Interaction of Cholera Toxin With Rat Intestinal Brush Border Membranes

RELATIVE ROLES OF GANGLIOSIDES AND GALACTOPROTEINS AS TOXIN RECEPTORS*

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David R. Critchley§, John L. Magnani¶, and Peter H. Fishman‡

From the Membrane Biochemistry Section, the Developmental and Metabolic Neurology Branch, National Institute of Neurological and Communicative Diseases and Stroke, and the Biochemistry Section, the Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolism and Digestive Diseases, the National Institutes of Health, Bethesda, Maryland 20205

Rat intestinal brush borders contained a small number (3–4 pmol/mg of protein) of high affinity (10⁻¹⁰ M) binding sites for cholera. Following extraction with chloroform/methanol solutions, the delipidated membranes lost 99% of their capacity to bind ¹²⁵I-cholera but retained their ability to bind ¹²⁵I-Ricinus communis agglutinin I (RCA I). When analyzed by thin layer chromatography, the major gangliosides in the lipid extract migrated differently than galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglycosylceramide (GM₁) and were neuraminidase-sensitive. Using a sensitive assay, however, small amounts of neuraminidase-resistant gangliosides with mobilities similar to GM₁ were detected. These latter gangliosides were labeled with ³²P when isolated from membranes treated with galactose oxidase and NaBH₄. Incorporation of ³²P into these gangliosides was reduced, however, when the membranes were first exposed to cholera. In contrast, cholera did not protect membrane galactoproteins from being labeled. When membranes containing bound ¹²⁵I-cholera were treated with neutral detergents, over 85% of the bound toxin was extracted. When the solubilized membranes were analyzed by sucrose density gradient centrifugation, the labeled toxin sedimented to the same region of the gradient as did cholera and cholera-GM₁ complexes and no higher molecular weight forms indicative of toxin-ganglioside complexes were detected. Membranes labeled by the galactose oxidase/NaBH₄ method were incubated with cholera, solubilized as above, and treated with antitoxin antibodies; the immune complexes were absorbed out with fixed Staphylococcus aureus and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Less than 1% of the ³²P associated with the membranes was immunoprecipitated, some of which corresponded to galactoproteins. Electrophoretograms of brush border membranes were analyzed directly, or after electrophoretic transfer of the proteins to nitrocellulose sheets, for iodinated RCA I or cholera binding. The galactoproteins were readily detected with RCA I but not with cholera. Cholera, however, did bind to the lipid-containing region of the electrophoretograms. We conclude that the predominant cholera receptors in rat intestinal brush borders are gangliosides with the characteristics of GM₁.

Cholera, an enterotoxin produced by Vibrio cholerae, is responsible for the clinical manifestations of the disease cholera (1). The toxin binds to specific receptors on the luminal surface of the intestinal mucosal cell and activates adenylate cyclase (2–5). Cholera can activate adenylate cyclase in other vertebrate cells and its mechanism of action has for the most part been elucidated (6, 7). The ganglioside GM₁ has been implicated as the receptor for the toxin (7, 9–13). The ability of GM₁ to be taken up by GM₁-deficient cells and thus sensitize the cells to cholera has clearly demonstrated that GM₁ can function as a toxin receptor (7, 14–16). Less is known about the role of GM₃ as the receptor in intestinal mucosal cells. Gangliosides isolated from intestinal mucosa blocked the action of cholera (10). A correlation between GM₃ content and toxin binding has been demonstrated in the small intestine of several species including man, and prior treatment of rabbit intestine with GM₁ increased the amount of toxin binding as well as the diarrheogenic action of the toxin (17). Recently, Morita et al. reported the presence of glycoproteins in rat intestinal membranes that bind cholera (18). We thus decided to re-examine the relative roles of GM₁ and glycoproteins as receptors for cholera in rat intestinal brush border membranes.

EXPERIMENTAL PROCEDURES

Materials—Cholera was obtained from Schwartz/Mann and iodinated as described (11). RCA I (castor bean lectin-129) was from P-L Biochemicals Inc. and was iodinated by the chloramine-T procedure in the presence of 0.2 μM d-Gal to protect the binding sites; the specific radioactivity was 1.7 mCi/mg. Na¹²⁵I and Na¹³¹I were obtained from Amersham Corp. Galactose oxidase was from Worthington Biochemical Co. V. cholerae neuraminidase and fixed Staphylococcus aureus (Pansorbin) were from Calbiochem-Behring Corp. Protease (Pronase Type VI) was obtained from Sigma. GM₁ was labeled with ¹²⁵I as described previously (14). Anti-cholera antigen raised in a burro was a generous gift of Dr. William Habig.

* Ganglioside nomenclature is according to Svennerholm (8).

† The abbreviations used are: RCA I, Ricinus communis agglutinin; NeuAc, N-acetylgalactosaminic acid; NeuNGc, N-glycolylneuraminic acid; GL-1a, glucosylceramide; GL-1b, galactosylceramide; GL-2, lactosylceramide; GL-3, galactosylgalactosylglycosylceramide; GL-4, N-acetylgalactosaminylgalactosylgalactosylglycosylceramide; PBS, phosphate-buffered saline, pH 7.4; SDS, sodium dodecyl sulfate.

‡ Visiting Scientist from the Department of Biochemistry, University of Leicester, Leicester, England.

§ Address correspondence and reprint requests to Dr. Peter H. Fishman, Building 10, Room 3D03, National Institute of Health, Bethesda, MD 20205.

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Bureau of Biologics, Food and Drug Administration; 1 ml neutralized 140 μg of toxin.

Preparation of Intestinal Brush Borders—Brush borders were prepared from the small intestine of male Sprague-Dawley rats (150-200 g) by the method of Miller and Crane (19). The sucrase activity of the brush border fraction was enriched 7-8-fold over the homogenate and assays for lactate dehydrogenase, succinate dehydrogenase, glucose-6-phosphatase, and RNA showed negligible contamination by other subcellular organelles.

Binding of Cholera and RCA I—Membranes were incubated in 0.2 ml of a solution containing 25 mM Tris-Cl (pH 7.4), 0.35% NaCl, 1 mM EDTA, 0.1% bovine serum albumin, and 200,000 cpm of [3H]-cholerae or -RCA I for 30 min at 25 °C. The samples were then filtered under vacuum on 0.2-μm Millipore EGWP filters (20). Non-specific binding was determined in the presence of 200 nM unlabeled cholerae or 0.2 M d-Gal. Binding of [3H]-cholerae was proportional up to 30 μg of membrane protein and 15 μg were used routinely; for RCA I, the corresponding amounts were 10 and 2 μg. Values have been corrected for nonspecific binding and are the mean of triplicate determinations; unless otherwise indicated, standard deviations were less than 10% of the mean.

Isolation and Analysis of Glycosphingolipids—Brush borders (up to 10 mg of protein) were extracted with 5 ml of chloroform:methanol (1:2, v/v) (21) and after centrifugation, the insoluble material was extracted with 2.5 ml of the same solvent at 45 °C for 1 h, sedimented by centrifugation, and washed with 1 ml of the same solvent. The combined extracts were analyzed directly for cholerae binding components as described below or further separated (21). Briefly, the extracts were taken to dryness, desalted on Sephadex G-25, and separated into neutral and acidic fractions on DEAE-Sephadex. The neutral fraction was treated with 0.2 ml NaOH in chloroform:methanol (2:1, v/v) to hydrolyze phospholipids; after neutralizing with acetic acid, the solution was partitioned and the resultant lower phase was washed once with the theoretical upper phase (22). The washed lower phase was then separated by thin layer chromatography on silica gel with the solvent system chloroform:methanol:water (65:25, v/v). The glycosphingolipids were detected by spraying the plates with a solution of 0.5% orcinol in ethanol: concentrated H2SO4 (8:2, v/v) and heated at 100 °C for 10 min. Gangliosides were isolated from the acid fraction following alkaline hydrolysis, desalting on Sephadex G-25, and column chromatography on Unisil (21). The gangliosides were separated by thin layer chromatography, visualized with resorcinol reagent, and quantified by scanning densitometry (21).

The total lipid extract and the purified ganglioside fraction were analyzed for cholerae binding components as described elsewhere (23). Briefly, portions were spotted along with standards on plastic-backed silica gel chromatography sheets (100 μm thick from Eastman Kodak Co.). The sheets then were clamped in a sandwich chamber and developed with chloroform:methanol:2.5% H2O (80:25, v/v). The sheets were dried and sprayed with a soln containing 25 m TrisCl (pH 7.4), 135 mM NaCl, 1 mM EDTA, and 3 mM NaN3. Then 1 μl of 10% bovine serum albumin and 2.5 μl of cholerae (1 mg/ml) were added to each sample. After 30 min at 4 °C, 20 μl of anti-cholerae antibodies were added. After 18 h at 4 °C, the immune complexes were absorbed by adding 0.1 ml containing 20 mg of fixed S. aureus that had been washed with 0.1% Triton X-100 in PBS. After 18 h at 4 °C, the samples were centrifuged at 10,000 X g for 2 min in a Beckman microfuge. The pellets were washed twice with 1 ml of the above solution and once with PBS. The pellets were extracted with chloroform: methanol (1:2, v/v). (After being desalted on Sephadex G-25 (21), the labeled gangliosides were separated by thin layer chromatography and detected by radioautography using a slab gel apparatus (Bio-Rad Laboratories Model 220). Gels were stained directly with iodinated cholerae or RCA I according to Burridge (27) or after electrophoretic transfer of proteins from the gel to nitrocellulose sheets (28). The gels or sheets were analyzed for radioactivity either by autoradiography or by slicing and counting 1-mm sections. Proteins were detected by staining the gels or sheets with Coomassie blue. Tritium was detected on gels by fluorography (29).

Binding of Cholerae to Brush Border Membranes—Binding of [3H]-cholerae to rat intestinal brush borders appeared to be to a single class of high affinity sites (Fig. 1).3 Binding of tracer amounts of iodotoxin was inhibited 50% by 0.3 nM unlabeled cholerae (Fig. 1A). Half-saturation of [3H]-cholerae binding occurred at 0.2 nM in one experiment (Fig. 1B) and at 0.3 nM in a second experiment with different preparations of membranes and of iodotoxin (data not shown). Solution of binding data obtained with [3H]-cholerae was found to be a two-site model. At saturation, the membranes bound 2.7 and 4.5 pmol of toxin per mg of protein for the two experiments.4 Concentrations of RCA I up to 0.2 nM had little effect on the binding of cholerae to the membranes (Fig. 2A). Above this concentration, binding was partially inhibited, a maximal effect occurring at 1 μM; at higher concentrations of RCA I, however, there was less inhibition.5 In contrast, cholerae at concentrations up to 1.2

3 Because cholerae is a multivalent ligand and the toxin-receptor complex does not readily dissociate (7, 11, 20), Scatchard analysis of the binding data is not appropriate (20). The smooth shapes of the binding curves shown in Fig. 1 are consistent with a single class of binding sites.

4 Walker et al. reported that rat intestinal microvillous membranes bound 10 fmol of cholerae per mg of protein and half-saturation occurred at 0.3 pM (9). We recalculated their data, and arrived at a lowest estimate of 560 fmol/mg of protein and 0.2 nM for half-saturation.

5 Gahmberg and Hakomori also observed that RCA I has heterogeneous effects on the surface structures of rat intestinal cell membranes (15). The effects of RCA I were different for different glycolipids (30). Thus, the lectin by binding to glycoproteins may mask the accessibility of GM1 to cholerae but at higher concentrations may cause aggregation of the glycoproteins and thus increase the exposure of GM1 to cholerae. It has also been shown that RCA I bound to GM1 incorporated into artificial lipid vesicles (31). Thus, RCA I either indirectly or directly could be inhibiting the binding of cholerae to GM1.
Receptors for Cholera Toxin in Rat Intestine

The lipid extract was separated into neutral and acidic lipids and gangliosides were isolated from the latter fraction as described under “Experimental Procedures.” The predominant neutral glycolipids migrated similarly to GL-1 and GL-3 on thin layer chromatography (Fig. 3A). Others had reported that GL-1a and GL-3 were the major neutral glycosphingolipids in rat intestinal epithelium (32, 33). There were several minor components that were less mobile than GL-4 as described previously (32, 33). The purified ganglioside fraction contained several closely migrating components with mobilities between GM1 and Gb2 and a minor component which migrated between GM1 and Gb3 on the chromatogram (Fig. 3B). In terms of lipid-bound sialic acid, there were 36 ± 1.6 and 2.4 ± 0.5 (n = 3) nmol/mg of protein of the major and minor gangliosides, respectively. Following treatment with neuraminidase, 95% of the gangliosides were hydrolyzed and several new resorcinol-negative bands with more rapid mobilities were observed (Fig. 3B). Further analysis of the hydrolysis products indicated that three neutral gangliosides which migrated between GL-2 and GL-3 were produced (Fig. 3C). These results are consistent with a previous study in which the predominant mucosal ganglioside was neuraminidase-sensitive and contained NeuNGc (32; see also Ref. 33).

As delipidation effectively removed cholera receptor activity from the intestinal brush borders, we next directly assayed for cholera binding components in the lipid extract using a sensitive method developed by Magnani et al. (23). The lipid extract was separated on silica gel-coated plastic sheets which were then overlayed with 125I-cholera (Fig. 4A); there was no cholera binding at the origin as would be expected if a cholera binding hydrophobic protein had been extracted from the membranes. Cholera binding appeared to be specific as it was blocked by excess unlabeled toxin (Fig. 4A). The purified ganglioside fraction also contained a cholera binding component which migrated as Gb3 and was resistant to neuraminidase (Fig. 4B). As we were unable to successfully separate Gb3 containing NeuAc from that containing NeuNGc on these chromatograms, we cannot state which form of Gb3 is present in the membranes.

Although the amounts of Gb3 in the brush borders were too small to quantitate by chemical means, we were able to estimate their amounts. Previous studies had shown that Gb3-deficient rat glial C6 cells took up Gb3 from the culture medium in proportion to its concentration and bound 125I-cholera in proportion to the amount of Gb3 taken up (34). C6 cells were suspended in medium containing increasing amounts of Gb3 or brush border gangliosides, incubated for 60 min at 37 °C, washed extensively, and assayed for cholera binding. Using this sensitive assay, we estimated that rat

![Image 1x0 to 589x809]
Fig. 3. Thin layer chromatograms of glycosphingolipids isolated from rat intestinal brush borders. Neutral glycosphingolipids (A) and gangliosides (B) were isolated from membranes, separated on thin layer silica gel, and visualized as described under "Experimental Procedures." A: lane 1, neutral glycosphingolipid standards; lane 2, neutral glycosphingolipids from 1 mg of brush border protein; lane 3, same as lane 2 except treated with neuraminidase (25 units for 24 h at 37°C); arrows indicated bands that are resorcinol-negative; lane 4, ganglioside standards. C: lane 1, neutral glycosphingolipids derived from brush border gangliosides treated with neuraminidase; lane 2, standards as in A.

Fig. 4. Binding of 125I-choleragen to components in the lipids and gangliosides isolated from rat intestinal brush borders. Total lipids (A) and purified gangliosides (B) from rat intestinal brush borders were separated on silica gel-coated plastic sheets which were then dried and overlayed with 125I-choleragen as described under "Experimental Procedures." The bound toxin was detected by autoradiography. A: lane 1, GM1; lane 2, total lipids from brush borders; lanes 3 and 4, same as lanes 1 and 2 except incubated in the presence of excess unlabeled toxin. B: lane 1, GM1; lane 2, gangliosides from brush borders; lane 3, same as lane 2 except gangliosides treated with neuraminidase as described in the legend to Fig. 2. Arrows indicate positions of from top to bottom GM3, GM2, GM1, and GD1a.

Intestinal brush borders contained 14 pmol/mg of protein of GM1, which is sufficient to account for the amount of cholera binding activity.

Effect of Choleragen on Labeling of Brush Borders by Galactose Oxidase/NaB3H4.—Consistent with the study by Morita et al. (18), a number of glycoproteins were labeled by exposing the brush border to galactose oxidase and NaB3H4 (Fig. 5b); there was very little incorporation of 3H in the absence of galactose oxidase (Fig. 5a). The labeled glycoproteins appeared to be those that bound 125I-RCA I (Fig. 5e); as expected, 0.2 M Gal totally inhibited RCA I binding to these glycoproteins (not shown). Prior exposure of the brush borders to a saturating amount of cholera, however, did not appear to have any effect on the labeling of these glycoproteins (Fig. 5c). The labeled brush borders were extracted and analyzed for labeled glycosphingolipids. Using the mild conditions of labeling (see "Experimental Procedures"), the ganglioside fraction was not effectively labeled. Using the more exhaustive conditions, substantial amounts of 3H were incorporated into the gangliosides of the brush borders (Fig. 6a). These gangliosides also were labeled when the membranes were exposed to galactose oxidase and the addition of choleraagen had no effect on their labeling (Fig. 6a). In contrast, gangliosides with mobilities similar to GM1 were labeled only with enzyme treatment and labeling was substantially reduced by prior treatment of the membranes with cholera. To further clarify the nature of these labeled gangliosides, portions were dissolved in 0.1% Triton X-100, incubated with cholera,
and subjected to immunoadsorption. The predominant labeled ganglioside isolated by this procedure corresponded to G\textsubscript{M1} and its labeling was effectively blocked by choleragen (Fig. 6B). The major neutral glycosphingolipids were labeled by the galactose oxidase/Na\textsubscript{3}H\textsubscript{4} procedure and prior exposure of the brush borders to choleragen had no effect on their labeling (data not shown).

**Analysis of Solubilized Choleragen-Receptor Complexes on Sucrose Density Gradients**—As indicated under "Experimental Procedures," Triton X-100 and NP-40 effectively extracted \( ^{125}\text{I} \)-choleragen bound to intestinal brush borders. When centrifuged on a linear 5-40% sucrose gradient, the detergent-extracted radioactivity sedimented in a symmetrical peak slightly ahead of \( ^{125}\text{I} \)-choleragen (data not shown). When brush borders containing bound iodotoxin were extracted with detergent and applied directly to the gradient without an initial centrifugation to remove insoluble material, some of the label also sedimented through a 60% sucrose cushion to the bottom of the tube (Fig. 7A). Most of the label sedimented slightly faster than \( ^{125}\text{I} \)-choleragen and the same as iodotoxin incubated with G\textsubscript{M1} in detergent. Results similar to those observed in Fig. 7A were obtained whether the brush borders were incubated first with 2.20 or 50 nm \( ^{125}\text{I} \)-choleragen or whether the detergent extracts were sedimented on 5-20, 5-30 or 5-40% linear sucrose gradients. There was no evidence of soluble choleragen-receptor complexes greater than 100,000 daltons. We were able to confirm the stability of toxin-receptor complexes as follows. Rat glial C6 cells were incubated with \( ^{3}\text{H} \)-G\textsubscript{M1} (16, 34), then with and without choleragen and then solubilized with Triton X-100. After centrifuging at 500 \( \times g \) for 10 min, the extracts were sedimented on linear sucrose gradients (Fig. 7B). In extracts from cells not exposed to toxin, all of the \( ^{3}\text{H} \)-G\textsubscript{M1} remained at the top of the gradient. Prior addition of choleragen to the cells caused the \( ^{3}\text{H} \)-G\textsubscript{M1} to sediment in the same position as detergent-treated iodotoxin-G\textsubscript{M1} complexes. Similar results were obtained when the \( ^{3}\text{H} \)-G\textsubscript{M1}-treated cells were exposed to the B component of choleragen or when the detergent extract of the cells was incubated with choleragen prior to centrifugation on the gradient (data not shown).

**Immunoadsorption of Brush Border Components**—Putative choleragen receptors were isolated from galactose oxidase/Na\textsubscript{3}H\textsubscript{4} labeled brush borders by immunoadsorption (24). The labeled membranes were extracted with NP-40 and choleragen was added to the detergent extracts followed by anticholeragen antibodies. The immune complexes then were absorbed to fixed S. aureus, washed extensively with detergent, solubilized in SDS and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 8). The major galactoproteins of the

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**Footnote:**

\( ^{\text{6}} \) Up to 50% of the choleragen bound to cultured cells including G\textsubscript{M1}-deficient cells treated with G\textsubscript{M1} is resistant to extraction by Triton X-100 and NP-40 depending on the extraction conditions. Some of the G\textsubscript{M0} taken up by G\textsubscript{M1}-deficient cells also is resistant to detergent and prior treatment of the cells with choleragen increases this amount of G\textsubscript{M1} (J. Hagmann and P. H. Fishman, unpublished observations).
brush border were effectively extracted by NP-40 (compare Fig. 8a with 8b). Galactoproteins of similar molecular weight to the major galactoproteins were adsorbed to the presence of cholera in Fig. 8d) although there was some nonspecific adsorption (Fig. 8c). Only 0.24% of the radioactivity in the NP-40 extracts was specifically absorbed. Recoveries of cholera receptors were obtained when brush borders were incubated with cholera and washed prior to detergent extraction or when no [3H]GM1 was added to the extracts.

Detection of Cholera and RCA I Receptors on SDS-Polyacrylamide Gel Electrophoreograms—Intestinal brush borders were solubilized in SDS and subjected to SDS-polyacrylamide gel electrophoresis. When the gels were incubated with [3H]-cholera in the presence of Burridge (27), the labeled toxin only bound to that region of the gel where the lipids migrated (Fig. 9). Binding was specific as it was blocked by excess unlabeled toxin. Cholera did not bind to other regions of the gel where the glycoproteins migrated. Identical results were obtained in several additional experiments including ones where the proteins were electrophoretically transferred to nitrocellulose sheets (28). With this latter technique, there was no cholera binding at all as the lipids were not effectively transferred to the sheets (inset, Fig. 9). The proteins and galactoproteins were effectively transferred as shown by the staining with Coomassie blue and the specific binding of [125I]-RCA I. In one experiment with the direct Burridge procedure, we did observe binding of [125I]-cholera to the major glycoproteins as well as to all other proteins including the molecular weight markers. The labeled cholera preparation used for this experiment had a large proportion of radioactivity that did not bind to membranes and we suspect that this material nonspecifically absorbed to the various proteins on the gel.

**DISCUSSION**

Our results clearly demonstrate that rat intestinal brush borders contain a small number of high affinity binding sites for cholera. These sites are resistant to proteases and neuraminidase but are extracted from the membranes with organic solvents. In contrast, the brush borders contain a large number of lower affinity binding sites for RCA I which are protease-sensitive and not extracted with organic solvents. Although we were unable to chemically detect GM1 in the lipid extracts, we were able to detect cholera binding components in the lipid extracts that had the same mobility as GM1 by thin layer chromatography. Using this very sensitive technique developed by Magnani et al. (23), we were able to show...
that these components were resistant to neuraminidase treat-
ment which is consistent with a GM₁-type structure. We also 
have provided indirect evidence that the brush borders con-
tain sufficient GMI to account for all of their cholera-
gen binding capacity. As NeuNGC is the predominant species 
of lipid-bound sialic acid (32, 33), this form of GMI may be 
present; previous studies have demonstrated that cholera-
gen can use GMI containing NeuNGC as a receptor (34).

When brush borders were labeled by the galactose oxidase/
NaB³H₄ procedure, a labeled ganglioside that corresponded to 
GMI was isolated from the labeled membranes. Incorporation 
of H into this ganglioside was substantially reduced when the 
membranes were exposed to cholera-
gen prior to being labeled. 

The ability of cholera-
gen to specifically protect GMI, from 
galactose oxidase has been shown previously for thyroid mem-

branes (35), cultured cells (36), and liposomes (37). 

Using a variety of techniques, we were unable to convinc-
ingly demonstrate a direct interaction between cholera-
gen and rat intestinal brush border glycoproteins. Although we 
used similar procedures, our results do not in general confirm 
a previous report by Morita et al. (18). In their studies, they 
reported the following.

(i) Glycoproteins from rat intestinal microvillus membranes 
could be separated from glycolipids by affinity chromatog-
raphy on RCA-agararose and could bind cholera-
gen as detected by gel filtration chromatography. Our studies indicated that 
cholera-
gen did not block RCA I binding to rat intestinal brush 
borders and that inhibition of cholera-
gen binding by RCA I 
was of low affinity and incomplete. In addition, detergent-
solubilized cholera-
gen-receptor complexes were separated ac-
cording to molecular size on sucrose density gradients; there 
was no evidence of complexes greater than 100,000 daltons. As 
the smallest glycoprotein detected by Morita et al. had a 
molecular weight of 69,000 (18), the smallest putative toxin-
glycoprotein complex would have a molecular weight of over 
150,000.

(ii) Using the Burridge technique, intestinal glycoproteins 
separated by SDS-polyacrylamide gel electrophoresis bound 
¹²⁵I-cholera-
gen and binding was blocked by 200 µg of unlabeled 
toxin (18). In several of our experiments, we observed that the 
iodotoxin only bound to the dye front region of the gels where 
the glycolipids migrate. In one experiment, we did detect 
cholera-
gen bound to membrane glycoproteins as well as other 
proteins, including the molecular weight markers. This unus-
ual binding also was blocked by a large excess of unlabeled 
cholera-
gen. We did find, however, that ¹²⁵I-RCA I bound in a 
specific way to many of the brush border glycoproteins either 
directly on the gels or after transfer to cellulose nitrate sheets. 
None of the galactoproteins bound ¹²⁵I-cholera-
gen by this 
latter technique.

(iii) When membranes labeled by galactose oxidase/NaB³H₄ 
were detergent-extracted and subjected to immunoadsorption 
by anti-cholera-
gen antibodies and S. aureus, at least 5 glyco-
proteins with molecular weights from 69,000 to 132,000 cor-
responding to the major galactoproteins were detected (18). We 
also observed several glycoproteins by this same procedure 
but they represented less than 0.25% of the total labeled 
membrane components. Prior incubation of the membranes 
with excess cholera-
gen, however, did not protect the glyco-
proteins from being labeled by galactose oxidase/NaB³H₄. The 
possibility that a small proportion of the total membrane 
galactoproteins are associated with GM₁ and therefore would 
appear to have been specifically immunoadsorbed must be 
considered (24).

One unusual feature of the studies by Morita et al. (18) is 
that most of the major glycoproteins of the intestinal mem-
brids that are labeled by galactose oxidase/NaB³H₄ appear 
to bind ¹²⁵I-RCA I and ¹²⁵I-cholera-
gen. In addition, their 
results suggest that most of the cholera-
gen binding is to these 
glycoproteins and not to the glycolipids although it is unfortu-
nate that their results were strictly qualitative and no 
quantitative data were presented. We believe that the strongest 
evidence in support of glycolipids being the predominant 
(if not only) receptors for cholera-
gen is the fact that 99% of 
the toxin binding activity is removed from the membranes by 
delipidation. Although it is possible that this procedure may 
denature putative cholera-
gen binding glycoproteins, one 
would anticipate that the binding determinants would reside 
in the carbohydrate portion of the molecules. In this regard, 
delipidation had no deleterious effects on the ability of these 
carbohydrate sequences to bind RCA I.

Although we used a different strain of rat and a different 
procedure for isolating intestinal membranes, we do not be-
that their results were strictly qualitative and no 
minor differences can account for the major 
differences between our results and those of Morita et al. (18).

Our results clearly indicate that rat intestinal mucosal mem-
brids have receptors for cholera-
gen with the characteristics 
of GMI and are thus consistent with numerous other studies 
demonstrating that GMI is the receptor for cholera-
gen.

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