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Binding of Cholera Toxin to Mucins and Inhibition by Gastric Mucin

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The effects of mucin on cholera toxin induced intestinal secretion was studied in the rat small intestine. Gastric mucin inhibited secretion when premixed with cholera toxin on an equal dry-weight basis. Neither salivary nor intestinal mucin inhibited the toxin's action in the intestine. Inhibition by gastric mucin was not reversed by a mucolytic agent or by hexoses or agents that bind hexoses. Plasma and serum did not inhibit the effects of cholera toxin in the intestine. Choleragen was labeled with ^{14}C and its binding to mucins and ganglioside was studied. Gastric mucin was shown to bind to choleragen, but less binding was observed when choleragen was dissociated into subunits by acid pH. Salivary and intestinal mucins bound intact choleragen but not the subunits at acid pH. Salivary mucin binding was reversed by *N*-acetyl neuraminic acid and neuraminidase. Ganglioside bound choleragen in both the intact and dissociated forms. Binding to the dissociated form was reversed by wheat germ agglutinin. Gastric mucin and ganglioside competed for binding to choleragen with the binding of intact choleragen greater for ganglioside and the affinity of the subunits greater for gastric mucin. Electrophoresis of labeled choleragen showed uniform labeling of the subunits dissociated by acid pH, but a major part of cholera toxin was found not to be labeled when fractionated with sodium dodecyl sulfate and 2-mercaptoethanol.

Rats have recently been used as a cholera model (14). Once thought to be resistant to the effects of cholera toxin placed in the intestine, this species can be used if it is prepared by first flushing the contents from the small intestine. The presence of the toxin in a flushed loop of small intestine then stimulates a copious secretion of intestinal fluid. The purpose of the present investigation was to study the nature of the toxin inhibition which is present in the nonflushed loop of rat intestine. This inhibition may be mediated by plasma substances which have been reported to inhibit cholera toxin (K. M. S. Aziz and P. W. Hochachka, Fed. Proc. 32:409, 1973). This would occur if plasma constituents were secreted into the lumen of the gastrointestinal tract. Mucins may act as inhibitory agents. They are an important secretion of the GI tract at three different major sites. These sources are the saliva, the stomach, and the small and large intestines. Mucins are glycoproteins which contain sugar moieties that are similar to those in the sialidase-resistant monosialosyl ganglioside (G_{M1}) that is a very potent inhibitor of the activity of cholera toxin (10). It has been shown that a free terminal galactosyl residue is necessary for a ganglioside to have this deactivating property. A sialidase-sensitive *N*-acetyl neuraminic acid (NANA) attached to the galactosyl end removes the deactivating property. The G_{M1} ganglioside also contains a *N*-acetyl galactosamine between the galactosyl and the lactosyl groups. The latter must possess a single sialidase-resistant NANA residue in order to have the deactivating property.

Salivary mucin consists of a protein core in the shape of a rod which comprises about 40% of the molecule and has polysaccharide prosthetic groups attached to it. (5). The carbohydrate portion (60%) consists primarily of hexosamines, hexoses, and NANA, which are suggested to be in the form of a disaccharide with the terminal residue being a sialidase-sensitive NANA. Thus, part of this substance is similar to the part of G_{M1} that deactivates cholera toxin.

Gastric mucin is more complex, which has made it more difficult to study (13). It has been characterized as consisting of three fractions which can be separated by gel filtration. The composition of each fraction is similar in that they contain about 28% hexose, 28% hexosamine, 10% fucose, less than 1% NANA, and 20 to 30% protein. A small variable amount of sulfate is also found in gastric mucin. The sequence of carbohydrates in gastric mucin is not known.

Intestinal mucin contains from 10 to 45% protein depending on the fraction and the method used to separate the fractions (1, 8). Total hexose is 20 to 25% and hexosamine content is the same. NANA is 0 to 10% and fucose is consistently found to be about 6% of the dry weight of the mucin. Sulfate is present in variable amounts less than 3%. Most of the NANA is suggested to be present as the terminal sugar on the oligosaccharides (8). In this way it resembles salivary mucin.

The major difference between gastric mucin and the other two is the NANA content. NANA is an important determinant of physical properties of mucin, and the differences between the properties of these three may have functional significance (8). The molecular weights of each of the three mucins are 1×10^6 to 4×10^6. Other physical properties have been measured with no major differences being reported.

MATERIALS AND METHODS

Animals. The stimulation of secretion of intestinal fluid by cholera toxin and its inhibition by mucin was studied in the rat by a method described previously (14). Male Sprague-Dawley rats, 200 to 300 g, were prepared for experiments by withholding food for 24 h but allowing them to drink a 5% glucose solution ad libitum. Under ether anesthesia the small intestine was ligated at a point just distal to the ligament of Treitz, and its contents were flushed into the cecum with 10 ml of 0.9% saline, which was introduced with a syringe and 27-gauge needle. After the intestine was emptied, the distal end was ligated.

A region in the middle of the jejunum was measured and ligated at each end to form one closed loop per rat into which cholera toxin and different mucins were injected. Five hours after injection, the loops were removed and weighed with and without their fluid contents.

Cholera toxin. Two preparations of cholera toxin were used. A freeze-dried whole-culture filtrate of *Vibrio cholerae* (Wyeth NIH lot 001) was used to prepare an aqueous solution (osmolality 300 mosmol) which was placed in intestinal loops. The concentration of dried filtrate in the solution was 30 mg/ml. One milliliter of toxin solution was used per 15 cm of loop. The other preparation was a highly purified cholera toxin, choleragen lot 1071, which was prepared under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by R. A. Finkelstein, University of Texas Southwestern Medical School, Dallas, Texas, according to a procedure described previously (6). It was reconstituted in 0.01 M phosphate-buffered saline (PBS) (pH 7.4). The concentration of choleragen in the buffer was 20 μg/ml, and 1 ml was used per 15 cm of intestine. Both toxins were kindly provided by Robert S. Northrup of the SEATO Cholera Research Program, NIAID. The activity of cholera toxin was measured by a method described by Craig (3) in which an intradermal injection of 0.1 ml of toxin is followed by an intravenous injection of pontamine blue and measurement of the blued area of skin.

^{14}C labeling of choleragen. Five milligrams of choleragen in 25 ml of PBS was labeled with ^{14}C. Unlabeled choleragen was first dialyzed against 0.1 M sodium borate, pH 9. It was then subjected to reductive alkylation by addition of 4.0 mg of sodium borohydride and 20 μCi of [^{14}C]formaldehyde having a specific activity of 59.2 mCi/mmol (New England Nuclear Corp.). The reaction was carried out over 30 min at 38 C. The labeled toxin was dialyzed against PBS (pH 7.4) until radioactivity was no longer detected in the dialysant. The specific activity of the labeled choleragen was 7.2 mCi/mmol. By inoculation into rat intestinal loops and into rabbits intradermally, it was verified that choleragen had not lost its toxicity in the labeling process. It was assumed that each subunit of choleragen was labeled to a comparable degree. To determine whether this was true, radioactivity of the subunits was measured. This was accomplished by electrophoretic separation of fragments of [^{14}C]choeleragen which was broken up by treatment with either sodium dodecyl sulfate (SDS) and 2-mercaptoethanol or acid pH. Dispersion by the former method involved incubating choleragen for 30 min at 37 C in 0.01 M sodium phosphate buffer (pH 7.1) containing 0.5% SDS and 0.5% 2-mercaptoethanol. Electrophoresis was performed on 10% polyacrylamide gels. Protein was detected by staining 2 to 3 h with Coomassie brilliant blue (0.25% in methanol)-water-acetic acid (5:5:1, vol/vol). They were destained in water-acetic acid-methanol (35:3:2, vol/vol) (16). For acid pH dispersion, choleragen was put in 5 M urea in acetate at pH 2.3 and separated on 7.5% polyacrylamide gels. They were stained and destained as the SDS-treated choleragen. The relative amount of protein in each band was estimated by scanning with a Gilford gel densitometer. The gels were sliced and each part was dissolved in 30% H_2O_2, after which Aquasol (New England Nuclear Corp.) was added and radioactivity was determined in a Packard liquid scintillation counter.

Mucins. Porcine gastric mucin was obtained from Sigma and is commercially prepared by pepsin-hydrochloric acid digestion of porcine stomach mucosa, the supernatant of which is subsequently treated with ethanol to fractionally precipitate the mucin. In this study any remaining lipids were removed by their extraction from 5 g of mucin with 1,000 ml of chloroform-methanol (2:1, vol/vol). The resultant mucin was lyophilized and put into a 10% solution with water. The pH was adjusted to 7.4 with 1 N NaOH and the solution was heated at 75 C for 60 min. Either the pH adjustment or the heating irreversibly denatures pepsin. The resulting gastric mucin was assayed for proteolytic activity by incubating a portion of mucin with 0.5% bovine serum albumin (wt/vol) in 0.01 N HCl (pH 2.0) at 37 C for 20 min. The reaction was terminated with 10% trichloroacetic acid and boiling which precipitated the intact proteins. The supernatant was analyzed for soluble proteins using the Lowry (12) method and L-tyrosine for the standard. The mucin possessed no proteolytic activity. Gastric mucin was also fractionated into an acidic and sulfated mucopolysaccharide fraction (AMPS) and a neutral glycoprotein fraction. Mucin in

solution was treated with 5% cetyltrimethyl ammonium bromide to precipitate the AMPS fraction which was collected by centrifugation at 10,000 × *g* for 30 min. The precipitate was solubilized with 3 M NaCl, precipitated with 3 volumes of ethanol, solubilized in water, dialyzed against water, precipitated with ethanol, and lyophilized. The glycoprotein fraction was precipitated with ethanol, solubilized with water, dialyzed against water, precipitated with ethanol, and lyophilized. Canine intestinal mucin was prepared from fluid secreted by closed loops of small intestine. Secretion was stimulated by placing a 20% mannitol solution in the lumen for 90 min. The secreted intestinal fluid was removed and centrifuged at 2,000 × *g* for 20 min to precipitate the insoluble mucin. The soluble mucin in the supernatant was precipitated with the addition of 3 volumes of ethanol and centrifugation at 10,000 × *g* for 30 min. The insoluble mucin was treated similarly, and after washing and lyophilization of each they were treated with chloroform-methanol (2:1, vol/vol) to extract any lipids. They were reconstituted in saline at a concentration of 50 mg/ml. Salivary mucin was obtained from Sigma. Equal weights (on dry-weight basis) of mucins and cholera toxin were premixed before inoculation into intestinal loops. *N*-acetyl glucosamine was also added on an equal weight basis to toxin and mucin after adjustment of pH to 7.4. Two milliliters of chitin solution (300 mosmol after pH adjustment) was mixed with 100 mg of Wyeth toxin in solution (about 3 ml), and *N*-acetyl cysteine was added so the final concentration was 40 mM in the studies on mucin inhibition.

Plant lectins. Wheat germ agglutinin (WGA) (from Sigma wheat germ lipase) was prepared by heat treatment as described by Burger and Goldberg (2). The concentration of agglutinin in the solution prepared was 25 mg/ml. Hemagglutinin assays were performed on human type A erythrocytes. One mg of this WGA preparation per ml had a titer of 1:100. One milligram of WGA was injected per centimeter of loop 15 min before cholera toxin was added in one group. Concanavalin A was obtained from Sigma.

Gangliosides and assay for sialic acid. Ganglioside (type II, bovine brain) was obtained from Sigma and used in a solution of PBS or was prepared into a water insoluble form by attachment to cerebrosides (17). When ganglioside was used in intestinal loop experiments, it was prepared in solution in PBS and premixed with toxin or added to loops 15 min after toxin at the ratio of 1.0 mg of ganglioside to 25 mg of Wyeth toxin. The mucins and ganglioside were assayed for NANA. They were incubated with *Vibrio cholerae* neuraminidase in pH 5.6, 50 mM acetate buffer with 10 mM $CaCl_2$ at 37 C. Free NANA was measured by the Warren method (15). Total sialic acid was determined by the same method after hydrolysis of the sample with 0.05 N H_2SO_4 at 80 C for 70 min. Neuraminidase (Sigma, 500 units/ml, *V. cholerae*) and sialic acid were obtained from Sigma.

In vitro binding of [^{14}C]choleragen. In vitro experiments were performed to study the binding properties of [^{14}C]choleragen. Labeled toxin in PBS was mixed with mucins or ganglioside-cerebroside in PBS. In some experiments different inhibitors were added. The mucins or ganglioside complex were separated from the unbound toxin by centrifugation.

Mucins were precipitated by adding 3 volumes of ethanol and centrifuging at 2,000 × *g* for 30 min. However, this procedure also precipitated 30 to 40% of choleragen when no mucin was present. Therefore, in some experiments choleragen was premixed with 0.02 M citrate buffer, pH 1.5, to give a medium with a pH of 3.0. At this pH choleragen is dissociated into subunits which do not precipitate at this rate of centrifugation. When ganglioside-cerebroside was the binding agent, it was precipitated by centrifugation in a PBS solvent system. Choleragen remained totally in the PBS supernatant when ganglioside-cerebroside was not present. The supernatant containing unbound labeled toxin was added to Aquasol and counted. Precipitated mucins and gangliosides were solubilized and counted after adding to Aquasol.

RESULTS

Effect of mucins, ganglioside, plasma, serum, and WGA on cholera toxin-stimulated intestinal secretion. A large amount of fluid was secreted when cholera toxin was placed in loops of small intestine (Table 1). When gastric mucin was premixed with toxin, practically no fluid was secreted. When this mucin was added 15 min after the toxin and mixed in the loop, no inhibition of secretion occurred. The inhibitory effect of the mucin was not reversed by *N*-acetyl glucosamine, chitin, or *N*-acetyl cysteine. *N*-acetyl glucosamine alone caused the secretion of a small amount of fluid. Neither salivary mucin or intestinal mucin inhibited cholera toxin (Table 2). Ganglioside premixed with toxin inhibited secretion, but when it was added 15 min after toxin was placed in the intestine little inhibition was observed. Neither plasma nor serum inhibited the secretory effect of toxin. Intestinal mucosa pretreated with wheat germ agglutinin still secreted fluid when cholera toxin was placed in the loops 15 min later.

Binding of [^{14}C]choleragen to mucins and ganglioside. [^{14}C]Choleragen was precipitated to some extent in 75% ethanol, but when it was dissociated under acid conditions none was precipitated (Table 3). Gastric mucin bound choleragen and thus precipitated all of it when the toxin was not disaggregated at pH 7. When choleragen was dissociated at pH 3, mucin bound and precipitated only two-thirds of that precipitated at pH 7. The glycoprotein and AMPS fractions of gastric mucin also bound toxin. None of the inhibitors reversed the gastric mucin binding to an appreciable extent. Salivary mucin and intestinal mucin both bound choleragen (Tables 4 and 5). However, neither mucin precipitated choleragen at pH 3. Only the binding of salivary mucin was reversed by neuraminidase treatment and NANA. Gan-

TABLE 1. *Effects of gastric mucin on the cholera toxin (Wyeth) stimulation of secretion of intestinal fluid*

Toxin	Inhibitor	Additive	N[a]	g of fluid secreted/g of intestine (wet wt)/h[b]
+			32	0.88 ± 0.04
+	Gastric mucin		24	0.03 ± 0.005[c]
+	Saline, 15 min after toxin		6	0.68 ± 0.06
+	Gastric mucin, 15 min after toxin		6	0.64 ± 0.06
+		*N*-acetyl glucosamine	6	0.80 ± 0.09
+	Gastric mucin	*N*-acetyl glucosamine	6	0.27 ± 0.09[c]
−		*N*-acetyl glucosamine	8	0.26 ± 0.04[c]
+		Chitin	8	1.02 ± 0.04
+	Gastric mucin	Chitin	8	0.06 ± 0.01[c]
−		Chitin	12	0.04 ± 0.01[c]
+		*N*-acetyl cysteine	6	0.78 ± 0.10
+	Gastric mucin	*N*-acetyl cysteine	6	0.03 ± 0.01[c]
−		*N*-acetyl cysteine	6	0.01 ± 0.01[c]

[a] Number of animals with one loop.
[b] Mean ± standard error of the mean.
[c] Versus control group (first in each set of two or three); $P < 0.001$.

TABLE 2. *Effects of salivary mucin, intestinal mucin, ganglioside, and serum or plasma on the cholera toxin (Wyeth) stimulation of secretion of intestinal fluid*

Inhibitor	N[a]	g of fluid secreted/g of intestine (wet wt)/h[b]
	32	0.88 ± 0.04
Salivary mucin	5	0.67 ± 0.07
Intestinal mucin, insoluble	5	0.80 ± 0.07
Intestinal mucin, soluble	6	0.84 ± 0.04
Plasma	6	0.74 ± 0.11
Serum	5	0.77 ± 0.04
Saline, 15 min after toxin	6	0.59 ± 0.05
Ganglioside with toxin	6	0.03 ± 0.01[c]
Ganglioside, 15 min after toxin	6	0.55 ± 0.06
Saline, 15 min before toxin	8	0.78 ± 0.06
WGA, 15 min before toxin	10	0.67 ± 0.05

[a] Number of animals with one loop.
[b] Mean ± standard error of the mean.
[c] Versus toxin with saline added 15 min before; $P < 0.001$. All other groups compared to controls (first in each set of two, three, or six); $P > 0.05$.

TABLE 3. *Binding of 20 μg of [^{14}C]choleragen to gastric mucin with and without inhibitors*

Gastric mucin (mg)	pH	Inhibitor	Counts in 75% ethanol (%)	
			Supernatant	Precipitate
	7		58.1	41.9
	3		97.2	3.8
10	7		3.7	96.3
5	7		6.5	93.5
2.5	7		7.1	92.9
1.0	7		8.7	91.3
10	3		31.2	69.8
5 (GP)	7		5.1	94.9
5 (AMPS)	7		7.4	93.6
5 (GP and AMPS)	7		3.8	96.2
10	7	WGA (2.5 mg)	13.4	86.6
10	7	Concanavalin (2.5 mg)	13.2	86.8
10	7	NANA (2.0 mg)	13.8	86.2
10	7	Neuraminidase	10.0	90.0

glioside bound and precipitated choleragen under neutral or acid conditions (Table 6). WGA slightly reversed the binding at pH 7.0 and greatly reversed the binding at pH 3.0. Concanavalin and neuraminidase partially reversed the ganglioside binding. The binding of choleragen to ganglioside was partially reversed by gastric mucin. Mucin alone resulted in precipitation of 27% of the labeled toxin while ganglioside alone precipitated 86%. A combination of mucin and ganglioside resulted in 70% of the choleragen being precipitated at pH 7.0. However, when subunits at pH 3.0 were added to gastric mucin and ganglioside, the amount in suspension and precipitate were more like when mucin alone was added than ganglioside alone. A part of gastric mucin which is soluble at pH

TABLE 4. *Binding of 20 μg of [^{14}C]choleragen to salivary mucin with and without inhibitors*

Salivary mucin (mg)	pH	Inhibitors	Counts in 75% ethanol (%)	
			Supernatant	Precipitate
	7		58.1	41.9
	3		97.2	3.8
5	7		8.9	91.1
5	3		88.3	11.7
5	7	WGA (2.5 mg)	9.8	91.2
5	7	Concanavalin (2.5 mg)	6.4	93.6
5	7	NANA (2.0 mg)	48.2	51.8
5	7	Neuraminidase	45.6	54.4

TABLE 5. *Binding of 20 μg of [^{14}C]choleragen to soluble intestinal mucin with and without inhibitors*

Intestinal mucin (mg)	pH	Inhibitor	Counts in 75% ethanol (%)	
			Supernatant	Precipitate
	7		58.1	41.9
	3		97.2	3.8
5	7		16.3	83.7
5	3		77.7	22.3
5	7	WGA (2.5 mg)	10.7	89.3
5	7	Concanavalin (2.5 mg)	15.2	84.8
5	7	NANA (2.0 mg)	15.3	84.7
5	7	Neuraminidase	13.7	86.3

7.0 becomes insoluble at pH 3.0; hence, the increased number of counts in the precipitate with mucin and toxin only.

Susceptibility of mucins to V. cholerae neuraminidase and NANA content. Neuraminidase released NANA from salivary mucin but did not release any from gastric mucin or its fractions, or from intestinal mucin. The relative amounts of NANA released from the mucins by acid digestion are compared to salivary mucin. Intestinal mucin contained 21% as much, glycoprotein 14%, gastric mucin 7%, and AMPS 3%.

Electrophoresis of [^{14}C]choleragen. Electrophoresis of labeled toxin at pH 2.3 resulted in two bands (Fig. 1a). The upper slow band contained 34.3% of the total radioactivity and 41% of the total protein. The lower fast band contained 65.7% of the radioactivity and 59% of the protein. Electrophoresis of labeled choleragen in SDS and 2-mercaptoethanol resulted in the formation of three bands (Fig. 1b). The radioactivity in the slow, medium, and fast bands (top to bottom) was 33.7, 4.4, and 61.9%, respectively, and the amount of protein was

TABLE 6. *Binding of 20 μg of [^{14}C]choleragen to ganglioside-cerebroside with and without inhibitors*

Ganglioside (μg)	pH	Inhibitor	Counts in PBS (%)	
			Supernatant	Precipitate
	7		98.4	1.6
50	7		13.9	86.1
100	7		13.7	86.3
100	3		8.4	91.6
100	7	WGA (2.5 mg)	18.7	80.3
100	3	WGA (2.5 mg)	80.8	19.2
100	7	Concanavalin (2.5 mg)	23.6	76.4
100	7	NANA (2.0 mg)	12.7	87.3
100	7	Neuraminidase	21.7	78.3
100	7	Gastric mucin (5 mg)	30.0	70.0
	7	Gastric mucin (5 mg)	73.0	27.0
100	7		13.7	86.3
100	3	Gastric mucin (5 mg)	42.2	57.8
	3	Gastric mucin (5 mg)	53.8	46.2
100	3		7.8	92.2

28.5, 29.8, and 41.7%, respectively. In both electrophoreses the recovery of radioactivity from the gels was greater than 90%.

DISCUSSION

The mixed ganglioside preparation from bovine brain contains about 20% G_{M1} (10), which is a potent deactivator of cholera toxin. This ganglioside preparation and all the mucins tested in this study bound and precipitated choleragen. However, only gastric mucin and the mixed ganglioside preparation inhibited the effects of the toxin in the intestine. It appears that choleragen binding to salivary mucin was via its NANA component since both neuraminidase treatment to remove its NANA and competitive inhibition with NANA reversed the binding. WGA has been reported to bind to NANA as well as to hexosamine (9), and it is surprising that this agglutinin did not bind to salivary mucin and reverse choleragen binding. Assuming the molecular weight of WGA to be 25,000 and that of salivary mucin to be 4×10^6, WGA was added to mucin at a high molar ratio compared to the ratio of choleragen to mucin. It is possible that salivary mucin bound to both but with a much greater affinity for choleragen than for WGA. Salivary mucin was unable to bind choleragen when it consisted of subunits at pH 3.0.

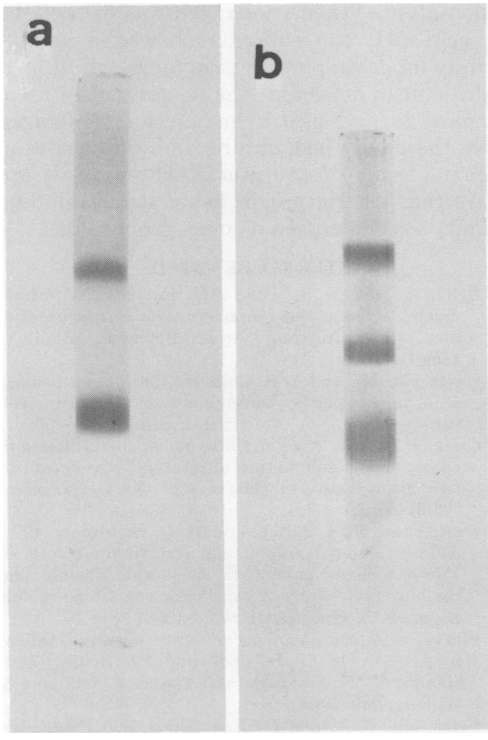

FIG. 1. *(a) Electrophoresis of [^{14}C]choleragen in 5 M urea at pH 2.3 on 7.5% polyacrylamide gel. (b) Electrophoresis of [^{14}C]choleragen in PBS with 0.5% SDS and 0.5% 2-mercaptoethanol on 10% polyacrylamide gel.*

Gastric mucin and intestinal mucin may bind choleragen via means other than NANA residues since neuraminidase and NANA did not reverse the binding to any sizable extent. At pH 3.0 the binding was reversed for both mucins but to different degrees, which indicates that either these mucins could not bind choleragen subunits as well as the intact toxin or that the mucin was changed so it could no longer bind the toxin. Concanavalin A did not reverse the binding of any of the mucins with toxin, which suggests that the toxin does not interact with membrane hexosepyranosides. The in vivo studies failed to show reversal of gastric mucin inhibition with a monohexosamine, a polyhexosamine, or a mild mucolytic agent, *N*-acetyl cysteine. These studies indicate that cholera toxin interaction with gastric or intestinal mucin resisted interference by a variety of agents which would normally inhibit binding of proteins to specific carbohydrates.

Much more cholera toxin is necessary to cause intestinal fluid secretion than to cause the intradermal capillary permeability changes. It has been shown that a high molar ratio of ganglioside to toxin of 300:1 is necessary to inactivate the toxin placed in the ileal loop (10). One explanation for this may be that there is already some inhibition of toxin by the mucin in the intestine so that less ganglioside is necessary to cause complete inhibition.

Intestinal mucin was not effective in inhibiting secretion in in vivo loops. The appearance of this mucin changed in the purifying procedure in that the mucin lost much of its viscosity. Thus, it is likely that some of its functional properties were lost and with this possibly the loss of a potential toxin inhibiting capacity could have occurred. It is apparent that the salivary mucin should not inhibit toxin in the loops since it is readily changed by neuraminidase in the crude toxin (11). It also appears that gastric mucin is not appreciably altered by any of the enzymes produced by *V. cholerae* in this preparation of crude toxin (11) since its inhibition persisted in the loops.

Ganglioside readily inhibited the toxin in loops but not when it was added 15 min after the toxin. Thus, the binding to the mucosal receptor is of a high order since swamping the mucosa with a binding competitor did not reverse the subsequent stimulation of secretion. The [^{14}C]choleragen bound to ganglioside that is released from binding to neuramindase-sensitive NANA by the enzyme should rebind to neuraminidase-resistant NANA. The enzyme caused more [^{14}C]choleragen to remain unbound, which suggests that neuraminidase may prevent rebinding of released toxin to other resistant NANA. Ganglioside was different from salivary mucin in that NANA had no effect to reverse ganglioside inhibition. This suggests that the ganglioside binding does not involve the NANA portion only and the affinity of choleragen for the binding site is not altered by swamping the reactants with NANA. The partial reversal by WGA and concanavalin A suggests that the binding sites may also involve the sugars other than NANA. WGA prevents choleragen binding to ganglioside to a much greater extent at pH 3.0 when choleragen is dissociated into subunits. On the other hand, the subunits of dissociated choleragen bound to ganglioside to a greater extent than to the intact toxin. The WGA reversal indicates that the subunits bind to a hexosamine or possibly a NANA unit. The intact choleragen has either a greater affinity for a specific binding site on ganglioside or it attaches to binding sites with no affinity for WGA. The affinity of WGA for ganglioside appears to be greater than the affinity of choleragen subunits for ganglioside. Ganglioside has been suggested to be the mucosal receptor for cholera toxin. The receptor binds intact choleragen and also choleragenoid, which when bound

to the membrane inhibits any subsequent effect of choleragen by preventing its binding.

In addition, the second fragment which with choleragenoid makes up the intact toxin has been suggested to be the active part of the molecule and it does not by itself appear to bind to the membrane receptor site (4). The results of the present study indicate that the subunits of choleragen dissociated by low pH bound ganglioside to a greater extent than the intact toxin. Thus, if ganglioside is the receptor site for cholera toxin it should bind choleragen, choleragenoid, and the active fragment with near equal affinity.

Choleragen has been shown to consist of subunits that can be studied after dissociation by pH changes to 3.5 or by treatment with SDS and 2-mercaptoethanol (7). There is not complete agreement on choleragen subunit sizes. It has been pointed out that caution should be exercised in interpreting subunit size from data derived from SDS-gel electrophoresis (7). The present studies support this conclusion. The SDS-gel electrophoresis showed that the dissociation of choleragen by SDS and 2-mercaptoethanol is different from that by acid pH. The former treatment resulted in a band of fragments that represented 30% of the choleragen molecule but less than 5% of the label. This suggests that these polypeptides are probably released from within the folded up intact choleragen and are not labeled with ^{14}C with the method used in this study. On the other hand, during dissociation with acid pH this inner part of the choleragen is disrupted differently so the label is present to the same degree as the protein on separated fragments. These results suggest that the above treatments split up choleragen along different planes and that a number of different types of subunits results. The unlabeled part may represent a part of choleragen that is necessary for toxicity and that is protected in the interior of the intact choleragen molecule.

The molar ratio of gastric mucin (molecular weight 1×10^6 to 2×10^6) to choleragen (molecular weight 90,000) was approximately 50:1 in the in vivo experiments where secretion of fluid was completely inhibited. In ileal loops of rabbit intestine a molar ratio of 3.5:1 of ganglioside to toxin inhibited secretion (10). In the present in vitro experiments the molar ratio of ganglioside to toxin was 300:1 and that of gastric mucin to toxin was 12:1. When the two inhibitors were mixed together with the toxin in PBS, a significant amount of [^{14}C]choleragen remained bound to the mucin in the supernatant. When choleragen was dissociated by acid pH, the subunits showed a greater affinity for gastric mucin than for ganglioside. It is difficult to determine the relative affinities of ganglioside and gastric mucin for choleragen since there may be a number of binding sites in each molecule of the mucin. However, it appears that gastric mucin has a strong binding affinity for choleragen as does ganglioside.

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