INFECTION AND IMMUNITY, Sept. 1986, p. 464–473
0019-9567/86/090464-10$02.00/0

Vol. 53, No. 3

Purification and Characterization of Type II Heat-Labile Enterotoxin of *Escherichia coli*

RANDALL K. HOLMES,* EDDA M. TWIDDY, AND CAROL L. PICKETT

Department of Microbiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814

Received 27 March 1986/Accepted 20 May 1986

Type II heat-labile enterotoxin (LT-II) of *Escherichia coli* has several biologic activities similar to cholera toxin (CT) and *E. coli* type I heat-labile enterotoxin (LT-I), but it is not neutralized by antiserum prepared against CT or LT-I. LT-II was purified from *E. coli* SA53 and from *E. coli* HB101(pCP3837), a strain that contains the cloned LT-II genes in a hybrid plasmid and produces up to 600 times more LT-II than does SA53. Purification involved sonic disruption of bacterial cells, ammonium sulfate fractionation, chromatography on Affi-Gel Blue, chromatofocusing, and gel filtration on Sephadex G-100. The LT-II purified to apparent homogeneity from HB101(pCP3837) had an isoelectric point of 6.8, induced increased vascular permeability in rabbit intracutaneous tests, caused rounding of cultured Y1 adrenal cells accompanied by increased intracellular cyclic AMP, and was 25 to 50 times more potent than CT or LT-I in the Y1 adrenal-cell assay. In contrast, purified LT-II did not cause secretion in ligated rabbit ileal segments at doses corresponding to CT controls that gave strongly positive reactions. LT-II was composed of two different polypeptides with M_rs of 28,000 (A) and 11,800 (B); treatment of LT-II with trypsin cleaved the A polypeptide to fragments A1 (M_r, 21,000) and A2 (M_r, 7,000). The activity of LT-II was not blocked by ganglioside GM1 at concentrations that inactivated LT-I or CT. Antiserum against the LT-II from *E. coli* HB101(pCP3837) completely neutralized purified LT-II and the LT-II in crude extracts of SA53, but it did not neutralize purified LT-I or CT.

Cholera enterotoxin (CT) is a heat-labile protein of *Vibrio cholerae* that causes secretory diarrhea by activating adenylate cyclase in the mucosa of the small intestine of patients with cholera (11). CT is the prototype for a group of heat-labile enterotoxins (LTs) of bacterial origin that are closely related in structure and share a common mechanism of action (12). There are several LTs of *Escherichia coli* that belong to this family. The LTs produced by strains of *E. coli* from humans (LTh) and from piglets (LTp) have both common and unique antigenic determinants and can be neutralized by antitoxin against CT (1, 2, 5, 14, 25, 28). These LTs have been designated serogroup I (LT-I) (41). *E. coli* SA53, isolated from a water buffalo in Thailand, produces a toxin that resembles LT-I with respect to several of its biologic activities but is not neutralized by antiserum against CT, LTh, or LTp (19, 20, 23, 26). This toxin, originally called LT-like toxin, is the prototype for a second serogroup of LTs (LT-II) (41).

The structural genes for LT-I are encoded by plasmids in *E. coli* (21). The genes for several members of the LT-I group have been cloned and sequenced, and the organization and expression of the LT-I operon have been analyzed in detail (7, 8, 42, 45, 46). In contrast, the structural genes for LT-II are not encoded by plasmids. The LT-II genes from *E. coli* were recently cloned, and the polypeptides that they encode were identified in minicells (41). The organization of the LT-II operon and the nucleotide sequence of its structural genes have not yet been determined.

Preliminary efforts to purify LT-II from *E. coli* SA53 yielded preparations with specific toxicities approximately 900-fold greater than that of crude sonic extract of the bacterial cells (19, 20, 23, 26), but such preparations were too impure to allow identification of the polypeptide subunits of LT-II. The studies reported here describe (i) conditions of cultivation favorable for production of LT-II by *E. coli* SA53, (ii) production of much greater amounts of toxin by *E. coli* HB101 carrying a hybrid plasmid with cloned LT-II genes, (iii) purification of LT-II to apparent homogeneity, and (iv) initial characterization of the structure, biologic activity, and immunochemistry of the purified toxin.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and media. The following bacterial strains were used in this study: *E. coli* SA53 (19), *E. coli* HB101(pCP3837) (41), *E. coli* HE12 (3), and *V. cholerae* 569B (24). Glucose syncase medium (13, 24), deferrated glucose syncase medium (40), Evans medium (10), LB medium (37), tryptone broth (37), and M-9 minimal salts (37) supplemented with 0.2% glucose or glycerol were prepared as described in the references cited. Penassay broth (Difco Laboratories, Detroit, Mich.) was prepared according to the instructions of the manufacturer. Unless otherwise stated, cultures were incubated at 37°C and aerated by vigorous rotary shaking at 250 rpm. Lincomycin (Sigma Chemical Co., St. Louis, Mo.) was added to glucose syncase medium at concentrations up to 200 μg/ml as specified for certain experiments.

Chemicals and buffers. All chemicals were reagent grade or the highest grade available. Ganglioside GM1 was purchased from Supelco, Inc. (Bellefonte, Pa.). Mixed gangliosides (Type III, lot no. 75C-8200), 3-isobutyl-1-methylxanthine, trypsin, and egg-white trypsin inhibitor were from Sigma. Affi-Gel Blue and low-molecular-weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were from Bio-Rad Laboratories (Richmond, Calif.). Polybuffer Exchanger PBE94, Polybuffer 96, Sephadex G-100, and Sephadex G-200 were from Pharmacia Fine Chemicals (Piscataway, N.J.). Chloramine-T trihydrate was from J. T. Baker Chemical Co. (Phillipsburg, N.J.). Carrier-free $Na^{125}I$ and cyclic AMP (cAMP) assay kits were from Amersham Corp. (Arlington Heights, Ill.). Ampholines (pH 5 to 8) were from LKB-

Produkter AB (Bromma, Sweden). Buffer 1 was 10 mM Tris chloride (pH 7.4). Buffer 2 was 25 mM Tris acetate (pH 8.3). Buffer 3 was composed of 50 mM Tris chloride (pH 7.5), 3 mM EDTA, 200 mM NaCl, and 0.02% (wt/vol) sodium azide.

Purification of LTs. CT was purified from supernatants of cultures of *V. cholerae* 569B (35). LTp was purified from sonic extracts of *E. coli* HE12 (24) and was used to represent the LT-I serogroup defined above.

LT-II was purified both from *E. coli* SA53 grown at 37°C for 18 h in glucose syncase medium with 25 μg of lincomycin per ml and from *E. coli* HB101(pCP3837) grown at 40°C for 18 h in glucose syncase medium plus 10 μg of thiamine per ml and without lincomycin. Throughout the purifications, quantitative assays for protein were performed by the method of Lowry et al. (33) with bovine serum albumin as the standard, and assays for toxicity were performed in the Y1 adrenal cell assay in microtiter plates as described below.

In a representative purification experiment with LT-II from *E. coli* HB101(pCP3837) 1-liter volumes of culture were grown in 4-liter Erlenmeyer flasks. Cells from 18 liters of culture were collected by centrifugation (13,000 × *g* for 10 min), washed once with buffer 1 at 4°C by suspension and centrifugation, and resuspended in 4 volumes of cold buffer 1. Samples containing approximately 100 ml of the bacterial slurry were placed in an ice bath to keep the temperature below 15°C and were disrupted by sonication in 1-min bursts for a total of 5 min with a model W185 Sonifier (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) operated at the highest setting. Particulate debris was removed by centrifugation at 27,000 × *g* for 60 min at 4°C, and all subsequent purification steps were also performed in the cold.

Ammonium sulfate was added to the pooled supernatants to 50% of saturation. The resulting precipitate was collected by centrifugation, dissolved, and dialyzed against several 4-liter volumes of buffer 1. Particulate debris was removed from the dialyzed sample by centrifugation at 27,000 × *g* for 20 min, and the supernatant was applied at a flow rate of 60 ml/h to a 295-ml-bed-volume column (5.0 cm diameter by 15 cm height) of Affi-Gel Blue equilibrated with buffer 1. The column was washed with 8 volumes of buffer 1 until the A_{280} was less than 0.1 and was then eluted with a 1.5-liter linear gradient of NaCl in buffer 1. Fractions containing 5.2 ml were collected. Fractions with the greatest biologic activity were pooled, concentrated to about 20 ml in a Diaflo concentrator with a UM-10 membrane (Amicon Corp., Lexington, Mass.), transferred to dialysis tubing, concentrated to 5 ml or less against dry Sephadex G-200, and dialyzed against 200 volumes of buffer 2.

This dialyzed sample was applied to a 17-ml-bed-volume column (0.9 cm diameter by 27 cm height) of Polybuffer Exchanger PBE 94 equilibrated with buffer 2. To generate a declining gradient from pH 8 to 6, the column was eluted with 300 ml of Polybuffer 96 (previously diluted 1:13 in distilled water and adjusted to pH 6 with glacial acetic acid) at a flow rate of 10 ml/h. Fractions containing 1.2 ml were collected, and fractions with the highest biologic activity were pooled, concentrated in dialysis tubing against dry Sephadex G-200 to approximately 1 ml, and dialyzed against buffer 3. This sample was applied to a 50-ml-bed-volume column (0.9 cm diameter by 78 cm height) of Sephadex G-100 equilibrated with buffer 3 and eluted with the same buffer at a flow rate of 5 ml/h. Fractions containing approximately 0.65 ml were collected, and the fractions with the highest biologic activity were pooled and stored at 4°C.

Bioassays for enterotoxins. Published methods were used to test for the ability of CT, LT-I, or LT-II to cause rounding of Y1 adrenal cells in monolayers in 96-well microtiter plates (19, 34), increased vascular permeability after intracutaneous inoculation in adult rabbits (6), and fluid secretion after inoculation into ligated ileal segments in adult rabbits (9). For each toxin, one unit of activity in the Y1 adrenal-cell assay was defined as the minimum dose required to give a 4+ reaction, corresponding to rounding of at least 75% of the cells in the monolayer (34). For experiments in which the intracellular cAMP concentration was to be determined, 3×10^5 mouse Y1 adrenal cells in 2 ml of RMPI 1640 medium (Microbiological Associates, Bethesda, Md.) supplemented with 10% fetal calf serum were seeded in 35-mm plastic Costar dishes (Costar Cambridge, Mass.). Fresh medium was provided on day 2, and monolayers were confluent on day 3. The confluent monolayers were rinsed twice with 2-ml samples of serum-free medium and then incubated for 30 min at 37°C in 1-ml samples of serum-free medium containing 0.5 mM 3-isobutyl-1-methylxanthine. Toxin was added to duplicate plates in the doses and for the times indicated for individual experiments. The monolayers were extracted for 5 min with 1-ml samples of acidic ethanol (1 ml of 1 N HCl/100 ml of ethanol). After centrifugation, the supernatants were collected, and the precipitates were washed once with 1-ml samples of ethanol-water (2:1). The supernatants from the extraction and the wash were combined and evaporated to dryness at about 55°C. The residues were dissolved and subjected to radioimmunoassays for cAMP with the cAMP assay kits according to the instructions of the manufacturer. The residues from the extracted cells were dissolved in 0.2 N NaOH, and assays for protein were performed by the method of Lowry et al. (33). The concentration of cAMP was expressed as picomoles of cAMP per milligram of protein.

Antitoxins, immunoassays, and ganglioside-blocking assays. Immunization of rabbits with purified CT (1), LTp-I (25), and LT-II (41) is described in the references cited. Assays for neutralizing activity of the antisera against homologous and heterologous antitoxins were performed in microtiter cultures of Y1 adrenal cells by a minor modification of published methods (19). Mixtures containing constant amounts of toxin and dilutions of antitoxin in RPMI 1640 medium with 10% fetal calf serum were incubated for 2 h at 37°C. Samples of 200 μl were transferred to microtiter wells containing monolayers of Y1 adrenal cells, and toxicity was scored after 16 h. Each sample contained a total of 4 U of toxin, and neutralization was considered to have occurred if the reaction to the toxin-antitoxin mixture did not exceed 1+ (corresponding to less than 25% rounded cells in the monolayer) (19, 34). Assays for toxin-blocking activity of gangliosides were similar to toxin neutralization assays with the following exceptions: gangliosides were substituted for antitoxin, and the toxin-ganglioside mixtures were incubated for 30 min at room temperature instead of 2 h at 37°C. Ouchterlony-type immunodiffusion tests in agar gels were based on published methods (22). Samples containing 100 μl of LT-I (200 μg/ml) or LT-II (88 μg/ml) were added to the center wells, and 100-μl samples of undiluted anti-CT, anti-LT-I, or anti-LT-II were added to the outer wells. Plates were incubated overnight at room temperature and examined for visible precipitates.

^{125}I labeling of LT-II. A modification of the chloramine-T method (29) was used. To a 100-μl volume containing 2 to 10 μg of purified or partially purified LT-II in buffer 3, 50 μl of chloramine-T trihydrate (0.5 mg/ml in H_2O) and 10 μl of carrier-free $Na^{125}I$ (1 mCi) were added. After incubation for 3 min on ice, the reaction was stopped by the addition of 100

TABLE 1. Effects of medium composition and lincomycin on the amount and specific activity of LT-II in sonic extracts of *E. coli* SA53

Medium	Lincomycin concn (μg/ml)	LT-II concn	
		U/ml[a]	U/μg of protein
Evans	0	9	0.027
Penassay	0	9	0.036
LB	0	3	0.018
Tryptone	0	27	0.079
M-9 + glucose	0	9	0.028
M-9 + glycerol	0	9	0.014
Glucose syncase	0	27	0.057
Deferrated glucose syncase	0	9	0.026
Glucose syncase	10	81	0.16
Glucose syncase	25	243	0.51
Glucose syncase	50	243	0.62
Glucose syncase	100	81	0.73
Glucose syncase	200	<1	<0.025

[a] Expressed per milliliter of original culture. LT-II activity was measured in the Y1 adrenal-cell assay.

μl of sodium bisulfite (0.58 mg/ml in H_2O). One minute later, 240 μl of buffer 3 containing 100 μg of bovine serum albumin per ml was added. Unbound ^{125}I was removed by exhaustive dialysis against several changes of buffer 3, and the radioiodinated protein was stored at 4°C.

Trypsin treatment of LT-II. Samples of ^{125}I-labeled LT-II at approximately 5 μg/ml were incubated for 1 h at 37°C with trypsin at 1 μg/ml. The reaction was stopped by the addition of egg-white trypsin inhibitor at 1.5 μg/ml.

PAGE and isoelectric focusing. Samples of proteins to be analyzed were boiled for 3 min in a sample treatment mixture containing 0.1% SDS, with or without 5% 2-mercaptoethanol, and subjected to electrophoresis in 13% polyacrylamide slab gels in the discontinuous buffer system described by Laemmli (31). The slab gels were fixed and stained for protein with Coomassie brilliant blue, or dried and exposed to Kodak XAR-5 film for preparation of autoradiographs, or both. The following proteins were included as molecular-weight standards: lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200), and phosphorylase *b* (92,500). Analytical isoelectric focusing of ^{125}I-labeled LT-II was performed in cylindrical 7% polyacrylamide gels with 2% (vol/vol) (pH 5 to 8; LKB Instruments, Inc., Rockville, Md.) ampholines by the method of Wrigley (44). After electrophoresis, the gels were sliced into 2-mm fractions. Each slice was broken into fragments in a small test tube containing 0.45 ml of distilled water, and the pH was measured. Then a 50-μl sample of 10× buffer 3 was added to each sample, and incubation was continued overnight at 4°C. The biologic activity of LT-II extracted from the gel slices was measured in Y1 adrenal-cell assays, and total radioactivity was determined by gamma scintillation counting. Eluted proteins in selected fractions from the isoelectric-focusing gels were also analyzed by SDS-PAGE.

RESULTS

Our initial investigations of LT-II demonstrated that the toxin was predominantly cell-associated in *E. coli* SA53 and that its activity in sonic extracts of strain SA53 was very low in comparison with the activity of LT-I in extracts of *E. coli* reference strains (19, 26). Preliminary experiments were performed to examine the effects of culture conditions on production of LT-II by *E. coli* SA53 (Table 1). The amount of toxin produced during overnight growth at 37°C varied from 3 U/ml in LB medium to 27 U/ml in glucose syncase or tryptone medium, and toxin production was not stimulated by removing iron from the medium. Addition of lincomycin at 25 to 50 μg/ml stimulated production of LT-II by approximately ninefold. Toxin production in glucose syncase medium at 30°C was three to nine times less than at 37 or 42°C, and no dramatic differences were noted between log-phase cultures and stationary-phase cultures (data not shown). Further efforts to purify LT-II from extracts of *E. coli* SA53 were performed with cells grown at 37°C in glucose syncase medium containing 25 μg of lincomycin per milliliter.

In an independent series of experiments, the structural genes for LT-II were cloned into the vector pMOB45. The hybrid plasmid constructed in this manner, named pCP3837, was transformed into *E. coli* HB101 (41). The pMOB45 vector has a temperature-sensitive defect that enables it to replicate to high copy numbers during growth in *E. coli* at 40°C. In glucose syncase medium at 40°C, with or without added lincomycin, *E. coli* HB101(pCP3837) produced up to 600 times more toxin than did SA53 grown in medium with lincomycin. LT-II was also purified from *E. coli* HB101(pCP3837) grown at 40°C in glucose syncase medium.

The present purification scheme was developed empirically during experiments with LT-II from *E. coli* SA53. After the more toxinogenic strain HB101(pCP3837) was developed, similar methods of purification were used successfully with toxin from that strain. The elution profile of LT-II from the genetically engineered strain HB101(pCP3837) was found to be similar to that of the toxin from the ancestral strain SA53 during Affi-Gel Blue chromatography (Fig. 1), chromatofocusing (Fig. 2), and gel filtration on Sephadex G-100 (Fig. 3). LT-II eluted from each of these chromatographic columns as a single peak, but in each case the specific activity of the HB101(pCP3837) toxin was at least 1,000-fold greater than that of the SA53 toxin. At the final step of purification of LT-II from HB101(pCP3837), the biologic activity corresponded with a major symmetrical peak of protein (Fig. 3B). In contrast, LT-II activity from strain SA53 did not correspond with a well-resolved protein peak (Fig. 3A). A sample of LT-II from SA53 was analyzed on a Sephadex G-100 column that had been calibrated with bovine serum albumin, ovalbumin, and chymotrypsinogen as molecular-weight standards. The LT-II activity eluted between the positions for bovine serum albumin and ovalbumin, corresponding to an apparent molecular weight of 50,000 to 60,000.

Quantitative data corresponding to the purifications shown in Fig. 1 to 3 are summarized in Table 2. Three independent purifications of LT-II from strain HB101(pCP3837) were performed. The final yield varied from 40 to 73% of the starting activity, and the specific activity of the purified LT-II varied from 0.85×10^6 to 2×10^6 U/μg. Because of the small amounts of purified LT-II available, all three lots of HB101(pCP3837) toxin were used for the experiments described below. To facilitate detection of very small amounts of protein, samples of LT-II were radioiodinated with ^{125}I. Control experiments demonstrated that radioiodination of LT-II by the chloramine-T method did not cause detectable loss of its biologic activity.

The most highly purified preparations of LT-II from strain SA53 and HB101(pCP3837) were analyzed by analytical isoelectric focusing (Fig. 4). The isoelectric point of the active toxin from each strain was approximately 6.8. The

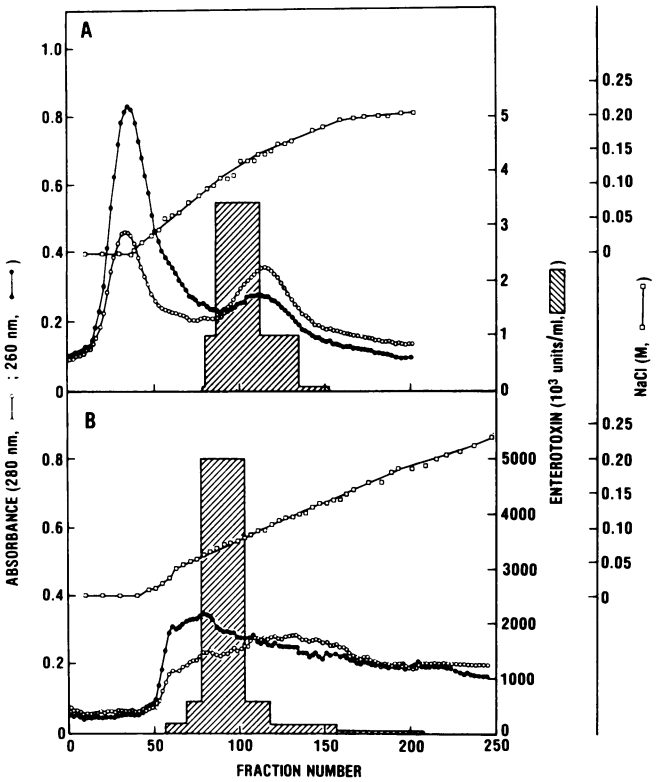

FIG. 1. Chromatography on Affi-Gel Blue of LT-II from *E. coli* SA53 (A) and *E. coli* HB101(pCP3837) (B). Samples designated fraction II in Table 2 were applied to the columns, which were washed with buffer 1 until the absorbance of the eluate returned to a low value. Most of the protein was recovered in the wash, but LT-II activity adhered to the columns and was eluted subsequently with linear gradients of NaCl. Fractions were collected and tested for enterotoxin in Y1 adrenal-cell assays, A_{280}, A_{260}, and conductivity. In panel A fractions 86 to 113 were pooled, and in panel B fractions 78 to 103 were pooled.

LT-II activity from strain HB101(pCP3837) comigrated with a single, asymmetric peak of radioactivity. In contrast, the active toxin from strain SA53 did not correspond to any of the major peaks of radioactivity, confirming the low degree of purity of the 12,300-fold-purified toxin from strain SA53. The toxins were also analyzed by SDS-PAGE. The partially purified toxin from SA53 contained a large number of different polypeptides; they were not analyzed further. In contrast, purified LT-II from HB101(pCP3837) contained two major polypeptides designated A (M_r, 28,000) and B (M_r, 11,800), plus smaller amounts of polypeptides designated A1 (M_r, 21,000) and A2 (M_r, 7,000) (Fig. 5A). In control experiments, the A and B polypeptides of LT-II comigrated with the A and B polypeptides of CT, but the A1 polypeptide of LT-II migrated slightly faster than the A1 fragment of CT (M_r, 23,000). The relative intensities of the bands corresponding to the various polypeptides of LT-II were similar both in autoradiographs and in samples stained for protein (data not shown), except for the A2 polypeptide, which was not visible except in the autoradiographs.

Fractions collected across the asymmetric peak of radioactivity from the isoelectric focusing gel (Fig. 4B) were also analyzed by SDS-PAGE (Fig. 5B). Fractions 30 and 31 (pI, 6.8) contained only the A and B polypeptides, whereas fractions 27 to 29 (pI, 6.6 to 6.7) contained the A1 and A2 polypeptides in addition to A and B. It seemed likely, therefore, that the asymmetric peak shown in Fig. 4B represented incompletely resolved peaks of intact and proteolytically nicked forms of LT-II that had slightly different isoelectric points. To investigate this possibility further, radioiodinated LT-II was treated with trypsin and analyzed by SDS-PAGE both before and after reduction with 2-mercaptoethanol (Fig. 5C). Treatment with trypsin did not affect the mobility of the A or B polypeptide in the absence of 2-mercaptoethanol, but reduction of the trypsin-treated LT-II demonstrated that the A polypeptide had been completely converted to fragments A1 and A2. LT-II holotoxin, which migrates as a single peak during gel filtration and isoelectric focusing, is therefore composed of A and B polypeptides that are noncovalently associated. Furthermore, the A polypeptide of LT-II can be cleaved by trypsin into A1 and A2 fragments that remain linked by one or more disulfide bonds in the absence of reducing agents.

Preliminary studies demonstrated that crude extracts or partially purified LT-II from *E. coli* SA53 caused rounding of cultured Y1 adrenal cells (19, 26), increased vascular permeability in intracutaneous tests in rabbits (26), fluid accumulation in ligated ileal segments in adult rabbits (26), and death of mice inoculated by the intraperitoneal route (unpublished observations). All of these activities except mouse lethality were similar to the activities of CT and LT-I. Crude extracts of *E. coli* HB101(pCP3837) had much higher specific activity in the Y1 adrenal-cell assay than did extracts from SA53, but they were not lethal for mice at doses that contained much

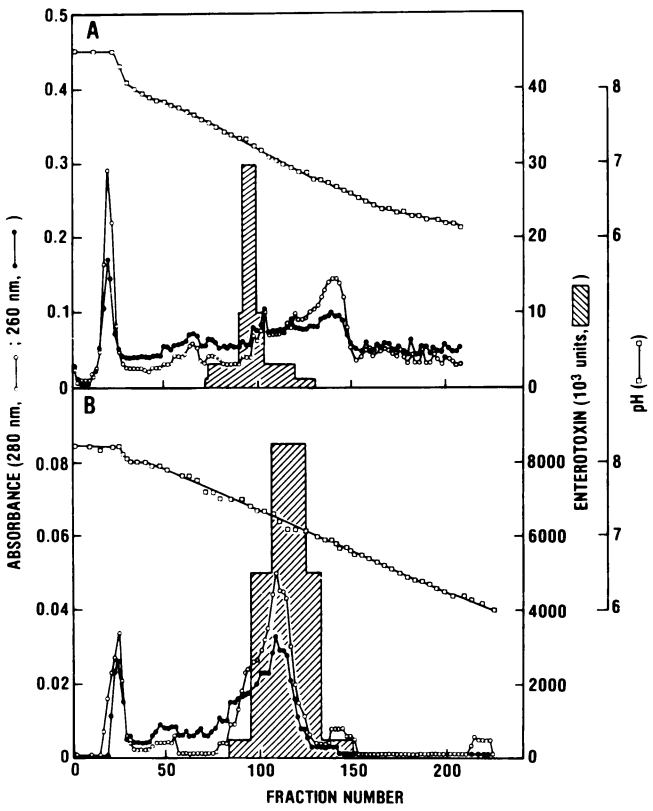

FIG. 2. Chromatofocusing of LT-II from *E. coli* SA53 (A) and *E. coli* HB101(pCP3837) (B). Samples designated fraction III in Table 2 were applied to chromatofocusing columns and eluted with gradients, pH 6 to 8. Fractions were collected and tested for enterotoxin in Y1 adrenal-cell assays, A_{280}, A_{260}, and pH. In panel A fractions 89 to 103 were pooled, and in panel B fractions 107 to 124 were pooled.

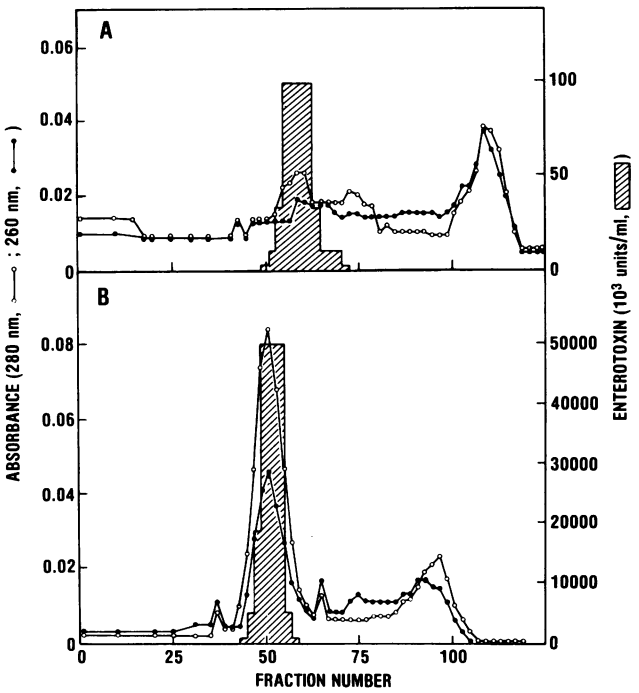

FIG. 3. Gel filtration on Sephadex G-100 of LT-II from *E. coli* SA53 (A) and *E. coli* HB101(pCP3837) (B). Samples designated fraction IV in Table 2 were loaded onto Sephadex G-100 columns and eluted with buffer 3. Fractions were collected and tested for enterotoxin in Y1 adrenal-cell assays, A_{280}, and A_{260}. In panel A fractions 53 to 63 were pooled, and in panel B fractions 48 to 56 were pooled.

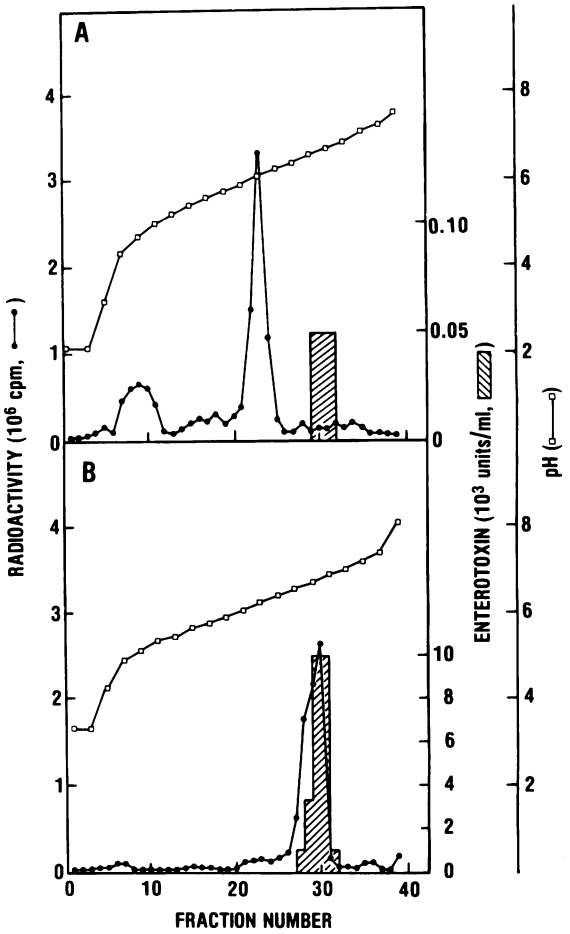

FIG. 4. Analytical isoelectric focusing of LT-II from *E. coli* SA53 (A) and *E. coli* HB101(pCP3837) (B). Samples designated fraction V in Table 2 were analyzed. In each experiment a sample of ^{125}I-labeled toxin was mixed with 25 μl of the same unlabeled toxin and subjected to isoelectric focusing with a gradient (pH 4 to 7) in a cylindrical polyacrylamide gel. Each gel was divided into approximately 40 equal slices, and each slice was extracted and tested for enterotoxin in the Y1 adrenal-cell assay, radioactivity, and pH. The data shown are representative of results from several experiments.

more LT-II activity than the lethal dose of SA53 extract. Therefore, the factor responsible for mouse lethality was not encoded by the recombinant plasmid pCP3837 and was clearly separable from the LT-II activity detected in the Y1 adrenal-cell assay. The lethal factor in crude extracts of strain SA53 has not yet been characterized.

The activity of highly purified LT-II from strain HB101(pCP3837) was examined in several different assay systems. The morphologic changes (rounding) induced in Y1 adrenal cells by LT-I and LT-II were indistinguishable (Fig.

TABLE 2. Purification of LT-II from *E. coli* SA53 and HB101(pCP3837)

Strain and fraction no.[a]	Vol (ml)	Protein (mg)	LT-II			
			Activity[b] (10^3 U)	Sp act (U/μg)	Yield (%)	Purification factor
SA53						
I	840	17,600	2,800	0.16	100	1
II	280	10,200	2,800	0.27	100	1.7
III	4.6	45	1,530	34	55	210
IV	1.5	0.73[c]	188	257	6.7	1,600
V	4.9	0.083	163	1,970	5.8	12,300
HB101(pCP3837)						
I	700	17,500	1,000,000	57	100	1
II	255	7,150	364,000	50.9	36	0.9
III	5.0	18.8	1,000,000	53,200	100	930
IV	1.5	0.85[c]	750,000	882,000	75	15,500
V	5.0	0.19	400,000	2,050,000	40	36,000

[a] Fractions were as follows: I, sonic extract of bacteria; II, 0 to 50% ammonium sulfate cut, after dialysis; III, pool from Affi-Gel Blue column, after dialysis; IV, pool from chromatofocusing column, after dialysis; V, pool from Sephadex G-100 column.

[b] Measured in the Y1 adrenal-cell assay.

[c] Estimated from A_{260} and A_{280}. Residual polybuffer in these samples interfered with protein determination by the Lowry method.

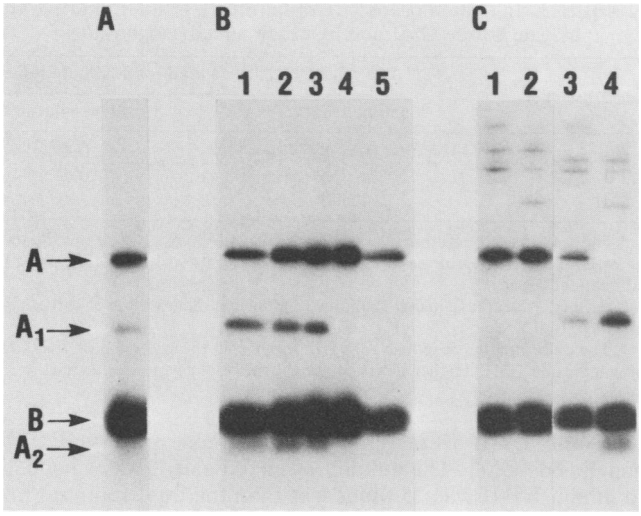

FIG. 5. Autoradiographs of SDS-polyacrylamide slab gels containing samples of LT-II from *E. coli* HB101(pCP3837). (A) ^{125}I-labeled purified LT-II (fraction V from Table 2) was electrophoresed in the presence of 2-mercaptoethanol (ME). (B) Lanes 1 to 5 contained samples eluted from fractions 27 to 31, respectively, of the analytical isoelectric focusing gel shown in Fig. 4B. Electrophoresis was performed in the presence of ME. (C) Cleavage of polypeptide A into fragments A1 and A2 by treatment with trypsin. Purified LT-II (specific activity, 0.9×10^6 U/μg in the Y1 adrenal-cell assay) was radioiodinated, and samples of trypsin-treated or untreated toxin were electrophoresed in the presence or absence of ME. Samples were as follows (lanes): 1, − trypsin, − ME; 2, + trypsin, − ME; 3, − trypsin, + ME; and 4, + trypsin, + ME. Small amounts of contaminating polypeptides with slower mobilities than polypeptide A were present in this sample of ^{125}I-labeled LT-II.

6). The minimal dose of LT-II that caused a 4+ morphologic response in microtiter cultures of Y1 cells was only 0.5 pg (2,050,000 U/μg = 1 U/0.5 pg [Table 2]). LT-II was 25 to 50 times more active than purified CT or LT-I (minimal reactive dose, 12.5 to 25 pg) when tests were performed simultaneously in Y1 cells. The minimal dose of LT-II needed to cause increased vascular permeability in rabbit intracutaneous tests was between 3 and 10 ng. This was comparable to the toxicity of LT-I and approximately 10 times less than the toxicity of CT in simultaneous tests. One preparation of purified LT-II (specific toxicity in the Y1 adrenal cells, 0.9×10^6 U/μg) was tested in ligated ileal segments of adult rabbits, but it did not cause fluid accumulation at doses up to 8.8 μg per segment. CT controls at 1 μg per segment produced strongly positive responses (>2 ml/cm). It was not feasible to test higher doses of LT-II for secretory activity with the amounts of purified toxin that were available for study.

The mode of action of purified LT-II was investigated and compared with that of purified LT-I in cultured Y1 adrenal cells. The concentration of cAMP increased in a dose-dependent manner in cells treated with LT-II or LT-I, but LT-II was about 10 times more potent than LT-I on a weight basis (Fig. 7). The time course for accumulation of cAMP in Y1 adrenal cells treated with equal doses by weight of LT-II and LT-I is shown in Fig. 8. Maximal accumulation of cAMP occurred over a period of approximately 6 h in cells treated with LT-II. The slower accumulation of cAMP in cells treated with LT-I reflected the lower specific activity of LT-I, but other experiments demonstrated similar time courses for accumulation of cAMP in cells treated with LT-II or LT-I at equipotent doses. Observation of the Y1 adrenal cells by phase-contrast microscopy demonstrated that rounding was a sensitive and early manifestation of intoxication by LT-II or LT-I. Maximal rounding responses were observed at the time that the intracellular concentration of cAMP was just beginning to rise. The samples of LT-II and LT-I used in this experiment contained both nicked and unnicked molecules. Pretreatment of the toxins with trypsin did not significantly change their potency, but a completely unnicked sample of LT-II was not available for testing before and after treatment with trypsin. Therefore, it is not yet known if nicking is required to activate the toxicity of LT-II.

Ganglioside GM1 can function as a plasma membrane receptor for CT and LT-I or, when it is added as a competing ligand, as an inhibitor of their toxicity (27, 43). Therefore, the ability of ganglioside GM1 to inhibit the activity of LT-I and LT-II was compared (Table 3). Monolayers of Y1 adrenal cells in microtiter plates were exposed to four minimal toxic doses of LT-I or LT-II in the presence of

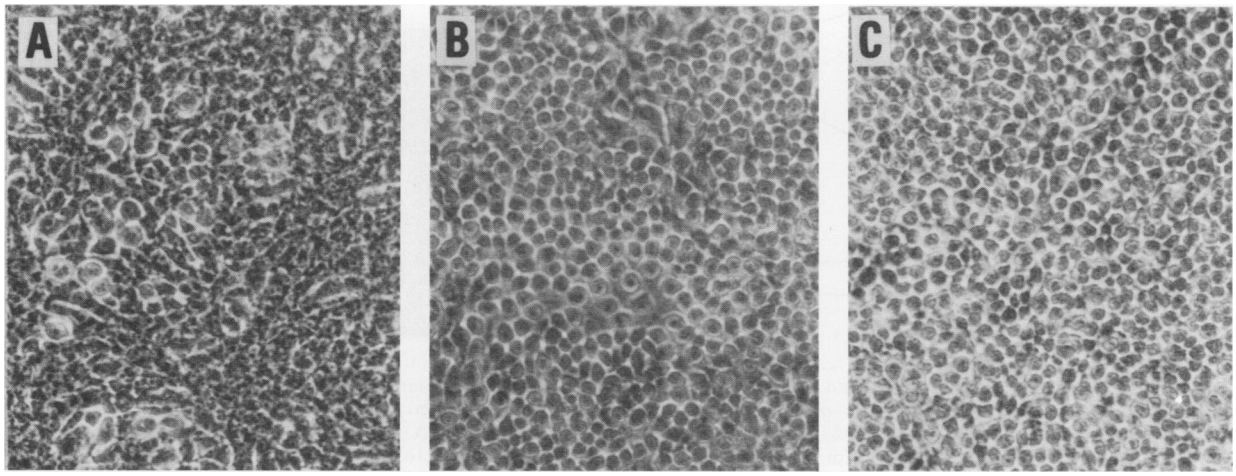

FIG. 6. Effects of LT-I and LT-II on morphology of cultured mouse Y1 adrenal cells. (A) Control monolayer, untreated. (B) 4+ Rounding response of monolayer treated with LT-II. (C) 4+ Rounding response of monolayer treated with LT-I. Monolayers were observed by phase-contrast microscopy and photographed at ×125.

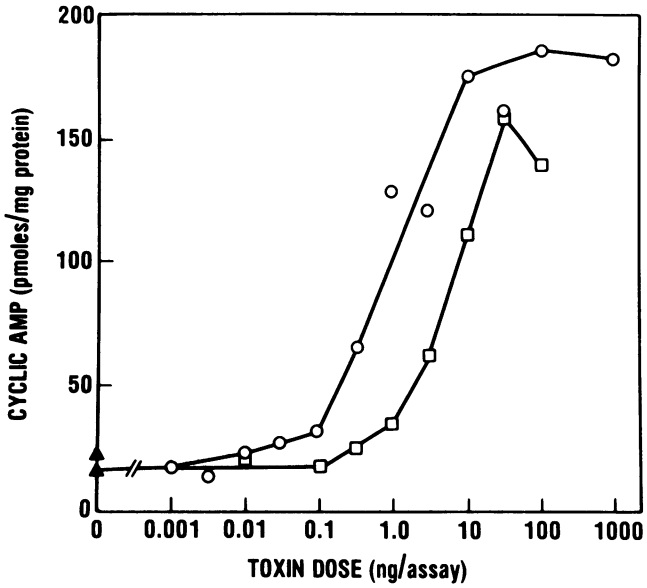

FIG. 7. Effects of LT-I and LT-II on cAMP in mouse Y1 adrenal-cell cultures. Confluent monolayers of Y1 adrenal cells in 35-mm plastic petri dishes containing 2 ml of medium were treated with purified LT-I (□) or purified LT-II (○) at the indicated doses for 16 h in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. At the end of the treatment period, assays were performed to determine the amounts of total cAMP and protein per monolayer. The results presented are averages from duplicate plates treated at each dose of LT-I or LT-II.

increasing amounts of ganglioside GM1 or crude mixed gangliosides. The amount of ganglioside GM1 required to inhibit LT-II was at least 125 times greater than that needed for comparable inhibition of LT-I, and only partial inhibition of LT-II was observed at the highest dose tested. In contrast, when a crude preparation of mixed gangliosides was tested for inhibitory activity, the relative potencies against

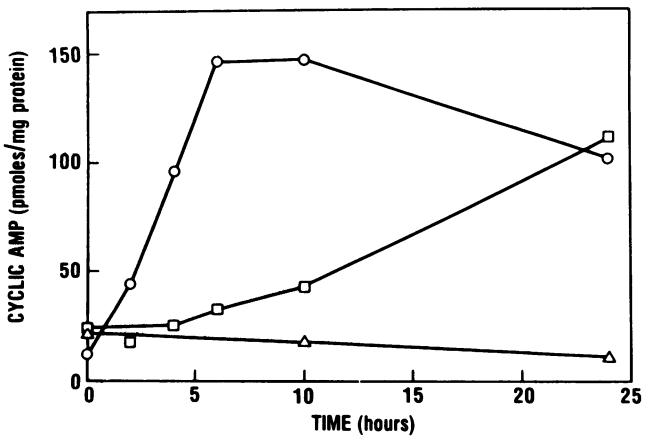

FIG. 8. Time course for accumulation of cAMP in mouse Y1 adrenal-cell cultures treated with LT-I and LT-II. Confluent monolayers of Y1 adrenal cells in 35-mm plastic petri dishes containing 2 ml of medium were treated with 2-ng doses of purified LT-I (□), purified LT-II (○), or no toxin (△) for the times indicated in the presence of 0.5 mM 3-isobutyl-1-methylxanthine. At the times indicated, duplicate plates were analyzed to determine the amounts of total cAMP and protein per monolayer, and the results presented are the average values.

TABLE 3. Inhibition of toxicity of purified CT, LT-I, and LT-II by ganglioside GM1 and by crude mixed gangliosides

Enterotoxin[a]	Amt of ganglioside required for inhibition (ng)[b]	
	Purified GM1	Mixed gangliosides
CT	8	1,000
LT-I	8	1,000
LT-II	>1,000[c]	40

[a] The test dose of enterotoxin per assay was as follows: 100 pg of CT, 100 pg of LT-I and 4.4 pg of LT-II (specific activity, 0.94×10^6 U/μg in the Y1 adrenal-cell assay).

[b] Defined as decrease in cell-rounding response in the Y1 adrenal-cell assay from 4+ to 0.

[c] The cell-rounding response to LT-II was 1+ in the presence of 1,000 ng of ganglioside GM1. Higher doses of ganglioside GM1 were not tested.

LT-I and LT-II were opposite from those observed with ganglioside GM1. The amount of mixed gangliosides needed to inhibit LT-II was 25 times less than the dose required for inhibition of LT-I. These experiments demonstrated that the most potent antagonist of LT-II in crude mixed gangliosides was not ganglioside GM1 and provided strong support for the hypothesis that the receptors for LT-II and for LT-I are different.

To investigate the immunochemistry of LT-II, rabbit antiserum was prepared against the most highly purified sample of LT-II from *E. coli* HB101(pCP3837) (Table 2). In immunodiffusion tests, purified LT-II formed a precipitate with this homologous antiserum (Fig. 9A), but it did not precipitate with antiserum against CT or LT-I. Conversely, purified LT-I precipitated with antiserum against CT or LT-I (Fig. 9B), but it did not precipitate with antiserum against LT-II. Quantitative titrations of neutralizing activity were also performed with these rabbit antisera and the homologous or heterologous toxin antigens in all pairwise combinations (Table 4). The antiserum against LT-II completely neutralized both purified LT-II from *E. coli* HB101(pCP3837) and the LT-II activity in crude extracts from *E. coli* SA53, confirming that the toxin encoded by the cloned genes in plasmid pCP3837 was antigenically indistinguishable from the LT-II toxin in the ancestral strain SA53. In contrast, purified LT-II was not neutralized by antiserum against CT or LT-I, and antiserum against LT-II had no neutralizing activity against CT or LT-I.

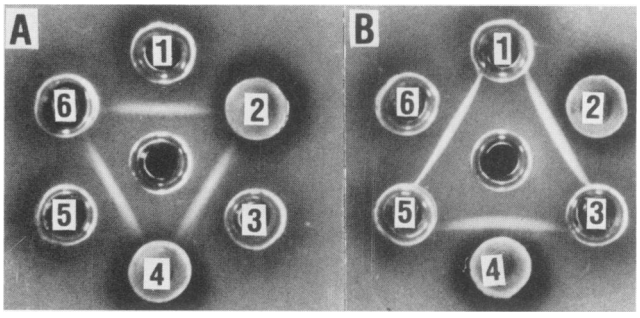

FIG. 9. Interactions of LT-I and LT-II with homologous and heterologous antienterotoxins in double immunodiffusion tests. Samples of purified toxin were added to the center wells as follows: 8.8 μg of LT-II (A) and 20 μg of LT-I (B). Antisera were added to the outer wells as follows: anti-LT-II, wells 1, 3, and 5; anti-CT, well 2; anti-LTp-I, well 4; and anti-LTh-I, well 6. LT-II formed a precipitin line only with the homologous antiserum. LT-I formed precipitin lines with the antisera against LTp-I, LTh-I, and CT but did not precipitate with anti-LT-II.

TABLE 4. Neutralizing activity of anti-CT, anti-LT-I, and anti-LT-II against homologous and heterologous LTs

Enterotoxin[a]	Neutralizing activity (U/μl) of:		
	Anti-CT	Anti-LT-I	Anti-LT-II
Purified CT	1,000	10	<0.1[b]
Purified LT-I	10	300	<0.1
Purified LT-II	<0.1	<0.1	300
Crude LT-II	<0.1	<0.1	100

[a] The test dose of toxin per assay was as follows: 100 pg of CT, 100 pg of LT-I, 1.8 pg of LT-II (specific activity, 2.05×10^6 U/μg in the Y1 adrenal-cell assay), or 10 nl of cell lysate from *E. coli* SA53.

[b] No neutralization was detected with 10 μl of antiserum.

DISCUSSION

The studies reported here describe purification to apparent homogeneity and initial characterization of LT-II of *E. coli*. The ancestral strain in which LT-II was discovered is SA53. By using gene cloning to construct the more highly toxinogenic strain HB101(pCP3837) and standard biochemical methods to purify the toxin, it was possible to obtain purified LT-II with specific activity 12,800,000 times greater than that of crude sonic extract from SA53 (Table 2). Only small amounts of pure LT-II were obtained, but the specific activity of the pure LT-II in the Y1 adrenal-cell assay was 25 to 50 times greater than that of pure CT or LT-I.

Previous structural studies demonstrated that CT and LT-I holotoxins are composed of one A polypeptide and five B polypeptides that are noncovalently associated, and the A polypeptide is cleaved by trypsin into A1 and A2 fragments (5, 14–16, 25, 28, 45). The molecular weight of the holotoxins is 84,000 to 86,000. The A and B polypeptide subunits of LT-II were similar in size to the corresponding subunits of CT and LT-I, and the A polypeptide of LT-II was also cleaved by trypsin (Fig. 5). The relative intensity of the bands formed by the A and B polypeptides of LT-II on SDS-polyacrylamide gels stained for protein was not detectably different from CT and LT-I controls (data not shown). Furthermore, when samples of purified ^{125}I-labeled LT-II were fractionated by isoelectric focusing (Fig. 4) or gel filtration (data not shown) and analyzed for radioactivity and biologic activity, all of the radioactivity was associated with the holotoxin; no free A or B polypeptides were detected. These findings were consistent with the possibility that LT-II holotoxin might also contain one A and five B polypeptides, but accurate estimation of the molecular weight of LT-II holotoxin will require further studies. The relative intensity of the A and B bands shown in the autoradiographs in Fig. 5 for LT-II differed from results obtained with ^{125}I-labeled CT or LT-I, because most of the counts in radioiodinated CT or LT-I were associated with polypeptide A or A1 instead of polypeptide B (25). This may reflect differences between LT-II and CT or LT-I with respect to the number or degree of exposure of tyrosine residues in the A or B polypeptide that can be iodinated.

CT and LT-I activate adenylate cyclase activity by NAD-linked ADP ribosylation of the G_s regulatory subunit of the cyclase complex (17, 18, 43). The biologic effects of CT and LT-I result from activation of adenylate cyclase and are mediated by cAMP. The increase of cAMP seen in Y1 adrenal cells treated with LT-II (Fig. 7 and 8) indicated that the biologic effects of LT-II, several of which resemble those of CT and LT-I, were also mediated by cAMP. Additional studies to be reported in detail elsewhere provided direct evidence that treatment of human fibroblasts with LT-II caused activation of adenylate cyclase by ADP ribosylation of G_s (P. P. Chang, J. Moss, E. M. Twiddy, and R. K. Holmes, Fed. Proc. **44**:701, 1985). These data demonstrated that CT, LT-I and LT-II are also homologous with respect to enzymatic activity and mechanism of activation of adenylate cyclase. The similarities in structure and mode of action summarized above provide a very strong basis for including LT-II in the cholera/*E. coli* family of LTs.

LT-II also had properties that differed very substantially from CT and LT-I. No antigenic cross-reactivity was detected between LT-II and CT or LT-I in immunodiffusion tests or quantitative neutralization tests (Fig. 9 and Table 4). These data support the previous proposal to classify the LTs of *E. coli* into two serogroups: LT-I, which is antigenically related to CT and can be neutralized by anti-CT antibodies, and LT-II, the prototype for which is the purified toxin described here (41). The partially cross-reacting variants of LT produced by *E. coli* strains from humans and from pigs are both neutralized by anti-CT and represent subgroups of LT-I (5, 14, 25, 28).

Evidence was obtained that ganglioside GM1 is not the physiologic receptor for LT-II on target cells. LT-II was much less susceptible than LT-I to inhibition by ganglioside GM1, whereas the converse was true for inhibition by crude mixed gangliosides (Table 3). Furthermore, LT-I adheres to agarose and can be eluted by D-galactose (4), whereas LT-II does not adhere to agarose under similar conditions (26). It is noteworthy that relatively large doses of LT-II did not cause secretion of fluid in ligated ileal segments of adult rabbits, particularly in view of the striking similarities described above between LT-II and CT or LT-I with respect to mode of action and activation of adenylate cyclase. This result indicates that the secretory activity observed previously in rabbit ileal segments treated with crude or partially purified extracts from *E. coli* SA53 (26) was probably not caused by the LT-II in the test samples. It is interesting to speculate that the lack of activity of purified LT-II in the adult rabbit ligated ileal segment assay might be a species-specific phenomenon related to the paucity or absence of specific receptors for LT-II in the rabbit gut. The heat-stable enterotoxins of *E. coli* provide a well-known example of species-specific activity in the intestine: ST-I is active both in mice and pigs, whereas ST-II is active in pigs but inactive in mice (20). It is also possible, however, that the biologic significance of LT-II is unrelated to its activity in the intestinal tract of infected hosts.

Previous studies demonstrated that LT-I and CT have an overall homology between 75 and 80% both in the amino acid sequences of their polypeptide subunits and in the nucleotide sequences of their structural genes (7, 32, 36, 42, 45). Within the LT-I serogroup, the LTh-I and LTp-I variants shared more than 95% homology both at the amino acid sequence level and at the nucleotide sequence level. DNA probes for LT-I genes will hybridize specifically with the structural genes for CT under conditions of moderate stringency (30). In contrast, no hybridization occurs between DNAs corresponding to the structural genes for LT-I and for LT-II under low-stringency conditions allowing up to 45% base-pair mismatch (19, 41). Failure of the LT-II and LT-I structural gene sequences to hybridize, however, does not rule out the possibility that they have homology at a level too low to be detected by the methods used. Determination of the nucleotide sequence of the LT-II genes is in progress in our laboratory, and the results of such studies should establish

conclusively the extent of genetic homology between the LT-II and LT-I toxins of *E. coli*.

The structural genes for the A and B polypeptides of LT-I are part of an operon located on plasmids in *E. coli* (8, 46). Transcription occurs from a single promoter which is adjacent to the structural gene for the A polypeptide. Transcription of the LT-I operon is not dependent on cAMP (38). Chromosomal genes can influence expression of the LT operon and determine a hypertoxinogenic phenotype (3), and subinhibitory concentrations of lincomycin can stimulate toxin production (47). *E. coli* K-12 harboring LT-I$^+$ plasmids from clinical isolates can produce as much as 10 μg of toxin per milliliter of bacterial culture (39). Production of LT-II by *E. coli* SA53 was also stimulated by lincomycin, but the amount of cell-associated toxin under optimal growth conditions did not exceed 0.1 ng/ml of culture, based on comparison of the specific activities of bacterial extracts and of pure LT-II. Even with the highly toxinogenic strain HB101(pCP3837) the activity of LT-II produced did not represent more than 50 ng/ml of bacterial culture. One of the most striking differences between regulation of the LT-I and LT-II genes in *E. coli*, therefore, is the much smaller amount of LT-II produced. Studies are in progress in our laboratory to analyze the organization of the LT-II operon and to determine the strength of its promoter.

Little is known about the prevalence of LT-II-producing strains among *E. coli* from humans, animals, or environmental sources. A DNA probe that appears to be specific for the LT-II structural genes has been prepared recently in our laboratory and has been used successfully to detect LT-II genes in strains of *E. coli* from animals, humans, and environmental sources in widely separated geographic areas (C. L. Pickett, E. M. Twiddy, B. C. Guth, L. R. Trabulsi, and R. K. Holmes, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, B18, p. 27). The development of rapid and accurate diagnostic tests for LT-II-producing bacteria will facilitate future investigations into their epidemiology and their possible role in pathogenesis of infectious diseases in humans and in animals.

ACKNOWLEDGMENTS

Work in our laboratory was supported in part by Public Health Service research grant 5-R22-AI14107 from the National Institute for Allergy and Infectious Diseases.

We thank Alex Hromockyj for technical assistance in experiments on optimizing culture conditions for production of LT-II, and we thank Richard L. Guerrant and Aldo A. M. Lima for testing purified LT-II in the rabbit ligated ileal segment assay.

LITERATURE CITED

1. **Belisle, B. W., E. M. Twiddy, and R. K. Holmes.** 1984. Characterization of monoclonal antibodies to heat-labile enterotoxin encoded by a plasmid from a clinical isolate of *Escherichia coli*. Infect. Immun. **43**:1027–1032.
2. **Belisle, B. W., E. M. Twiddy, and R. K. Holmes.** 1984. Monoclonal antibodies with an expanded repertoire of specificities and potent neutralizing activity for *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **46**:759–764.
3. **Bramucci, M. G., E. M. Twiddy, W. B. Baine, and R. K. Holmes.** 1981. Isolation and characterization of hypertoxinogenic (*htx*) mutants of *Escherichia coli* KL320(pCG86). Infect. Immun. **32**:1034–1044.
4. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxins with high specific activity from *Escherichia coli* cultures. Infect. Immun. **24**:760–769.
5. **Clements, J. D., D. C. Flint, and F. A. Klipstein.** 1982. Immunological and physicochemical characterization of heat-labile enterotoxins isolated from two strains of *Escherichia coli*. Infect. Immun. **38**:806–809.
6. **Craig, J. P.** 1971. Cholera toxins, p. 189–254. *In* S. Kadis, T. C. Montie, and S. J. Ajl (ed.), Microbial toxins. Bacterial protein toxins, vol. 2A. Academic Press, Inc., New York.
7. **Dallas, W. S.** 1983. Conformity between heat-labile toxin genes from human and porcine enterotoxigenic *Escherichia coli*. Infect. Immun. **40**:647–652.
8. **Dallas, W. S., D. M. Gill, and S. Falkow.** 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. **139**:850–858.
9. **De, S. N., and D. N. Chatterje.** 1953. An experimental study of the mechanism of action of *Vibrio cholerae* on the intestinal mucous membrane. J. Pathol. Bacteriol. **66**:559–562.
10. **Evans, D. J., Jr., D. G. Evans, S. H. Richardson, and S. L. Gorbach.** 1976. Purification of the polymyxin-released heat-labile enterotoxin of *Escherichia coli*. J. Infect. Dis. **133**(Suppl.):97–102.
11. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**:553–623.
12. **Finkelstein, R. A.** 1976. Progress in the study of cholera and related enterotoxins, p. 53–84. *In* A. W. Bernheimer (ed.), Mechanisms in bacterial toxinology. John Wiley & Sons, Inc., New York.
13. **Finkelstein, R. A., P. Atthasampunna, M. Chulasamaya, and P. Charunmethee.** 1966. Pathogenesis of experimental cholera: biological activities of purified procholeragen A. J. Immunol. **96**:440–449.
14. **Geary, S. J., B. A. Marchlewicz, and R. A. Finkelstein.** 1982. Comparison of heat-labile enterotoxins from porcine and human strains of *Escherichia coli*. Infect. Immun. **36**:215–220.
15. **Gill, D. M.** 1976. The arrangement of subunits in cholera toxin. Biochemistry **15**:1242–1248.
16. **Gill, D. M., J. D. Clements, D. C. Robertson, and R. A. Finkelstein.** 1981. Subunit number and arrangement in *Escherichia coli* heat-labile enterotoxin. Infect. Immun. **33**:677–682.
17. **Gill, D. M., and R. Meren.** 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. USA **75**:3050–3054.
18. **Gill, D. M., and S. H. Richardson.** 1980. ADP-ribosylation of adenylate cyclase catalyzed by the heat-labile enterotoxin of *Escherichia coli*: comparison with cholera toxin, p. 142–147. *In* Proceedings of the fifteenth joint conference on cholera, publication 80-2003. National Institutes of Health, Bethesda, Md.
19. **Green, B. A., R. J. Neill, W. T. Ruyechan, and R. K. Holmes.** 1983. Evidence that a new enterotoxin of *Escherichia coli* which activates adenylate cyclase in eucaryotic target cells is not plasmid mediated. Infect. Immun. **41**:383–390.
20. **Guerrant, R. L., R. K. Holmes, D. C. Robertson, and R. N. Greenberg.** 1985. Roles of enterotoxins in the pathogenesis of *Escherichia coli* diarrhea, p. 68–73. *In* L. Leive, P. F. Bonventre, J. A. Morello, S. Schlesinger, S. D. Silver, and H. C. Wu (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.
21. **Gyles, C., M. So, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**:277–283.
22. **Holmes, R. K., and R. B. Perlow.** 1975. Quantitative assay of diphtherial toxin and of immunologically cross-reacting proteins by reversed passive hemagglutination. Infect. Immun. **12**:1392–1400.
23. **Holmes, R. K., C. L. Pickett, B. W. Belisle, E. M. Twiddy, J. W. Newland, N. A. Strockbine, S. F. Miller, and A. D. O'Brien.** 1986. Genetics of toxinogenesis in *Escherichia coli* relating to vaccine development, p. 68–73. *In* J. Holmgren, A. Lindberg, and R. Mollby (ed.), Development of vaccines and drugs against diarrhea, 11th Nobel Conference, Stockholm 1985. Studentlitteratur AB, Lund, Sweden.
24. **Holmes, R. K., and E. M. Twiddy.** 1983. Characterization of monoclonal antibodies that react with unique and cross-reacting determinants of cholera enterotoxin and its subunits. Infect. Immun. **42**:914–923.
25. **Holmes, R. K., E. M. Twiddy, and M. G. Bramucci.** 1983. Antigenic heterogeneity among heat-labile enterotoxins from

Escherichia coli, p. 293–300. *In* S. Kuwahara and N. Pierce (ed.), Advances in research on cholera and related diarrheas. Martinus Nijhoff Publishers, Boston.
26. **Holmes, R. K., E. M. Twiddy, and R. J. Neill.** 1985. Recent advances in the study of heat-labile enterotoxins of *Escherichia coli*, p. 125–131. *In* Y. Takeda and T. Miwatani (ed.), Bacterial diarrheal diseases. Martinus Hijhoff Publishers, Boston.
27. **Holmgren, J., P. Fredman, M. Lindblad, A.-M. Svennerholm, and L. Svennerholm.** 1982. Rabbit intestinal glycoprotein receptor for *Escherichia coli* heat-labile enterotoxin lacking affinity for cholera toxin. Infect. Immun. **38**:424–433.
28. **Honda, T., T. Tsuji, Y. Takeda, and T. Miwatani.** 1981. Immunological nonidentity of heat-labile enterotoxins from human and porcine enteropathogenic *Escherichia coli*. Infect. Immun. **34**:337–340.
29. **Hunter, W. M., and F. C. Greenwood.** 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature (London) **194**:495–496.
30. **Kaper, J. B., S. L. Moseley, and S. Falkow.** 1981. Molecular characterization of environmental and nontoxigenic strains of *Vibrio cholerae*. Infect. Immun. **32**:661–667.
31. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
32. **Lockman, H., and J. B. Kaper.** 1983. Nucleotide sequence analysis of the A2 and B subunit of *Vibrio cholerae* enterotoxin. J. Biol. Chem. **258**:13722–13726.
33. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
34. **Maneval, D. R., Jr., R. R. Colwell, S. W. Joseph, R. Gray, and S. T. Donta.** 1980. A tissue culture method for the detection of bacterial enterotoxins. J. Tissue Cult. Methods **6**:85–90.
35. **Mekalanos, J. J., R. J. Collier, and W. R. Romig.** 1978. Purification of cholera toxin and its subunits: new methods of preparation and the use of hypertoxinogenic mutants. Infect. Immun. **20**:552–558.
36. **Mekalanos, J. J., D. J. Swartz, G. C. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
37. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Neill, R. J., and R. K. Holmes.** 1983. Expression of plasmid genes encoding *Escherichia coli* heat-labile enterotoxin in bacterial strains with different genetic backgrounds, p. 201–206. *In* S. Kuwahara and N. F. Pierce (ed.), Advances in research on cholera and related diarrheas. Martinus Nijhoff Publishers, Boston.
39. **Neill, R. J., E. M. Twiddy, and R. K. Holmes.** 1983. Synthesis of plasmid-coded heat-labile enterotoxin in wild-type and hypertoxinogenic strains of *Escherichia coli* and in other genera of *Enterobacteriaceae*. Infect. Immun. **41**:1056–1061.
40. **O'Brien, A. D., G. D. LaVeck, M. R. Thompson, and S. B. Formal.** 1982. Production of *Shigella dysenteriae* type 1-like cytotoxin by *Escherichia coli*. J. Infect. Dis. **146**:763–769.
41. **Pickett, C. L., E. M. Twiddy, B. W. Belisle, and R. K. Holmes.** 1986. Cloning of genes that encode a new heat-labile enterotoxin of *Escherichia coli*. J. Bacteriol. **165**:348–352.
42. **Spicer, E. K., and J. A. Noble.** 1982. *Escherichia coli* heat-labile enterotoxin: nucleotide sequence of the A subunit gene. J. Biol. Chem. **257**:5716–5721.
43. **Vaughan, M., and J. Moss.** 1978. Mechanism of action of choleragen. J. Supramol. Struct. **8**:473–488.
44. **Wrigley, C. W.** 1971. Gel electrofocusing. Methods Enzymol. **22**:559–564.
45. **Yamamoto, T., T. Nakazawa, T. Miyata, A. Kaji, and T. Yokota.** 1984. Evolution and structure of two ADP-ribosylation enterotoxins, *Escherichia coli* heat-labile toxin and cholera toxin. FEBS Lett. **169**:241–246.
46. **Yamamoto, T., T. Yokota, and A. Kaji.** 1981. Molecular organization of heat-labile enterotoxin genes originating in *Escherichia coli* of human origin and construction of heat-labile toxoid-producing strains. J. Bacteriol. **148**:983–987.
47. **Yoh, M., K. Yamamoto, T. Honda, Y. Takeda, and T. Miwatani.** 1983. Effects of lincomycin and tetracycline on production and properties of enterotoxins of enterotoxigenic *Escherichia coli*. Infect. Immun. **42**:778–782.