Specific Binding of Cholera Toxin to Isolated Intestinal Microvillus Membranes

(enterotoxin/intestinal antibodies/microvillus membranes)

W. ALLAN WALKER*, MICHAEL FIELD†, AND KURT J. ISSELBACHER*

The Departments of Medicine and Pediatrics, Harvard Medical School, and the Gastrointestinal Units of the Massachusetts General,* and the Beth Israel Hospitals, Boston† Mass. 02114

Contributed by Kurt J. Isselbacher, October 9, 1973

ABSTRACT A sucrose density gradient assay was used to demonstrate the specificity and saturation of the binding of [3H]cholera toxin to isolated intestinal microvillus membranes from rat small intestine. When the toxin is first complexed to antitoxin and then exposed to intestinal membranes, the binding of cholera toxin is inhibited. To emphasize the physiologic importance of these observations, similar concentrations of [3H]cholera toxin were shown to stimulate the accumulation of cyclic AMP in mucosal homogenates and to increase the secretion of fluid into intestinal loops, whereas the same concentrations of toxin mixed with antitoxin had no effect on cyclic AMP accumulation. These studies suggest that cholera toxin attaches to brush border binding sites before exerting its biologic effect and that local intestinal antibody protection against cholera toxin may be due to inhibition of toxin attachment to these binding sites.

Cholera toxin (CT) affects the small intestine by stimulating the active secretion of chloride ion into the intestinal lumen (1–3). This secretory mechanism is probably mediated by an activation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1.] activity and a concomitant elevation in cyclic AMP (cAMP) levels within the intestinal mucosa (4–6). It is assumed that the toxin exerts its effect on the intestinal microvillus membrane, since experimental studies indicate that the Vibrio cholera organism does not invade the intestinal mucosal barrier (7) and that the enterotoxin released by the organism does not penetrate the luminal epithelial surface in detectable quantities (8). Adenylate cyclase is a membrane-associated enzyme (9) and the toxin binds with specific affinity to cell membranes with adenylate cyclase activity (10, 11). Because of the polar nature of the intestinal epithelial cell, it is not yet understood whether the toxin attaches to a specific receptor site on intestinal microvillus membranes and affects adenylate cyclase activity at that location or whether it modifies adenylate cyclase activity elsewhere on the cell surface. Indirect evidence obtained with animal studies supports the association between intestinal binding of CT and its biologic action (2, 6, 12). Using secretion as an indicator for intestinal CT binding, the toxin has been noted to attach rapidly and efficiently to intestinal epithelial cells. Once exposure has occurred, the biologic effect cannot be reversed with specific antibodies (13) or by using chlorelleopoid (natural toxoid) to displace the membrane attachment of the toxin (12).

Specific immunization by either oral exposure or parenteral exposure to a toxoid or attenuated toxoid represents a potential means of controlling the effects of CT on the small intestine. In experimental studies, both means of immunization have proven effective in producing a significant and prolonged protection from the biologic effect of CT on small intestinal secretion (14, 15). However, the mechanism whereby antibodies interfere with toxin activity has not been elucidated. A possibility is that the antitoxin interferes with or modifies the attachment of the toxin to the receptor site on the surface of the intestinal epithelium. Although indirect evidence exists to support the concept that antibodies interfere with the biologic action of CT by inhibiting its binding to intestinal epithelium (13–15), direct demonstration of antibody interference with CT binding has not been reported previously.

In the present study, a newly developed method has been used to demonstrate the specificity and saturation of CT binding to isolated microvillus membranes of rat small intestine. Antitoxin has been shown to interfere directly with CT binding to intestinal surface membranes. This inhibitory effect would appear to represent the mechanism by which immunization blocks the stimulation of intestinal secretions by CT.

METHODS

Preparation of Intestinal Microvillus Membranes. Each specimen of purified intestinal microvillus membrane was prepared from the small intestinal scrapings of four female Sprague–Dawley rats (Holtzman Co., Madison, Wisc.) weighing approximately 175 g. Animals fasted overnight were given 0.5 ml of saline (0.13 M NaCl) containing 50 μCi of [3H]glucosamine (New England Nuclear Corp., Boston, Mass.) by intraperitoneal injection. As determined previously (16), the optimum incorporation of this radiolabeled sugar into rat microvillus membrane occurs 3 hr after injection. Accordingly, 3 hr after injection and under ether anesthesia the animals were subjected to laparotomy and microvillus membranes were prepared from mucosal scrapings of small intestine according to the method of Hopfer et al. (17). The purity of the preparations was evaluated morphologically by using phase contrast microscopy and biochemically by comparing the specific activity of sucrase (β-D-fructofuranose fructohydrolase EC 3.2.1.26) (18) in the final preparation with that in the initial homogenate. Only membrane preparations in which sucrase specific activity increased greater than 30-fold were used in binding experiments.

Membrane-protein concentration was determined by the method of Lowry et al. (19), and [3H]glucosamine radioactivity was counted in a Tricarb model 3775 scintillation
TABLE 1. Effects of toxin-containing compounds on cAMP levels in rabbit ileal mucosa and epinephrine-treated turkey erythrocytes

<table>
<thead>
<tr>
<th>Concentration of cAMP (µg/mg of ileal mucosal protein or µg/µl of erythrocyte cells)</th>
<th>Concentration of toxin-containing compound (µg/ml of incubation medium*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit ileal mucosa</td>
<td></td>
</tr>
<tr>
<td>Unlabeled toxin</td>
<td>5.4</td>
</tr>
<tr>
<td>[125I]CT†</td>
<td>5.4</td>
</tr>
<tr>
<td>[125I]CT-antiCT‡</td>
<td>5.4</td>
</tr>
<tr>
<td>Turkey erythrocytes + 0.1 µM epinephrine</td>
<td></td>
</tr>
<tr>
<td>Unlabeled toxin</td>
<td>7.2</td>
</tr>
<tr>
<td>[125I]CT†</td>
<td>7.2</td>
</tr>
<tr>
<td>[125I]CT-antiCT‡</td>
<td>7.2</td>
</tr>
</tbody>
</table>

Each number represents the mean result of duplicate determinations for one experiment. The turkey erythrocytes were incubated with CT-containing preparations for 5 min at 4°C, then washed and incubated in HCO3-Ringers solution containing 40 mM glucose and 0.1% BSA for 3.5 hr at 38°C. At 10 min before the end of incubation, epinephrine (0.1 µM) was added and cAMP levels were determined (4). All three toxin-containing compounds were derived from the same lot of toxin and handled in identical fashion except for the specific procedures used to alter the molecules.

* Initial concentration of medium. There were about 150 mg of wet weight of rabbit ileal mucosa in 4 ml of medium and 0.1 ml of packed erythrocytes in 1 ml of medium.
† Numbers for comparison of unlabeled toxin and [125I]CT are representative of three experiments.
‡ Numbers for comparison of unlabeled toxin and [125I]CT-antiCT are representative of four experiments.

Preparation of [125I] CT. Purified CT, supplied by Dr. Robert Northrup (SEATO Cholera Research Program, National Institute of Allergy and Infectious Diseases), was prepared according to the method of Finkelstein et al. (21). [125I]CT was prepared with Na125I (New England Nuclear Corp., Boston, Mass.) according to the method of Greenwood et al. (22). A linear relationship was noted between CT radioactivity and protein concentration of CT at several dilutions of radiolabeled toxin preparation. Furthermore, it was estimated that 30-40% of CT molecules were labeled with 125I using calculations based on the original specific activity of Na125I, a molecular weight of 84,000 for CT (23), and the final protein concentration of the labeled toxin. Some [125I]CT preparations were tested for biologic activity using the turkey erythrocyte method (Table 1) and the rabbit-ileal-mucosal homogenate assay of Kimberg et al. (4). No appreciable biological activity was lost in the process of radiolabeling CT (Table 1). [125I]CT was also identified immunologically using rabbit anti-CT serum and radioautography techniques (24). Choleragenoid (the natural toxoid of Vibrio cholera) was kindly supplied by Dr. Richard Finkelstein, Southwestern Medical School, Dallas, Texas (23).

**Fig. 1.** Precipitin reaction for CT and rabbit anti-CT serum. Increasing amounts of [125I]CT and unlabeled CT(CT[µg]) were mixed with a constant volume of antiserum (0.3 ml). The percent of total CT (O—O) present in the precipitate at various CT concentrations is determined from specific activity of CT. Maximum precipitation occurs at 10 µg of CT. No free CT (O—O) is present in the reaction mixture until the amount of antigen available exceeds the complexing capacity of the antiserum.

**Antibodies to Cholera Toxin.** Albino male rabbits (D. Gaulitz Farms, Franklin, Mass.) weighing approximately 5 kg were initially given footpad injections of a partially purified preparation of CT (provided by Dr. Thomas Hendrix, the Johns Hopkins Medical School, Baltimore, Md.) emulsified in a complete Freund's adjuvant (Difco Co., Detroit, Mich.) (0.5 ml per footpad of 4.0 mg of CT/ml) and subsequently given paraspinal-intramuscular booster injections of toxin and incomplete Freund's adjuvant (0.5 ml of 4 mg of CT/ml) at 2 week intervals. Beginning 6 weeks after the initial injection, rabbits were bled at weekly intervals and the serum was pooled for CT-antiCT studies.

**CT-antiCT Complexes.** A standard quantitative precipitin reaction (25, 26) was done to determine the appropriate concentration of CT and antitoxin necessary to produce antigen-antibody complexes for binding studies (Figure 1). In subsequent binding experiments, toxin-antitoxin complexes were prepared at twice the optimum antibody concentration. However, insoluble complexes prepared according to standard conditions migrate to the same position on the sucrose gradient as microvillus membrane fractions (W. A. Walker, unpublished observations) and thus cannot be used to demonstrate any possible effect of antitoxin on CT binding. Therefore, attempts were made to prepare only soluble small molecular weight toxin-antitoxin complexes by shortening the period of CT-antiCT exposure to 30 min and separating soluble from insoluble complexes using Sephadex G-200 gel filtration. Toxin-antitoxin complexes were assayed for biologic activity and noted to have no stimulatory effect on cAMP accumulation compared to similar concentrations of free CT (Table 1). Additional experiments also demonstrated that under these preparative conditions, toxin-antitoxin complexes were free of contamination by polymers of free CT and by nonspecific CT-protein binding complexes.

**Assay for Biologic Activity in [125I]CT and [125I]CT-antiCT Preparations.** Toxin activity was assayed by determining the
Enterotoxin and epinephrine with tubes containing centrifugation, after Field, Instruments, Inc., prepared mixture was saline, from ethanesulfonic acid (HEPES) (0.001 M) protein and 10^3 - 10^4 cpm per sample. The mixture was applied to a linear 10-60% sucrose gradient prepared in cellulose nitrate centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). The gradients were spun at 105,000 x g for 18 hr using either an SW 50.1 or SW 39 rotor in a Spinco ultracentrifuge (Beckman Instruments, Inc.) according to the method of Jones et al. (27). After centrifugation, the bottom of each tube was punctured and successive two drops of aliquots were collected in separate tubes containing 0.5 ml of saline for radioactive counting; effects of toxin-containing preparations on cAMP levels in rabbit ileal mucosa (4) and in turkey erythrocytes (1) (Table 1).

**Assay for CT Binding.** Aliquots (0.1-ml) of purified radiolabeled microvillous membranes (10-50 μg of membrane protein and 10^4 - 10^6 cpm) in Tris-HEPES-mannitol buffer (0.001 M Tris with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.1 M mannitol, pH 7.5) were incubated for 5 min at 20° with varying concentrations of [125I]CT mixed with unlabeled CT (1-3500 pg in 0.1 ml of saline, from 1 x 10^4 to 50 x 10^4 cpm per sample). The mixture was applied to a linear 10-60% sucrose gradient in cellulose nitrate centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). The gradients were spun at 105,000 x g for 18 hr using either an SW 50.1 or SW 39 rotor in a Spinco ultracentrifuge (Beckman Instruments, Inc.) according to the method of Jones et al. (27). After centrifugation, the bottom of each tube was punctured and successive two drops of aliquots were collected in separate tubes containing 0.5 ml of saline for radioactive counting; effects of toxin-containing preparations on cAMP levels in rabbit ileal mucosa (4) and in turkey erythrocytes (1) (Table 1).

**Assay for CT Binding.** Aliquots (0.1-ml) of purified radiolabeled microvillous membranes (10-50 μg of membrane protein and 10^4 - 10^6 cpm) in Tris-HEPES-mannitol buffer (0.001 M Tris with N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 0.1 M mannitol, pH 7.5) were incubated for 5 min at 20° with varying concentrations of [125I]CT mixed with unlabeled CT (1-3500 pg in 0.1 ml of saline, from 1 x 10^4 to 50 x 10^4 cpm per sample). The mixture was applied to a linear 10-60% sucrose gradient in cellulose nitrate centrifuge tubes (Beckman Instruments, Inc., Palo Alto, Calif.). The gradients were spun at 105,000 x g for 18 hr using either an SW 50.1 or SW 39 rotor in a Spinco ultracentrifuge (Beckman Instruments, Inc.) according to the method of Jones et al. (27). After centrifugation, the bottom of each tube was punctured and successive two drops of aliquots were collected in separate tubes containing 0.5 ml of saline for radioactive counting; effects of toxin-containing preparations on cAMP levels in rabbit ileal mucosa (4) and in turkey erythrocytes (1) (Table 1).

**Fig. 2.** Sucrose gradient separation of CT and isolated microvillous membranes from rat small intestines. [14C]Glucosamine activity, representing intestinal microvillous membranes, appeared in a distinct zone at the bottom of the gradient (2A). [125I]CT was found in a different zone, which was also distinct from the intestinal membranes (2C). The numbers on the ordinates of 2B and 2C have been multiplied by 10^-4.

**Fig. 3.** Sucrose gradient separation of CT-antiCT complexes and isolated microvillous membranes from rat small intestine. [14C]Glucosamine appeared in a distinct zone at the bottom of the gradient (3A). [125I]CT-antiCT complexes mixed with membranes appeared in a biphasic intermediate zone distinct from the membranes (3B). Complexes alone appeared in the same biphasic intermediate zone of the gradient (3C). (The numbers on the ordinates of 3B and 3C have been multiplied by 10^-4.)

three separate binding experiments were done using various concentrations of CT, CT-antiCT complexes, and choleragenoid.

To ascertain any differences in CT binding as a function of time, samples were incubated for 5 and 30 min before placement on the gradient. No difference in CT binding could be demonstrated under these conditions; therefore, the 5 min incubation period was used in subsequent experiments.

[14C]Glucosamine activity was corrected for overlap of [125I]CT counts in the scintillation counter. CT binding to microvillous membrane was calculated from the specific activities of the membranes and CT.

**RESULTS**

**CT Binding.** CT was found to bind consistently to purified preparations of rat intestinal microvillous membranes (Fig. 2). The membrane fractions, represented by [14C]glucosamine counts, appeared in a small, well-defined zone near the bottom of the gradient (Fig. 2A). When [125I]CT was incubated with microvillous membranes and placed on the gradient, two peaks were apparent (Fig. 2B). The first peak, representing 95% of total CT, was located in the same position as the membrane fraction at the bottom of the gradient, and the other peak, representing 5% of total CT, was in the upper zone and clearly distinct from microvillous membranes; these two peaks appeared to represent membrane-bound and...
unbound fractions of CT, respectively. In contrast, when CT alone was placed on the gradient, radioactivity appeared only in the upper zone, a location clearly separate from the membrane fractions (Fig. 2C) and in the same position as the second, smaller CT peak observed after exposure of CT to the membranes (Fig. 2B).

To examine the specificity of CT binding, microvillous membranes were preincubated with sufficient unlabeled CT (600 pg) to saturate the membrane binding-sites and then mixed with [125I]CT under typical experimental conditions. No radioactivity appeared in the membrane fraction of the gradient suggesting that the process of radiolabeling CT did not contribute to binding activity and also suggesting that any 125I dissociated from the toxin was not by itself attached to the membranes. As an additional control for nonspecific binding, equivalent quantities of radiolabeled (125I) bovine serum albumin (BSA) were mixed with microvillous membranes. Under these conditions, none of the BSA appeared in the membrane fraction of the gradient. Furthermore, when BSA was preincubated with membranes and [125I]CT then added, no interference in CT binding to microvillous membranes was noted, which suggests specific CT binding to the membrane.

**Inhibition of Binding with Specific Antibodies.** When soluble [125I]CT-antiCT complexes (10 pg CT) mixed with microvillous membranes were placed on a sucrose gradient and compared with equivalent amounts of free [125I]CT mixed with membranes, the binding of CT was inhibited (Fig. 3). The toxin-antitoxin complexes appeared in an intermediate zone of the gradient (Fig. 3B) which was clearly separated from the membrane fraction (Fig. 3A). Less than 15% of the total radioactivity in the complex fraction (Fig. 3B) appeared in the membrane fraction (Fig. 3A). This amount probably represents the overlap of larger complexes migrating to a lower position in the gradient rather than the partial binding of complexes to membranes, since a similar amount (10%) of complex alone overlapped into the membrane zone of the gradient (Fig. 3C). The bimodal distribution of complexes undoubtedly represent a heterogenous sizing of complexes at various stages of aggregation. To eliminate the possibility that rabbit gamma globulin competed for CT binding sites on intestinal membranes, microvillous membranes preincubated with gamma globulin from nonimmunized animals were mixed with [125I]CT. No inhibition of CT binding was noted. In addition, concentrations of radiolabeled (125I) rabbit immunoglobulin G (IgG) similar to antibody concentrations used to prepare CT-antiCT complexes were obtained from normal animals and those immunized with CT (prepared by Dr. Joseph Perrotto, Gastrointestinal Unit, Massachusetts General Hospital). These immunoglobulins did not bind to microvillous membranes when placed on the sucrose gradient under the usual experimental conditions.

**Inhibition of CT Binding with Choleragenoid.** When aliquots of microvillous membranes were preincubated with excess quantities of unlabeled choleragenoid (100 µg) for 5 and 30 min and then washed in saline and mixed with [125I]CT (10 pg), more than 90% of the total CT appeared in the upper zone of the gradient (unbound CT fraction) as shown previously (Fig. 2B). This suggested that choleragenoid can effectively inhibit toxin attachment to microvillous membrane binding sites.

![Figure 4](image4.png)

**Fig. 4.** CT bound to microvillous membrane (molecules X 10^4 bound per µg of membrane protein) compared to increasing concentrations of CT. Saturation of binding occurred at a concentration of 0.39 pM. The number of binding sites per unit membrane protein was calculated to be 0.0 X 10^6 sites per µg of protein based on the concentration at saturation and on a molecular weight of 84,000 for CT.

**Binding Characteristics of CT.** CT binding (per µg of membrane protein) was determined when increasing concentrations of CT were mixed in constant amounts of microvillous membrane (Fig. 4). A linear relationship between binding and concentration occurred up to a concentration of 0.39 pM CT. Above this concentration, CT binding reached a plateau suggesting saturation of the microvillus-membrane binding-sites. The number of CT binding-sites at saturation was 6 X 10^6 per µg of membrane protein.

When microvillus membrane mixed with [125I]CT (10 pg) was washed in saline and then incubated for 5 and 30 min with excess unlabeled CT (600 pg), no radiolabeled CT was found in the unbound fraction of the gradient. This suggested that the association between CT and intestinal brush border membrane is extremely strong. Furthermore, when mixtures of [125I]CT and unbound CT similar to the mixtures used in binding experiments were placed in rat intestinal loops as described previously (28), excessive secretion occurred as compared to control loops without toxin. These observations indicated that the CT concentrations used in these experiments were sufficient to cause a typical biologic response. To provide further support for the physiologic significance of these studies, [125I]CT activity was measured in rat intestinal loops at CT concentrations known to cause altered secretion. Residual radioactivity was present in luminal contents 5 and 30 min after exposure to CT, which suggests saturation of intestinal binding sites. When the CT concentration was increased 10-fold, no further fluid accumulation occurred in the loops, which indicates that the maximum stimulatory effect of CT had been reached. These results demonstrated that the CT binding characteristics correlated directly with the physiologic response.

**DISCUSSION**

It has been shown that CT binds to isolated rat intestinal microvillus membranes at concentrations which produce the usual biologic response of increased intestinal secretion. The binding capacity appears to be saturable, which suggests that a finite number of CT binding sites exist per µg of microvillus membrane. Furthermore, when two other proteins (BSA and IgG) were mixed with intestinal membranes and placed on a sucrose gradient, no binding could be demon-
intestinal epithelial cells of CT involves space. It has been observed that CT can increase adenylate cyclase activity in crude homogenates of small intestinal mucosa. Comparative effects of CT have been observed in other cellular systems with known membrane-associated adenylate cyclase. Cuatrecasas (10) has shown that CT binds specifically to intact adipose tissue, and Boyle et al. (11) demonstrated that CT binds specifically to rat thymocytes and produces a tenfold increase in cAMP. However, Parkinso et al. (5) reported that CT exposure produced an inhibition of sodium and potassium-dependent adenosine triphosphatase (ATP phosphohydrolase, EC 3.6.1.3) activity in the lateral-basal plasma membrane of intestinal cells. They also found adenylate cyclase activity in microvillous membranes to be much lower than in lateral-basal membranes. Furthermore, Stombeck (29) reported that cholchinic, an inhibitor of cellular microtubular function, can inhibit the biologic effect of CT after the toxin has acted at sites other than the brush border and that intracellular transport of CT may occur.

When CT was complexed with anti-CT in amounts sufficient to inhibit the stimulatory effect on cAMP accumulation, there was an interference in the attachment of CT to intestinal binding sites. This suggests that the mechanism whereby active immunization interferes with the biologic action of CT on the intestine may involve interference with CT attachment to the intestinal cell surface. Although the conditions of these in vitro experiments may not be entirely analogous to the conditions occurring in the intact small intestine exposed to CT, the results strongly suggest that when intestinal antibodies are present within the intestinal lumen or the glycoctalyx of microvillous membranes they either (a) combine with CT such that they cover the region of the molecule which specifically attaches to the membrane binding site or (b) block the binding sites and thus prevent a direct interaction or attachment of CT to the surface membrane.

The present study confirms the observations of Pierce (12) that choleraeogonid (toxoid) inhibits CT binding to intestinal microvillous membranes. Pierce (12) proposed that the binding and biologic activities of CT might be due to two separate portions of the CT molecule, since toxoid interferes with binding but has no apparent effect on intestinal secretion. Pierce also found that toxoid had to be incubated with intestinal loops for prolonged periods before competitive inhibition of CT binding could be demonstrated; he therefore suggested that choleraeogonid was less tightly bound to intestine than CT. However, we found choleraeogonid to inhibit CT binding even when incubated with membranes for short periods (5 min). This may be due to the 10-fold greater amounts of toxoid used to inhibit the CT effect.

These studies indicate that CT initially interacts with the microvillous membrane of intestinal absorptive cells before exerting its biologic effect. However, whether CT is taken up into the cell after its membrane attachment and subsequently has direct intracellular actions remains to be determined.

We are grateful to Miss Laura Davenport for expert technical assistance. This work was supported by a grant from the National Institutes of Health (AM-16269) and a U.S. Army Medical Research and Development Command Contract (DADA-17-70-C-0113). Dr. Walker is a recipient of a Research Fellowship from the Medical Foundation, Inc., Boston, Mass.