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Immunology

Antibodies against synthetic peptides of the B subunit of cholera toxin: Crossreaction and neutralization of the toxin

(synthetic vaccines/cholera antigen/tetanus toxoid/vascular permeability)

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ABSTRACT Six peptides corresponding to various segments of the B subunit of cholera toxin have been synthesized and covalently linked to tetanus toxoid. Of the antibodies raised in rabbits against these conjugates, four crossreacted to different extents with the intact B subunit and whole native cholera toxin. Antisera to the peptide of sequence 75–85 were not crossreactive, whereas elongation by six amino acid residues resulted in a peptide (69–85) leading to antibodies crossreactive with the cholera toxin. Of most interest was peptide CTP3 (50–64), which was the only one that reacted with antisera to cholera toxin and which led to antibodies reacting with the cholera toxin to a similar level as its homologous peptide-antipeptide reaction. Indeed, antisera to CTP3 neutralized significantly the biological activity of cholera toxin, as followed by skin vascular permeability and by fluid accumulation in ligated small intestinal loops of rabbits.

Synthetic peptides, attached to appropriate carriers, may lead to antibodies capable of crossreacting with proteins containing the peptides in their sequences. We have shown it to be true for hen egg-white lysozyme (1), for the coat protein of the bacteriophage MS2 (2), and for influenza hemagglutinin (3). Other laboratories have reported similar results on protein M of *Streptococcus pyogenes* (4), diphtheria toxin (5, 6), hepatitis virus (7–13), and foot and mouth disease virus (14, 15). In several cases of viruses and toxins (3–5, 13–15), the antibodies formed were capable of neutralizing the biological activities.

The purpose of the present study was to prepare synthetic peptides capable of provoking antibodies neutralizing efficiently cholera toxin. The toxin of *Vibrio cholerae* is composed of two subunits, A and B. Subunit A activates adenylate cyclase, which triggers the biological activity, whereas subunit B is responsible for binding to cell receptors and expresses most immunopotent determinants. Antibodies to the B subunit are capable of neutralizing the biological activity of the intact toxin. The B subunit (choleragenoid) is a pentamer, each of the chains containing 103 amino acid residues (16).

In the present report, we describe the synthesis of several peptides derived from the B subunit of the cholera toxin and show that at least one of the peptides synthesized leads to antibodies neutralizing the intact cholera toxin.

MATERIALS AND METHODS

Cholera toxin was purchased from Schwarz/Mann. Separation of subunits and isolation of the B subunit were performed by gel filtration on Sephadex G-75 in 5% formic acid according to Lai (16). *t*-Butyloxycarbonyl derivatives of the various amino acids were purchased from Sigma. All other reagents were of analytical grade or the best grade available.

Peptide Synthesis. Peptides were synthesized by the solid-phase method according to Merrifield (17). The side chain protecting groups of the *t*-butyloxycarbonyl derivatives were as follows: benzyl ethers for the hydroxyls of serine and threonine, dichlorobenzyl ether for the phenolic hydroxyl of tyrosine, and carbobenzoxy for the ε-amino group of lysine. Asparagine and glutamine were protected at the α-carboxyl group by the *p*-nitrophenyl ester. The nitroguanidino group of arginine and the imidazole group of histidine were protected by tosyl. The initial amino acid resin was prepared by esterification of the relevant *t*-butyloxycarbonyl amino acid to chloromethylated resin (polystyrene-1% divinylbenzene).

The progress of synthesis was monitored by ninhydrin analysis. Two cycles of coupling were performed whenever the coupling reaction was <99% complete by the ninhydrin test and amino acid analysis. For the synthesis of CTP6, 5% (vol/vol) 1,2-ethanedithiol was added to the trifluoroacetic acid to prevent oxidation of tryptophan. The protecting groups were removed and the peptides were cleaved from the resin at 0°C with anhydrous hydrogen fluoride containing 10% anisole and 1% 1,2-ethanedithiol as scavengers.

Crude peptides recovered after cleavage from the resin were purified on a Sephadex G-25 column. Purity of peptides was analyzed by amino acid analysis and by reversed-phase HPLC or high-voltage paper electrophoresis (or both).

Conjugation of Peptides with Tetanus Toxoid. Two methods of conjugation were used. (*i*) 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride was used as the coupling agent as described (3). (*ii*) While still on the resin, the peptide was elongated by *t*-butyloxycarbonyl *p*-aminophenyl-acetic acid prior to the HF cleavage. *p*-Aminophenylacetic acid peptides were dissolved in cold 2 M HCl, diazotized by addition of ice-cold aqueous sodium nitrite (0.1 M), and added to a solution of tetanus toxoid in $NaHCO_3$ (0.5 M), while maintaining the pH at 8.5 by addition of concentrated Na_2CO_3. After 10 hr at 4°C, the mixture was dialyzed against 10 mM ammonium carbonate and lyophilized. The peptide content of the conjugates was determined by amino acid analysis and by trace labeling with ^{125}I, when possible.

Immunization Procedure. Rabbits were immunized by multisite intradermal injections of 1 mg of conjugate dissolved in 0.5 ml of phosphate-buffered saline (P_i/NaCl) and emulsified in 0.5 ml of complete Freund's adjuvant, with several booster injections, as described (3).

Solid-Phase Radioimmunoassay (RIA). RIA was performed on antigen-coated (0.5–1.0 μg per well) V-bottom flexible microtiter plates [precoated with glutaraldehyde (0.2%) whenever peptides were used as antigen] by addition of a 3-fold serial dilution of the tested serum, followed by addition of ^{125}I protein

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Abbreviation: RIA, radioimmunoassay.

A labeled by the Bolton–Hunter reagent (10^5 cpm/50 μl per well). The washed and dried wells were cut and the radioactivity was counted in a gamma counter.

In competitive inhibition assays the antigen-coated wells were incubated with 1:10 serial dilutions of the tested inhibitor peptide solution in P_i/NaCl containing 0.1% bovine serum albumin prior to addition of a constant dilution of the antipeptide serum.

Enzyme-Labeled Immunosorbent Assay. The assay was carried out similarly to the RIA, except that flat-bottom plates were used, and a β-galactosidase conjugate of protein A (Amersham) was used instead of the radioactive label. After addition of the substrate (*o*-nitrophenyl-β-D-galactopyranoside) the plates were read in an automatic reader (Titertek Multiskan, Flow Laboratories).

Immunoprecipitation. Cholera toxin was labeled with ^{125}I by the chloramine-T method (18). Immunoprecipitation was performed basically according to Kessler (19) with slight modifications. ^{125}I-Labeled cholera toxin was preadsorbed on intact *Staphylococcus* protein A and then reacted with various antisera. The precipitates obtained after addition of fixed *Staphylococcus* protein A were analyzed by 5–15% $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by autoradiography.

Electrophoretic Blotting Procedure. Cholera toxin, separated into its subunits on a 5–15% $NaDodSO_4$/polyacrylamide gel, was transferred to a nitrocellulose sheet according to the method of Towbin *et al.* (20). To reduce nonspecific binding of antiserum, the blot was incubated for 1 hr with 9 mM Tris·HCl buffer (pH 7.4) containing 0.9 M NaCl and 3% (wt/vol) bovine serum albumin and then was cut into strips. The strips were incubated for 3 hr in room temperature with a 1:50 dilution of different antisera.

After thorough washing, the strips were incubated for 2 hr with ^{125}I-labeled goat anti-rabbit IgG (5×10^5 cpm/ml). The washed and dried blots were autoradiographed.

Toxin Neutralization Activity. *Vascular permeability assay.* The assay was performed essentially according to Craig (21) and Lai (16) with slight modifications. Briefly, serial dilutions of the test antisera were mixed with a constant amount of cholera toxin and incubated for 1 hr at room temperature; 0.1 ml of the mixtures was injected intradermally (in triplicate) into the shaved skin of an adult New Zealand White rabbit. After 18 hr the rabbit was injected intravenously with 1 ml of 5% Evans blue per kg and the diameter of the resulting blue induration was measured 1 hr later. Neutralization end point was taken to be the highest dilution of serum that prevented the blueing phenomenon. Both positive (no antiserum) and negative (no cholera toxin) controls were included for each rabbit.

Ligated ileal loop assay. The assay was performed essentially as described by Fujita and Finkelstein (22). Briefly, rats or adult rabbits fasted for 12 hr were anesthesized with ether, the abdomen was opened, and the small intestine was ligated in 3- to 4-cm-long loops starting ≈10 cm from the duodenum. The loops were injected with different dilutions of the tested sera previously incubated with a constant amount of cholera toxin, and the abdomen was closed. Food and water were withheld and the animals were sacrificed after 5 hr. Fluid accumulation per centimeter of loop was determined by measuring the length and weight of each loop. Both positive (no antiserum) and negative (no cholera toxin) controls were included for each animal.

RESULTS AND DISCUSSION

Cleavage of the B Subunit. To locate the regions participating in antigenic reactivity, the B subunit was cleaved by CNBr to yield three fragments with the following sequences: 1–37 and 69–101 linked by a disulfide bridge between cysteine residues at positions 9 and 86, 36–68, and 102–103. The fragments were separated on a Sephadex G-50 column, and the two larger peptides were tested for reactivity with antisera prepared against cholera toxin. The largest peptide was partially crossreactive with anticholera toxin sera, whereas the peptide 38–68, although incapable of binding directly to the antisera, was capable of inhibiting the toxin-antitoxin homologous antisera (results not shown).

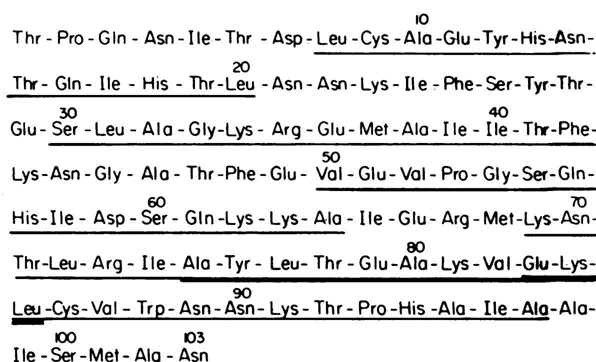

FIG. 1. The amino acid sequence of the B subunit of cholera toxin as quoted by Lai (16). Regions of the protein selected for synthesis are underlined.

Selection of Peptides for Synthesis. Considerations based on the above results obtained with the CNBr fragments of the B subunit, as well as several previous studies on antigenically important regions of this protein (23–26), dictated the selection of peptides for chemical synthesis. Thus, the peptide 30–42 (CTP2) was synthesized, because it was suggested that Arg-35 or the region surrounding it may be involved in antibody and receptor binding activity (23). The peptide 83–97 (CTP6) containing tryptophan at position 88 was synthesized, because chemical modification of this residue had resulted in loss of galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglucosylceramide (GM_1) binding to cholera toxin (24). We have also synthesized the peptide containing the highest local average hydrophilicity (residues 79–84), because such sequences are thought to be located in or immediately adjacent to antigenic determinants (25). Based on the reported role of tyrosine

Table 1. Properties of various peptide conjugates with tetanus toxoid (M_r 150,000)

			Peptide/carrier ratio	
Peptide	Position	Method of coupling	Used for coupling, mol/mol	In the conjugate, mol/mol
CTP1	8–20	*p*-Aminophenylacetic acid*	95	54
		EDCl	40	27
CTP2	30–42	EDCl	45	11
CTP3	50–64	*p*-Aminophenylacetic acid*	94	63
		EDCl	41	14
CTP4	69–85	EDCl	40	19
CTP5	75–85	EDCl	35	10
CTP6	83–97	*p*-Aminophenylacetic acid*	75	25
		EDCl	49	24

EDCl, 1-Ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride.

* Attached to the peptide via an amide bond and coupled to the protein after diazotization.

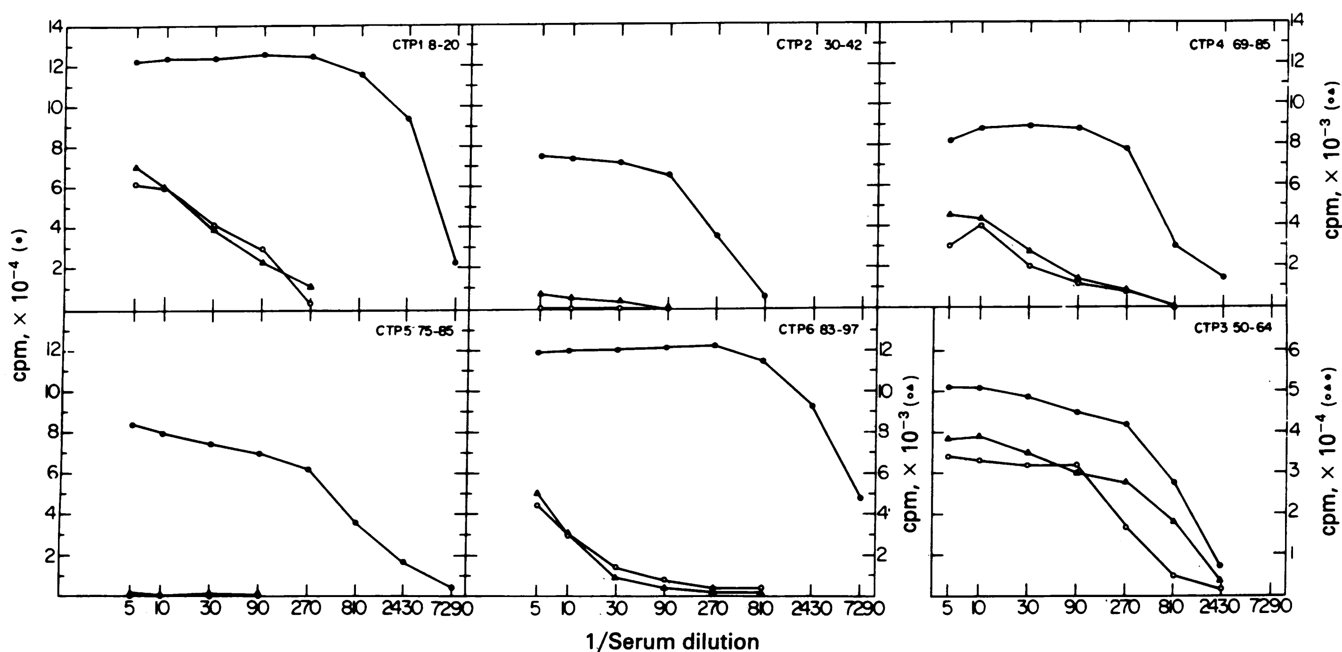

FIG. 2. Antibody response of rabbits to different peptides of the B subunit of cholera toxin: reactions with homologous peptides (○), the B subunit of cholera toxin (▲), and cholera toxin (●). Note difference in the scales of the reactions with the peptide and intact proteins in the case of all peptides except CTP3.

residues for antigenicity (26), peptides containing, respectively, Tyr-12 and Tyr-76—namely, CTP1 (residues 8–20) and CTP5 (residues 75–85)—were included. Because the latter peptide contained only 11 residues, we prepared an additional peptide with a longer sequence (residues 69–85, denoted CTP4). Peptide 50–64 (CTP3) was synthesized because it is part of the inhibitory CNBr fragment 38–68.

Synthesis and Conjugation of Peptides. The peptides synthesized correspond to several regions of the B subunit of cholera toxin (Fig. 1). The only change from the native sequence was the replacement of cysteines at positions 9 and 86 (in CTP1 and CTP6, respectively) by alanine to avoid formation of aggregates.

The results of amino acid analyses of the peptides were in good agreement with the expected values for the various amino acid residues. The purity of the peptides was further established by reversed-phase HPLC or high-voltage paper electrophoresis (or both), indicating <5% impurities in the end products. The purified peptides were conjugated to tetanus toxoid either by using a water-soluble carbodiimide as a coupling agent or through an azo bond, when *p*-aminophenylacetic acid derivatives of the peptides were employed. The advantage of binding via the *p*-aminophenylacetic acid residue is the specificity of the conjugation that occurs only between the NH_2-terminal amino group of the *p*-aminophenylacetic acid and histidine or tyrosine residues on the carrier (27). Both methods of coupling yielded adequate conjugates (Table 1).

Immunological Reactivity. The conjugates of tetanus toxoid with all six peptides induced antibodies specific towards the respective homologous peptide (Fig. 2). As shown, the highest response was observed with CTP1 and CTP6. Four of the antisera were also crossreactive to a different extent with the intact B subunit and whole native cholera toxin. This crossreactivity

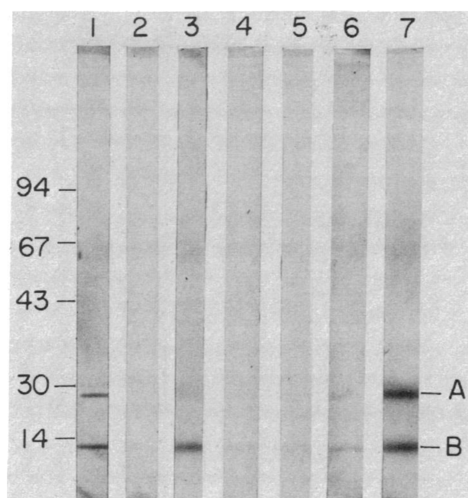

FIG. 3. Reactivity of the antisera against the synthetic peptide with cholera toxin subunits as detected by the immunoblotting technique. The polyacrylamide gel-electrophoresed intact cholera toxin was transferred to a nitrocellulose sheet, strips of which were reacted with the following antisera: anti-CTP1 to anti-CTP6 (lanes 1–6, respectively); anticholera toxin (lane 7). The positions of the A and B subunits are indicated. The molecular weights of the markers are indicated on the left.

Table 2. Immunoprecipitation of ^{125}I-labeled cholera toxin by different antipeptide sera

	^{125}I-Labeled cholera toxin precipitated	
Serum sample	cpm	% of total radioactivity
Anti-CTP1	41,880	4.4
Anti-CTP2	4,956	0.5
Anti-CTP3	66,498	7.0
Anti-CTP4	9,119	0.9
Anti-CTP5	2,980	0.3
Anti-CTP6	47,813	5.0
Anticholera toxin	221,560	23.0
Preimmune serum	2,694	0.28

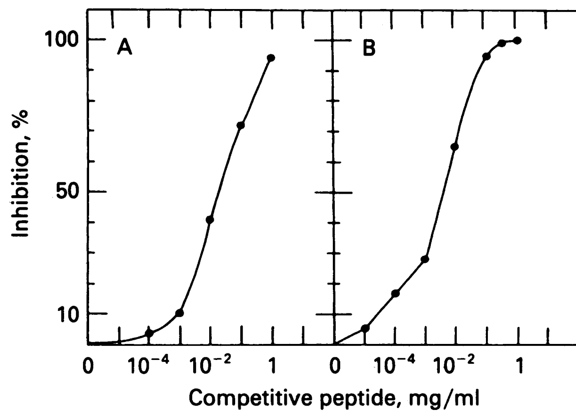

FIG. 4. Inhibition of the reactions CTP3-anti-CTP3 (*A*) and cholera toxin-anti-CTP3 (*B*) by the CTP3 peptide, as performed by solid-phase RIA.

was demonstrated by three assays: solid-phase RIA (Fig. 2), immunoblotting technique (Fig. 3), and immunoprecipitation (Table 2). All three methods showed similar results.

As depicted, antisera against CTP1 and CTP6, although giving the highest titer of homologous reaction, showed only slight reactivity with the intact proteins, several orders of magnitude lower than their reactivity with the homologous peptides. On the other hand, peptide CTP3 induced antibodies, which, though of lower absolute titer, gave a very strong crossreactivity with the intact toxin, similar in its level to that of the homologous peptide-antipeptide reaction (Fig. 2). Both the homologous peptide-antipeptide reaction and the crossreactivity are indeed specific, because they can be completely inhibited by excess of the free CTP3 peptide (Fig. 4). Furthermore, CTP3 is the only peptide of those investigated that reacted with antiserum against the intact native cholera toxin (Fig. 5). Thus, the crossreactivity with a native protein is not a characteristic of just any peptide segment derived from it.

This is further confirmed by a comparison of the related peptides CTP4 (69–85) and CTP5 (75–85). Antiserum against CTP5 was incapable of crossreacting with either the B subunit or whole toxin, although it had a high titer towards the homologous peptide. Elongation of this peptide by six amino acid residues resulted in CTP4, which elicited antibodies crossreactive with the

Table 3. Inhibition of anti-CTP3 peptide of cholera toxin-induced vascular permeability in rabbit skin

Challenge, ng of toxin	Vascular permeability reaction*†		
	Anti-cholera toxin	Anti-CTP3	Preimmune serum
0.5	–	–	+++
1.0	–	+	+++
2.0	–	+++	+++
3.0	–	+++	+++

* +++, Strong blue induration; +, faint blue induration; –, no blue color.

† All sera were used at a 1:10 dilution.

intact proteins, even though the homologous antipeptide titer was not significantly higher than in the case of CTP5.

The results of the immunoprecipitation experiment (Table 2) present the quantitative aspect of the crossreactivity between the antipeptides and cholera toxin. As clearly emerges from the data, the antipeptide CTP3 indeed gives the highest crossreactivity, amounting to about 30% of the homologous toxin-antitoxin reaction. In accordance with the results of the RIA, anti-CTP1 and anti-CTP6 are significantly crossreactive with the intact toxin, whereas the remaining peptides show only slight reactivity.

The immunoblotting experiment serves as another confirmation of these results and raises an additional point of interest: because in this case the intact cholera toxin was separated by electrophoresis into its two subunits prior to the interaction with the various antisera, it is clear from the results (Fig. 3) that antiserum to CTP1 reacts not only with the B subunit but also, to an appreciable extent, with the A subunit of cholera toxin. This phenomenon and its significance in relation to the possible crossreactivity between the A and B subunits of cholera toxin (28, 29) must be further investigated.

Neutralization of Biological Activity. The sera against the various peptides were evaluated for their capacity to neutralize biological effects of cholera toxin. For this purpose two *in vivo* assays of the cholera toxin activity—namely, a skin test measuring the increased vascular permeability induced by the toxin, as well as the fluid accumulation induced by cholera toxin in ligated small intestinal loops of adult rabbits—were employed. The only antiserum that was capable of neutralizing the toxin

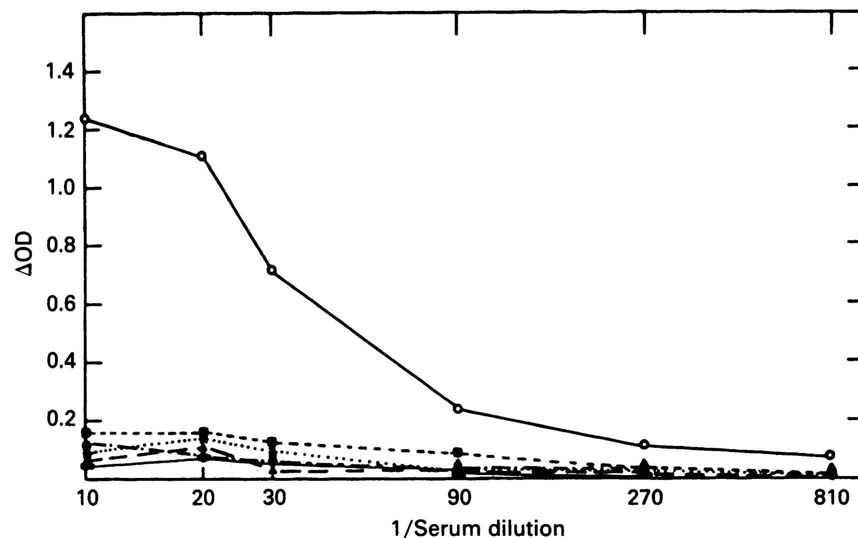

FIG. 5. Crossreaction of anticholera toxin with the various synthetic peptides. *·······*, CTP1; ■---■, CTP2; ○——○, CTP3; ●-·-●, CTP4; ▲---▲, CTP5; and ■——■, CTP6. OD measured at 405 nm.

Table 4. Neutralization of cholera toxin by antisera to peptide CTP3 (50–64)

Cholera toxin, μg	Serum	Dilution	Ligated ileal loop: Weight, g/cm of loop	Ligated ileal loop: Reduced secretion, %*
0.0	None (saline)		0.20	
1.5	None (saline)		1.30	
1.5	Anticholera toxin	1:20	0.45	
1.5	Anti-CTP3	1:2	1.02	40
1.5	Normal rabbit serum	1:2	1.25	
3.0	None (saline)		1.36	
3.0	Anticholera toxin	1:20	0.51	
3.0	Anti-CTP3	1:2	1.08	33
5.0	None (saline)		1.42	
5.0	Anticholera toxin	1:20	0.58	
5.0	Anti-CTP3	1:2	1.32	12
7.5	None (saline)		1.42	
7.5	Anticholera toxin	1:20	0.60	
7.5	Anti-CTP3	1:2	1.32	12
10.0	None (saline)		1.50	
10.0	Anticholera toxin	1:20	0.69	
10.0	Anti-CTP3	1:2	1.50	0

*Assuming that the reduction effected by antiserum to cholera toxin is 100%.

activity was anti-CTP3. The results of these two experiments are presented in Tables 3 and 4. In both assays anti-CTP3 caused partial inhibition of the toxin activity. Although less effective than antiserum against native cholera toxin, the anti-CTP3 reduced quite significantly—up to 40%—the fluid accumulation in rabbit intestine. These two assays, although performed *in vivo*, demonstrate only the presence of antibodies in the sera of the immunized rabbits. They do not provide any indication about cellular or local immunity in their gastrointestinal tract. However, the results obtained thus far indicate that peptide CTP3, which showed the strongest immunological crossreaction with cholera toxin, has an appreciable ability to induce antibodies capable of neutralizing the biological activity of cholera toxin and could therefore serve as a suitable candidate for induction of protective immunity towards the toxin.

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