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In Vitro Formation of Hybrid Toxins Between Subunits of *Escherichia coli* Heat-Labile Enterotoxin and Those of Cholera Enterotoxin

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Heat-labile enterotoxin (LT) was purified from cells of enterotoxigenic *Escherichia coli* isolated from a patient with traveller's diarrhea. Purified LT was separated into A and B subunits by treatment with 6 M urea solution in 0.1 M propionic acid (pH 4.0). Biologically active toxin was reconstituted from isolated A and B subunits of LT. Hybrid toxins with biological activity were obtained in vitro from the A subunit of cholera enterotoxin and B subunit of LT, and from the A subunit of LT and B subunit of cholera enterotoxin. The hybrid toxins show a similar toxicity to that of the parent toxins from which the A subunits were derived. The in vitro formations of the hybrid toxins were confirmed by polyacrylamide gel disk electrophoresis.

The molecular structure of heat-labile enterotoxin (LT) from enterotoxigenic *Escherichia coli* is very similar to that of cholera enterotoxin (CT). Dallas and Falkow (7) showed that LT synthesized by using minicells containing Ent plasmid deoxyribonucleic acid was composed of two distinct proteins with molecular weights of 25,500 and 11,500. These values were very similar to those of the A and B subunits of CT (CTA and CTB, respectively). Clements and Finkelstein (3) demonstrated more directly that the subunit structure of purified LT was remarkably similar to that of CT; that is, LT consisted of two subunits, A and B (LTA and LTB, respectively), with molecular weights of 28,000 and 11,500, respectively. Furthermore, similarities in the amino acid sequences of CTA, CTB, LTA, and LTB were reported by Spicer et al. (29) and Dallas and Falkow (8). The immunological similarities of LT and CT are also well established (1, 2, 10, 11, 17, 18, 27, 28). From these findings, it was concluded that hybrid toxins between subunits of CT and LT could be formed in vitro under appropriate experimental conditions. In this paper, we report the in vitro formation of biologically active hybrid toxins from CTA and LTB and from LTA and CTB.

MATERIALS AND METHODS

Bacterial strain and culture of cells. *E. coli* 536-5, a strain which produces LT and which was isolated from a patient with traveller's diarrhea, was cultured in CAYE medium (23, 30) with vigorous shaking at 37°C for 24 h. CAYE medium contained 2% Casamino Acids (Difco Laboratories, Detroit, Mich.), 0.6% yeast extract (Difco), 0.25% NaCl, 0.871% K_2HPO_4, 0.25% glucose, and 0.1% (vol/vol) trace salt solution (5% $MgSO_4$, 0.5% $MnCl_2$, 0.5% $FeCl_3$, and 0.001% H_2SO_4). The medium was supplemented with 90 μg of lincomycin hydrochloride per ml (Lincocin Injection, Japan Upjohn Ltd., Tokyo) to enhance the synthesis of LT (20, 21).

Preparation of crude LT. Crude LT was prepared from *E. coli* cells because it has been reported that whole-cell lysate is the richest source of LT (3). About 9 liters of bacterial cultures were centrifuged at 10,000 rpm for 20 min, and pelleted cells were suspended in about 300 ml of 0.01 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride buffer (pH 8.6) containing 0.9% NaCl. The suspension was sonicated in a Branson Sonifier model 185 (Branson Sonic Power Co., Nanbury, Conn.). The sonicated suspension was centrifuged at 15,000 rpm for 30 min, and the supernatant was further centrifuged at 25,000 rpm for 3 h. The supernatant was collected, and solid ammonium sulfate was added to give 65% saturation. The precipitate was collected by centrifugation at 10,000 rpm for 30 min and suspended in about 200 ml of 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetate, 3 mM sodium azide, and 0.2 M NaCl (TEAN buffer) (14). It was then dialyzed twice against 2 liters of TEAN buffer and used as crude LT preparation.

Purification of LT. LT was purified essentially as described by Clements and Finkelstein (3). The crude LT preparation (200 ml) was applied to a Bio-Gel A5m column (90 by 2 cm) equilibrated with TEAN buffer. The column was washed with 400 ml of TEAN buffer, and LT was then eluted with 400 ml of 0.3 M D-galactose solution in TEAN buffer. Fractions containing LT, assayed by both the Ouchterlony double-gel diffusion test and passive immune hemolysis (31) with

anti-purified CT antisera, were collected and concentrated to about 3 ml by Amicon PM-10 membrane filtration (Amicon Corp., Lexington, Mass.). The preparation was then applied to a Sephacryl S-200 column (90 by 2 cm) equilibrated with TEAN buffer. The column was eluted with the same buffer, and the fractions containing LT were collected, concentrated by Amicon PM-10 membrane filtration, and used as purified LT preparation.

Purified CT. Purified CT, obtained from *Vibrio cholerae* 569B by the method of Ohtomo et al. (24), was purchased from Sanko Junyaku Co., Tokyo.

Separation of A and B subunits of LT and CT. Procedures for separation of LTA, LTB, CTA, and CTB were essentially as described by Ohtomo et al. (25). Toxin solution (10 to 15 mg in 5 ml) was dialyzed first against 200 ml of 6 M urea solution in 0.1 M propionic acid (pH 4.0) for about 16 h and then against 200 ml of the same solution for 6 h. Spectra/por 6 membrane tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.), which cuts off molecules with molecular weights of less than 1,000, was used for dialysis. The dialyzed preparation was applied to a Sephadex G-75 column (100 by 2.5 cm) equilibrated with 6 M urea solution in 0.1 M propionic acid (pH 4.0), and material was eluted with the same solution.

Polyacrylamide gel disk electrophoresis. Polyacrylamide gel disk electrophoresis was carried out as described by Davis (9) by using 7% acrylamide. Electrophoresis was performed at a constant current of 2 mA per tube for 2 to 2.5 h at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel disk electrophoresis in 0.1% sodium dodecyl sulfate was carried out as described by Fairbanks et al. (12) with 8% acrylamide. Electrophoresis was performed at a constant current of 5 mA per tube for 3 h.

Gels were stained with Coomassie brillant blue and then destained as described previously (12).

Vascular permeability test. The vascular permeability test was carried out essentially as described by Craig (5, 6). The hair was clipped from the backs of Japanese white rabbits, and 0.1-ml volumes of threefold serial dilutions of samples in borate-gelatin buffer (50 mM H_3BO_3, 120 mM NaCl, and 0.1% gelatin) were injected intracutaneously into two or four randomized sites in the back of two rabbits. After 24 h, the animals were inoculated intravenously with 1.2 ml of a 5% Diphenyl Brilliant Blue-Supra FF (Ciba-Geiby) solution in phosphate-buffered saline (pH 7.0) per kg of body weight. After 1 h, the intensity and diameter of the blue lesions in the skin were measured. The diameters to the nearest 0.5 mm were plotted against the logarithm of the dilution of the test sample, and the toxic potency of each preparation was expressed as the 4-mm blueing dose per ml. One 4-mm blueing dose was defined as the amount of toxin producing a mean lesion diameter of 4 mm under the conditions described above.

Chinese hamster ovary cell assay. The Chinese hamster ovary cell assay was carried out as described previously (19). Eagle minimal essential medium was used in place of the F12 medium originally described by Guerrant et al. (16) because it decreased the extent of elongation of the cells in the absence of toxin.

Rabbit ileal loop test. The rabbit ileal loop test was carried out essentially as described by Gorbach et al. (15). Japanese white rabbits, weighing about 2 kg, were starved for 48 h and anesthetized. From 6 to 10 intestinal loops, each 8 to 12 cm long, were constructed, and 1-ml volumes of toxin solution in phosphate-buffered saline (pH 7.0) were injected. The animals were sacrificed after 18 h, and the fluid accumulation (in milliliters) and the length of the loop (in centimeters) were measured. LT activity was expressed as the ratio of fluid accumulated to the length of the loop (milliliters per centimeter).

RESULTS

Homogeneity of purified LT. The homogeneity of the purified LT was examined by polyacrylamide gel disk electrophoresis. As shown in Fig. 1, the purified LT gave a single stained band on the gel. To demonstrate that the stained band corresponded to LT itself, the unstained polyacrylamide gel was cut into 2-mm sections, and each was suspended in TEAN buffer to extract the toxin. Assays of the extracts for LT activity showed that LT activity migrated in the same position as the stained band (Fig. 1). These data show that LT was purified almost to homogeneity.

Separation of A and B subunits. A 10-mg amount of purified LT was treated with 6 M urea solution in 0.1 M propionic acid (pH 4.0) as described in the text and separated into A and B subunits on a Sephadex G-75 column equilibrated with 6 M urea solution in 0.1 M propionic acid (pH 4.0). A typical profile of the separation is shown in Fig. 2. The pattern of separation of LT was almost identical to that of 15 mg of CT treated in the same way. LTA was eluted slightly

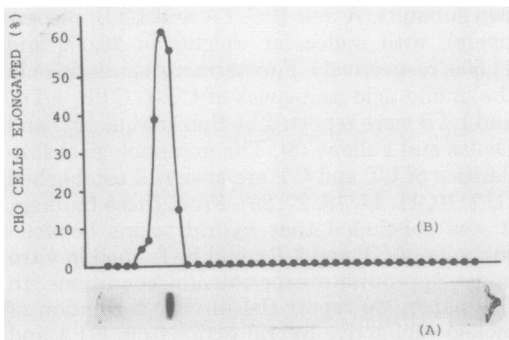

FIG. 1. *Polyacrylamide gel disk electrophoresis of purified LT. Electrophoresis of 10 μg of purified LT was carried out as described in the text. (A) Gel stained with Coomassie brilliant blue showing one stained band. (B) Unstained gel was sliced into 2-mm sections, and LT activity that was extracted from each slice was assayed with Chinese hamster ovary cells (CHO) as described in the text.*

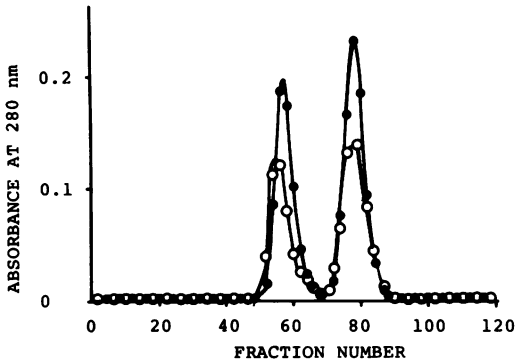

FIG. 2. *Separation of subunits of LT and CT by Sephadex G-75 gel filtration. Purified LT (10 mg) and CT (15 mg) were treated with 6 M urea solution in 0.1 M propionic acid (pH 4.0) as described in the text, and Sephadex G-75 gel filtration of the treated materials was carried out as described in the text.* ●, *CT;* ○, *LT.*

earlier than CTA. The homogeneity of the isolated LTA and LTB was demonstrated by sodium dodecyl sulfate-polyacrylamide gel disk electrophoresis (Fig. 3).

Reconstitution of biologically active toxin. For reconstitution, fractions eluted from the Sephadex G-75 column (Fig. 2) with 6 M urea solution in 0.1 M propionic acid (pH 4.0) with the highest absorbance at 280 nm (A_{280}) (fractions 58 and 79 in Fig. 2 for CTA and CTB, respectively, and fractions 56 and 79 in Fig. 2 for LTA and LTB, respectively) were used. Equal volumes of fractions 57 (A_{280} = 0.126) and 79 (A_{280} = 0.142) from the LT column were mixed and dialyzed against TEAN buffer for 16 h, with one change of the buffer after 12 h, and the biological activities of the preparations were examined. As shown in Table 1, biologically active toxins was recovered. All of the biological activities examined, that is, the rabbit skin vascular permeability activity, the ability to cause morphological changes in Chinese hamster ovary cells, and the ability to accumulate fluid in a rabbit ileal loop, were more or less the same as those of intact LT. Neither LTA nor LTB alone had significant activities.

Similarly, biologically active toxin was reconstituted from CTA and CTB. When equal volumes of fractions 58 (A_{280} = 0.200) and 79 (A_{280} = 0.232) from the CT column were mixed, dialyzed against TEAN buffer, and examined for biological activities, it was found that reconstituted toxin with almost the same toxicity as CT was formed (Table 1).

In vitro formation of biologically active hybrid toxins. An attempt to obtain hybrid toxins was successful, as shown in Table 1. When equal volumes of fraction 58 (CTA) and fraction 79 (LTB) were mixed and dialyzed against TEAN buffer, a hybrid toxin was formed which showed almost the same toxicity as that of CT. On the other hand, when equal volumes of fraction 56 (LTA) and fraction 79 (CTB) were mixed and dialyzed against TEAN buffer, the hybrid toxin formed showed a similar toxicity to that of

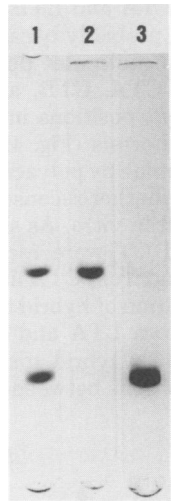

FIG. 3. *Sodium dodecyl sulfate-polyacrylamide gel disk electrophoresis of purified LT and its subunits. Purified LT, LTA, and LTB, isolated as for Fig. 2, were each heated at 100°C for 5 min, and sodium dodecyl sulfate-polyacrylamide gel disk electrophoresis was carried out as described in the text. 1, purified LT; 2, isolated LTA; 3, isolated LTB.*

TABLE 1. *In vitro formation of biologically active reconstituted and hybrid toxins from CTA, CTB, LTA, and LTB*

Toxin	BD_4 (ng)[a]	CHO activity (ng)[b]	Ileal loop activity (ml/cm)[c]
LTA	640	6,800	0
LTB	1,400	35,000	0
LT	4	320	1.04
LTA + LTB	15	88	1.14
CTA	5.8	1,300	0
CTB	12	2,000	0
CT	0.016	6.2	1.09
CTA + CTB	0.023	4.8	0.86
CTA + LTB	0.092	3.5	1.11
LTA + CTB	3.7	180	0.64

[a] The amount of toxin was expressed as the protein content measured as described by Lowry et al. (22); BD_4, 4-mm blueing dose.

[b] Chinese hamster ovary cell (CHO) activity was defined as the amount of toxin causing 50% elongation of CHO cells.

[c] Ileal loop activity was examined with 5 μg of toxin.

LT. These data suggest that the hybrid toxins have similar toxicities to those of the parent toxins from which the A subunits were derived.

Demonstration of reconstituted and hybrid toxin formations by polyacrylamide gel disk electrophoresis. The formation of reconstituted toxin and hybrid toxin was confirmed by polyacrylamide gel disk electrophoresis. The mobilities of LTA, LTB, and LT differed, although LTA and LTB did not migrate as a single band, probably because of denaturation of the toxin molecules during urea treatment (Fig. 4A); CTA, CTB, and CT also migrated to different positions in polyacrylamide gel disk electrophoresis (Fig. 4B). Thus, it was possible to determine by polyacrylamide gel disk electrophoresis whether reconstituted or hybrid toxin was formed in vitro. As shown in Fig. 4A and 4B, LT and CT were reconstituted from LTA and LTB and from CTA and CTB, respectively. The formation of hybrid toxins from CTA and LTB and from LTA and CTB is demonstrated in Fig. 4C. Hybrid toxins migrated to intermediate positions between CT and LT.

DISCUSSION

In vitro reconstitution of biologically active CT from the subunits of CT has been reported by Finkelstein et al. (13). They separated CTA and CTB and demonstrated reconstitution by polyacrylamide gel disk electrophoresis and activity of the reconstituted toxin both in causing fluid accumulation in young rabbit intestine and in increasing rabbit skin vascular permeability. The present study confirmed these results and also demonstrated the reconstitution of LTA and LTB as well as the formation of hybrid toxins of CTA and LTB and of LTA and CTB.

LT used in this experiment was isolated from cells of *E. coli* by sonication, and LTA was unnicked (data not shown). This might be the reason for the lower specific activity of LT compared with that of CT as reported by Clements and Finkelstein (3). Actually, unnicked LT was nicked by trypsin treatment, and the specific activity of nicked LT was substantially greater as reported by Rappaport et al. (26) (data not shown). However, use of unnicked LT resulted in the interesting finding that the hybrid toxins

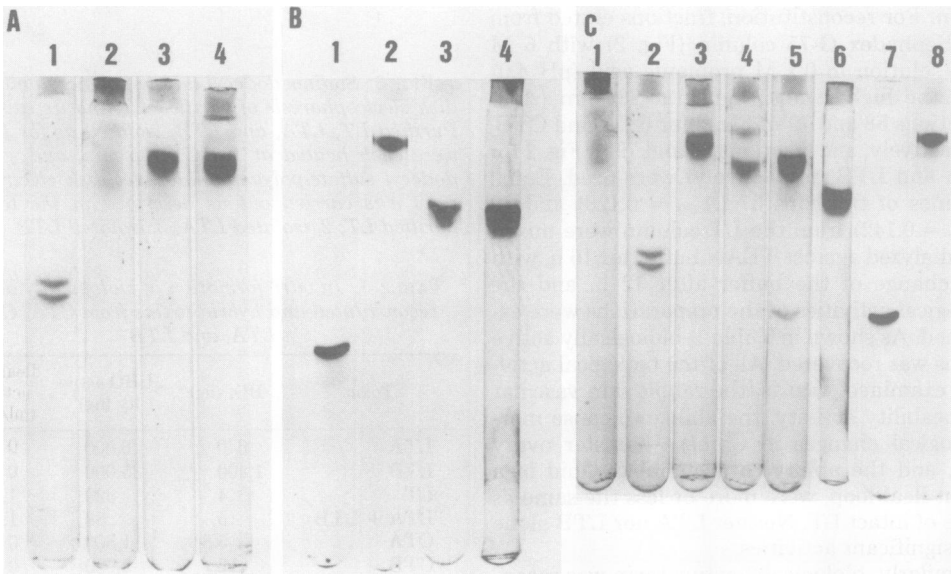

FIG. 4. *Polyacrylamide gel disk electrophoresis of reconstituted and hybrid toxins. Polyacrylamide gel disk electrophoresis was carried out as described in the text. (A) Reconstituted toxin from LTA and LTB. Fraction 56 alone (LTA), fraction 79 alone (LTB), and a mixture of equal volumes of fractions 56 and 79 were dialyzed separately against TEAN buffer for 16 h with one change of the buffer after 12 h, and 80 μl of each sample were analyzed by polyacrylamide gel disk electrophoresis. 1, LTA; 2, LTB; 3, reconstituted toxin; 4, purified LT. (B) Reconstituted toxin from CTA and CTB. Fraction 58 (CTA) and fraction 79 alone (CTB) and a mixture of equal volumes of fractions 58 and 79 were dialyzed separately as described above, and 80 μl of each sample was analyzed by polyacrylamide gel disk electrophoresis. 1, CTA; 2, CTB; 3, reconstituted toxin; 4, purified CT. (C) Hybrid toxins. Conditions of the analysis were as described above. 1, LTB; 2, LTA; 3, purified LT; 4, hybrid toxin from LTA and CTB; 5, hybrid toxin from CTA and LTB; 6, purified CT; 7, CTA; 8, CTB.*

show similar toxicities to those of the parent toxins from which the A subunits were derived.

In polyacrylamide gel disk electrophoresis, the mobilities of CTA, CTB, LTA, and LTB were quite different. This is consistent with the report of Clements et al. (4) that the isoelectric points of CT and LT were different and probably reflects the immunological nonidentity of these two molecules (1, 2, 10, 11, 17, 18, 27, 28). We do not know why LTA and LTB did not migrate as one sharp band because the same preparations migrated as a single band in sodium dodecyl sulfate-polyacrylamide gel disk electrophoresis (Fig. 3). It is probable that partial denaturation of LTA and LTB during their separation in the presence of 6 M urea may have caused this, but we do not think that it was due to contamination or nonhomogeneity of the subunits. The migration pattern of LTB suggests an alternative explanation; that is, the pH of the gel is nearly at the isoelectric point of LTB, thus resulting in diffuse migration.

The formation of hybrid toxins of LTA, LTB, CTA, and CTB suggests that these subunits have similar structures for association of A and B subunits. It will be interesting to determine which fragments of the subunit molecules are necessary for association of their A and B subunits and whether these fragments of CT and LT really have the same molecular structures.

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