli* LT, which toxin was found to bind significantly to the delipidized tissue fraction of intestinal cells as well as of brush-border membranes (Fig. 4). Delipidized duodenal mucosa had a slightly stronger LT-binding activity than the delipidized jejunal and ileal fractions, but the difference was only twofold (50% LT-inhibitory

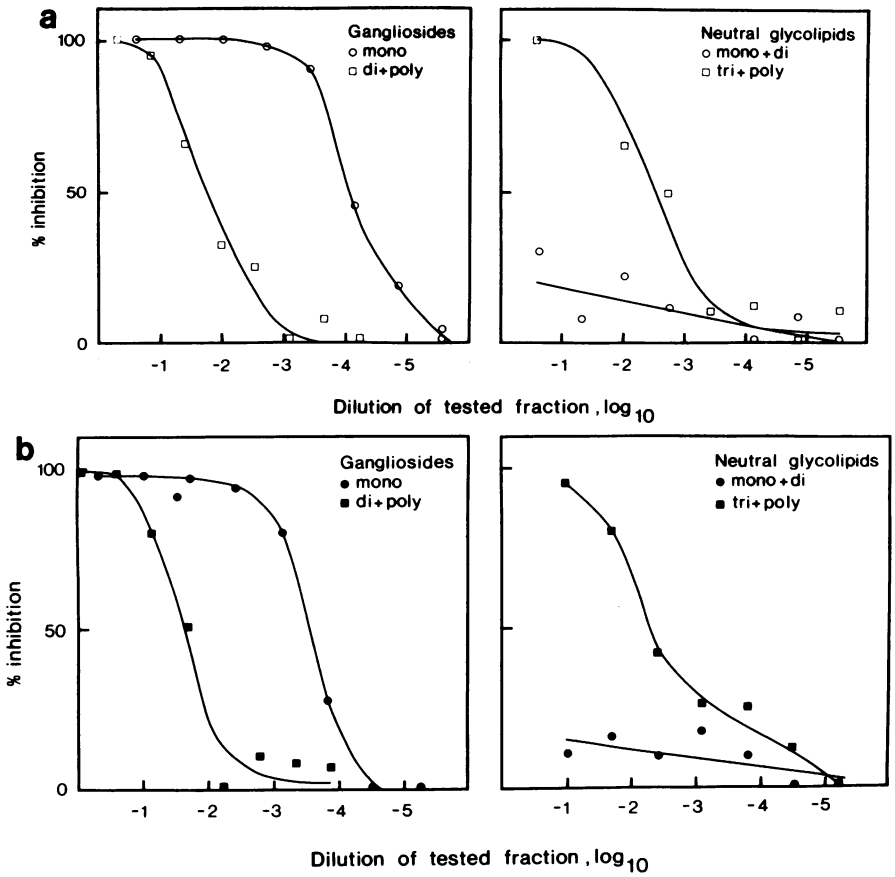

FIG. 3. Capacity of ganglioside and neutral glycolipid fractions from 1 g of packed rabbit duodenal epithelial cells to inhibit cholera toxin (a) or *E. coli* LT (b) binding to GM1-coated polyvinyl wells. Fractions were preincubated with 0.025 μg of the respective toxin per ml, and after centrifugation 0.1 ml of the incubation mixture was tested for uninhibited toxin by the GM1-ELISA method. The percentage inhibition of toxin binding was calculated by comparison with results obtained with buffer-toxin mixtures. Results are the means of duplicates.

titer of duodenal fraction being 1/400 as compared with 1/200 for jejunal and ileal fractions).

Periodate treatment of delipidized tissue fractions completely inactivated the binding sites for *E. coli* LT, whereas extensive digestion with pronase or heat treatment did not reduce the LT-binding titer appreciably. However, the proteolytic digestion resulted in a solubilization of previously tissue-bound binding sites for *E. coli* LT as shown by the isolation on a Sephadex G-10 column of a soluble heat-stable LT-inhibitory material from the pronase-digested, delipidized brush-border membranes.

DISCUSSION

This study provides two main sets of results. First, the data support earlier studies by showing that the GM1 ganglioside is the main, if not the only, specific binding receptor for cholera toxin in rabbit intestine. The second set of data, on the other hand, sheds new light on the nature of the receptors for *E. coli* LT in rabbit intestine by indicating the presence of a main glycoprotein receptor for this toxin.

All of the cholera toxin binding activity of rabbit intestinal epithelium was lipid extractable, and more than 95% of it was recovered in the monosialoganglioside fraction. These findings agree with those of Critchley et al. (6), who observed more than 99% of the cholera toxin binding receptors of rat microvillous membranes to be lipid extractable and to have chromatographic and other properties indicating that the receptor-active glycolipid indeed was GM1 ganglioside. Morita et al. (28), on the other hand, found significant cholera toxin-binding activity in glycoproteins extracted from rat microvillous membranes, but Critchley et al. (6), who used similar methods, could not confirm the presence of any direct interaction between cholera toxin

TABLE 2. Toxin inhibitory titers of ganglioside and neutral glycolipid fractions of epithelial cells from different parts of rabbit small intestine

Cell source	Fraction	Cholera toxin		*E. coli* LT	
		Titer[a]	nmol of GM1[b]	Titer	nmol of GM1
Duodenum	Gangliosides[c]				
	Mono	10,000	2.5	4,500	2.3
	Di + poly	50		45	
	Neutral glycolipids[d]				
	Mono + di	<2		<2	
	Tri + poly	350		200	
Jejunum	Gangliosides				
	Mono	11,400	2.8	5,400	2.7
	Di + poly	200		50	
	Neutral glycolipids				
	Mono + di	<2		<2	
	Tri + poly	280		120	
Ileum	Gangliosides				
	Mono	16,000	4.0	8,000	4.0
	Di + poly	200		320	
	Neutral glycolipids				
	Mono + di	<2		<2	
	Tri + poly	260		100	
	Reference GM1 ganglioside	0.25 nM		0.50 nM	

[a] Dilution of fraction (as prepared from 1 g of packed epithelial cells and tested in 1-ml volume) or concentration of reference GM1 ganglioside (purified from human brain [39]) giving 50% inhibition of 0.025 μg of the respective toxin per ml.

[b] Titer translated to quantity of GM1 ganglioside based on comparisons with purified reference GM1.

[c] Mono, Gangliosides with one sialic aid; Di + poly, gangliosides with two or more sialic acids.

[d] Mono + di, glycosphingolipids with one or two sugars; Tri + poly, neutral glycosphingolipids with three or more sugars.

and rat intestinal brush border glycoproteins. Our present findings do not support any interaction between cholera toxin and glycoproteins in rabbit intestinal epithelium, since epithelial cells which had been delipidized by a procedure which does not extract glycoproteins to any appreciable extent completely lacked cholera toxin-binding activity irrespective of whether the cells were from duodenum, jejunum, or ileum. Furthermore, our results indicate that the cholera toxin receptor concentration of rabbit intestinal epithelium closely agrees with the content of chromatographically identifiable GM1 ganglioside. Thus, the cholera toxin-inhibitory activity of intestinal glycolipid fractions per nanomole of mucosal GM1 was essentially the same as the specific toxin-inhibitory activity of highly purified reference GM1 ganglioside. These findings confirm our previous studies which demonstrated a close agreement between the number of cholera toxin binding sites and GM1 ganglioside content in small intestinal mu-

TABLE 3. Ganglioside, neutral glycolipid, and phospholipid contents of rabbit epithelial cells from duodenum, jejunum, and ileum[a]

Cell source	Gangliosides[b] (nmol/g of packed cells)		Neutral glycolipids[c] (nmol/g of packed cells)		Phospholipids (μmol/g of packed cells)
	Mono	Di + poly	Mono + di	Tri + poly	
Duodenum	4.1[d]	3.5	116	359	6.8
Jejunum	4.3[e]	4.8	99	276	5.8
Ileum	6.3[f]	7.8	105	273	6.0

[a] The lipids were extracted, separated and quantified as described in the text.

[b] Mono, Gangliosides with one sialic acid; Di + poly, gangliosides with two or more sialic acids in the molecule.

[c] Mono + di, glycosphingolipids with one or two sugars; Tri + poly, neutral glycosphingolipids with three or more sugars.

[d] Of this, 1.5 nmol/g was GM1.

[e] Of this, 2.0 nmol/g was GM1.

[f] Of this, 3.9 nmol/g was GM1.

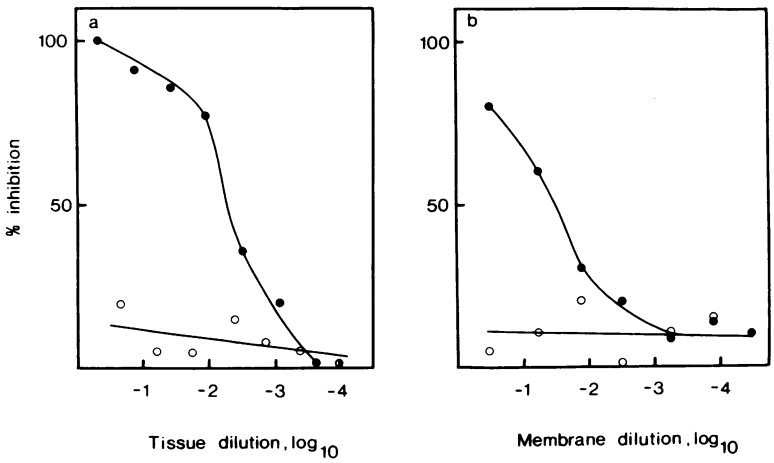

FIG. 4. Capacity of delipidized tissue residue from 1 g of packed rabbit duodenal epithelial cells (a) and of delipidized brush-border membranes (2.5 mg of membrane protein) (b) suspended in 1 ml to inhibit cholera toxin (○) or *E. coli* LT (●) binding to GM1-coated polyvinyl wells. Fractions were preincubated with 0.025 μg of the respective toxin per ml, and after centrifugation 0.1 ml of the incubation mixture was tested for uninhibited toxin by the GM1-ELISA method. The percentage of inhibition of toxin binding was calculated by comparison with results obtained with buffer-toxin mixtures. Results are the means of duplicates.

cosa of various species (23).

The results concerning the nature of the receptors for *E. coli* LT indicate that only a smaller fraction of the LT receptors are shared by cholera toxin and have GM1 ganglioside properties, whereas a larger fraction appears to be of a glycoprotein nature. Holmgren (18) showed that *E. coli* LT bound specifically to GM1 ganglioside in vitro, although the binding affinity seemed to be lower than between GM1 and cholera toxin. Several studies have confirmed that *E. coli* LT is efficiently inactivated by free GM1 ganglioside (10, 44). Furthermore, Moss et al. (30) recently described that incorporation of exogenous GM1 into the membrane of ganglioside-deficient fibroblasts specifically sensitized these cells to the action of *E. coli* LT. All of these findings are consistent with the notion that GM1 ganglioside indeed may function as receptor for *E. coli* LT. However, at the same time the early in vivo studies by Holmgren (18) and Pierce (33) indicated that choleragenoid or cholera B subunit, even though effectively inhibiting cholera toxin action, presumably by blocking GM1 mucosal receptors, did not inhibit the fluid loss-inducing action of *E. coli* LT preparations in rabbit intestine. This apparent discrepancy between findings in fibroblasts and rabbit intestine has remained unexplained until now.

The present results confirm the inability of cholera B subunits to inhibit *E. coli* LT action in rabbit small intestine even when used in as much as a 100-fold excess to the amount giving complete inhibition of a similar challenge dose with cholera toxin and added to the intestine 15 min before the LT challenge. The LT used in these experiments, in contrast to the earlier studies (18, 33) which were open to criticism on these points, was produced by a well-defined LT-only *E. coli* strain and was purified to apparent homogeneity. Control experiments showed that the LT action on fluid transport in rabbit intestine was completely inhibited by exposing the LT to heating, specific anti-LT or anti-cholera toxin antiserum, or GM1 ganglioside. Furthermore, the LT action on rabbit intestinal fluid movement was specifically blocked by the homologous B subunits (LT-B), a finding which also excludes the possibility that the non-GM1 interaction of LT with intestinal epithelium was mediated, for instance, via the A subunit and therefore not blocked by cholera B subunits. Interestingly, the LT-B preparation blocked the action of cholera toxin, too. These results suggest that whereas CT-B binds only to GM1 ganglioside and thus completely blocks the receptors used by cholera toxin, LT-B binds both to the GM1 cholera toxin receptors and to other receptor sites used by *E. coli* LT but not cholera toxin. In view of the fact that complete blocking of GM1 (cholera toxin) receptors by cholera B subunit did not give any detectable inhibition of *E. coli* LT, the non-GM1 LT receptors would appear to predominate in number or efficiency as compared with LT receptors of the GM1 type.

The suggested presence of additional binding sites for *E. coli* LT as compared with cholera toxin on rabbit enterocytes was confirmed by the in vitro binding experiments. As a mean the epithelial cells of small intestine or isolated

brush-border membranes bound LT 10 to 15 times better than cholera toxin, as determined by titration experiments in which the toxins were incubated with serial dilutions of cells or membranes. Studies with chemical fractions of intestinal cells indicated that the non-GM1 LT-only receptors are not glycolipids, as little, if any, LT binding activity in lipid extracts was found that could not be accounted for by GM1 ganglioside. Instead, the delipidized tissue fraction of rabbit duodenal, jejunal, and ileal epithelial cells and brush-border membranes retained substantial binding activity for *E. coli* LT, even though it contained no detectable lipid and no cholera toxin-binding activity either.

The nonganglioside receptor for *E. coli* LT retained in delipidized intestinal brush-border membranes had properties consistent with it being a glycoprotein. It was resistant to boiling at neutral pH, but was inactivated by periodate oxidation. Proteolytic digestion of the delipidized tissue did not destroy the LT-inhibitory (binding) activity, but rather solubilized it in a molecular form which could be isolated as a heat-stable LT inhibitor from a Sephadex G-10 column, consistent with the release of a specific-binding oligosaccharide from a membrane glycoprotein.

Whereas the GM1 ganglioside concentration was about twice as high in ileal as compared with duodenal epithelium, the nonlipid LT receptors seemed to be slightly more abundant in the duodenum than in the ileum as judged from results with delipidized cells from different parts of the intestine. Of greater importance than these relatively minor variations is the question of whether the present results in rabbits extend to other species, especially to the human intestine. Nalin and McLaughlin (32) described that in dogs, choleragenoid consisting of cholera B subunit blocked *E. coli* LT-induced and cholera toxin-induced ileal fluid secretion, suggesting that the majority of the functional LT receptors in this species were shared by cholera toxin, a finding contrasting with our results in rabbits. Cholera B subunit can now be produced in quantities amenable to testing its effect as a prophylactic cholera blocking agent in population groups at especially high risk (19). This makes it of practical as well as theoretical interest to clarify whether LT receptors in human intestine would be blocked to any significant extent by cholera B subunit, or, as in the rabbit intestine, would require the preparation and use of the homologous LT B subunits.

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