Comparison of Heat-Labile Enterotoxins from Porcine and Human Strains of *Escherichia coli*

STEVEN J. GEARY†, BENEDICT A. MARCHLEWICZ, AND RICHARD A. FINKELSTEIN*

Department of Microbiology, School of Medicine, University of Missouri, Columbia, Missouri 65212

Received 31 August 1981/Accepted 1 December 1981

Heat-labile enterotoxins (LTs) from porcine (EWD₂₉₉) and human (H74-114) enterotoxigenic strains of *Escherichia coli* were isolated by a single-step galactose elution procedure. Although both strains had similar amounts of LT in their whole-cell lysates, H74-114 yielded a smaller quantity of purified LT than did EWD₂₉₉. Immunodiffusion studies with specific antisera revealed that although the two LTs shared major antigenic determinants each also had unique antigens. Both also had shared and unique specificities in comparison with the cholera enterotoxin (cholera toxin). Differences also exist in the apparent molecular weights of their B-subunit oligomers (coligenoid) as well as in the B-subunit monomers. The monomer molecular weights are 11,500 for EWD₂₉₉ porcine LT and 12,700 for H74-114 human LT. The results suggest that either this isolated human LT has a tetrameric coligenoid or it moves differently in sodium dodecyl sulfate gels for other reasons. The A-subunits of both LTs were similar in size (28,000 daltons), and both LTs were activated by mild proteolytic processing. Amino acid analysis showed a threefold increase in the level of tryptophan and two- and fourfold decreases in the levels of glutamic acid and methionine, respectively, in H74-114 LT compared with EWD₂₉₉ LT. These structural and antigenic differences may prove to be significant in immunoprophylaxis of the cholera-coli family of enterotoxins. Further studies to define the extent of evolutionary drift of these toxins are needed.

Some enteropathogenic strains of *Escherichia coli* elaborate a heat-labile enterotoxin(s) (LT) which is structurally, functionally, and immunologically similar to cholera enterotoxin (also known as cholera toxin) elaborated by *Vibrio cholerae* (3-5, 14, 16). These enterotoxins are responsible for the diarrhea which follows infection of the small intestine with the respective organisms. Both enterotoxins activate adenylate cyclase (9, 22), and both are believed to bind, through their B-regions, with host cell membrane receptors containing ganglioside G_{M1} (15, 20).

Recently, Clements and Finkelstein (3) developed a single-step procedure for the isolation of homogeneous *E. coli* LT. This procedure involved the elution of LT, which was bound to an agarose column, by 0.2 M D-(+)-galactose. The isolated LT, when activated by protease treatment, had a specific activity equivalent to that of pure cholera toxin in several biological assays.

This study was undertaken to determine whether this same procedure is applicable for the isolation of LTs from strains other than the *E. coli* 711(F₁LT), which was used by Clements

and Finkelstein. The isolation of LTs from two enterotoxigenic strains of *E. coli* and the subsequent structural, antigenic, and amino acid analyses are presented.

MATERIALS AND METHODS

Bacterial strains. The two strains used were *E. coli* EWD₂₉₉, a transformed K-12 derivative which contains multiple copies of the LT gene(s) of the Ent plasmid from the porcine strain P307 (6) (kindly provided by S. Falkow and W. Dallas); and *E. coli* H74-114 (O8:H9), an LT-only producing, serologically smooth, human pathogen (kindly provided by R. Rapaport) (21).

Culture conditions. Three and four 9.5-liter fermentors (Magnaferm fermentors, New Brunswick Scientific, Inc., Edison, N.J.) of tryptic soy broth (Difco Laboratories, Detroit, Mich.) were inoculated with 10⁷ organisms of strains H74-114 and EWD₂₉₉, respectively, per ml and cultured at 37°C with vigorous aeration and agitation. In confirmation of the observations of Levner et al. (18), preliminary studies revealed that lincomycin significantly enhanced production of LT. Lincomycin (Sigma Chemical Co., St. Louis, Mo.), at a concentration of 100 µg/ml, was added at 2 h, and incubation was continued for an additional 18 h. Foaming was controlled by the use of antifoam B emulsion (Sigma), 0.4 ml/liter.

Purification of LT. Cells were harvested by continu-

† Present address: Burns-Biotec Laboratories, Inc., Omaha, NE 68103.

ous-flow centrifugation (Sorvall TZ-28; Ivan Sorvall Inc., Norwalk, Conn.) at $40,000 \times g$ and 4°C and then resuspended to 1% of the original volume in pH 7.5 TEAN buffer (0.05 M Tris, 0.001 M EDTA disodium salt, 0.003 M NaN_3 , 0.2 M NaCl) (12). Cells were lysed in a French press (Wabash hydraulic press; Wabash Metal Products Co., Inc., Hydraulics Div., Wabash, Ind.) at 18,000 to 20,000 lb/in². The crude lysate was clarified by centrifugation at $12,500 \times g$ for 30 min at 4°C , and the supernatant was saved. This whole-cell lysate was precipitated with 60% saturated $(\text{NH}_4)_2\text{SO}_4$ at 4°C for 4 h. The precipitate was harvested by centrifugation at $8,000 \times g$ for 20 min at 4°C and dialyzed against several changes of TEAN buffer. The preparations (750 ml containing 212 mg of toxin antigen for EWD₂₉₉ and 525 ml containing 131 mg of toxin antigen for H74-114) were centrifuged at $12,500 \times g$ for 20 min and then applied to a column (28 by 5.5 cm) of agarose A-5m (Bio-Rad Laboratories, Richmond, Calif.), which had been equilibrated with TEAN at 4°C . The column was washed with TEAN until the optical density (280 nm) of the effluent returned to base line. Bound LT was then eluted with 0.2 M D-(+)-galactose in TEAN (3). Absorbance was recorded, and fractions (10 ml) were collected in a Gilson fraction collector (Gilson Medical Electronics, Inc., Middleton, Wis.). The galactose-eluted material was concentrated by ultrafiltration on PM-10 membrane filters (Amicon Corp., Lexington, Mass.) and applied to a column of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), which had been equilibrated with TEAN at 4°C . This was to remove any residual galactose, aggregated material, and other trace contaminants (3).

Mouse Y-1 adrenal cell assay. The mouse Y-1 adrenal cell assay was performed essentially according to the modification of the technique of Donta et al. (8) described by Sack and Sack (23). Samples were tested with and without trypsin treatment (3) to compare activity. Trypsin (Sigma) was added to samples (1 mg/ml in TEAN) to a final concentration of 1 $\mu\text{g}/\text{ml}$, and the samples were incubated at 37°C for 1 h. Soybean trypsin inhibitor (Sigma) in TEAN was then added at a final concentration of 1 $\mu\text{g}/\text{ml}$.

Preparation of antiserum. A young adult male goat was inoculated in two hind sites subcutaneously with 1.0 mg of purified H74-114 LT in 5.0 ml of Freund complete adjuvant. Three weeks later the goat was reinoculated as before. Antiserum was harvested starting 2 weeks after the second inoculation.

Antigen determinations. A modified (12) Mancini (19) radial immunodiffusion test was utilized to determine antigen concentrations. Immunodiffusion plates (Hyland Division, Travenol Laboratories, Inc., Costa Mesa, Calif.) were prepared with 2.5 ml of 1% Noble agar (Difco), 1% sodium azide, and 1% goat anti-711(F₁LT) toxin. Standards of the appropriate pure LT preparation were included in each assay. Wells (2.5 mm) cut in the agar plates were filled with 4 μl of the sample to be assayed, and the plates were then incubated, in a sealed plastic bag, at room temperature for 12 h. Diameters of the zones of precipitation were measured with a binocular stereoscope fitted with an optical reticle.

Immunodiffusion. Ouchterlony-type immunodiffusion assays were performed in 7.5 ml of 1% Noble agar (Difco) with 1% sodium azide added as a preservative in 50-mm petri plates. Wells (5.5 mm) cut in the agar

(9.0 mm center to center) were filled with 50 μl of sample and incubated at room temperature. Antisera utilized were goat anti-H74-114 LT, prepared as described above, goat anti-711(F₁LT) prepared by Clements et al. (4), and equine anticholera toxin (10).

PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 12% gels according to a modified procedure of Laemmli (17). The following samples were examined: SDS-PAGE low-molecular-weight standards (Bio-Rad), 711(F₁LT) (prepared by Clements and Finkelstein [3]), EWD₂₉₉ LT, and H74-114 LT. All of the LT samples were run unheated and nonreduced, heated (100°C for 2 min), and heated and reduced (2-mercaptoethanol, 3.8%). Gels 1.5 mm thick were stained with 0.2% Coomassie blue R for 2 h and were destained in a solution of 40% methanol-4% acetic acid.

Amino acid analysis. Cholera toxin (purified by the technique of Finkelstein and LoSpalluto [12] as modified [11]) and the LTs were subjected to amino acid analysis after hydrolysis in 6 N hydrochloric acid in vacuo for 22 h at 110°C . Analyses were performed by automated cation-exchange chromatography (1). Protein concentrations were determined by using the Bradford technique (2).

RESULTS

Judging from the results of radial immunodiffusion tests, EWD₂₉₉ produced only slightly more LT antigen (approximately 5.3 mg/liter) in whole-cell lysates than did strain H74-114 (4.4 mg/liter), but yielded 15 times more purified LT (4.6 versus 0.3 mg/liter) after the galactose elution step. The H74-114 LT antigen that did not bind to the A-5m column on the first application was collected and reapplied to the same column, after it had been reequilibrated with TEAN buffer. No additional toxin bound. The poorer yield of purified H74-114 LT may be due to a more firm association of this LT with outer membrane components, such as lipopolysaccharide. It should be noted that this "complexed LT" produced precipitin bands which developed more slowly and were more diffuse than purified LT. Thus, quantitative comparisons with crude materials may not be entirely reliable in this assay.

In the mouse Y-1 adrenal cell assay, both EWD₂₉₉ LT and H74-114 LT rounded the cells at a level of 10 pg per 250- μl well after mild trypsinization. Cholera toxin rounded cells at a level of 7 pg per well.

SDS-PAGE (Fig. 1) revealed that the unheated and nonreduced porcine LTs, derived from strains EWD₂₉₉ and 711(F₁LT), each formed a broad protein-staining region in the area of 58,000 daltons, whereas the LT from the human strain, H74-114, formed a narrow sharper band in the 48,000-dalton region. It has been suggested (13) that this broad band formation may be due to a conformational change resulting from

FIG. 1. SDS-PAGE of porcine and human LTs. Lanes (left to right) contain: 1, low-molecular-weight Bio-Rad standards—phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400); 2, 711(F_1 LT) LT (5 μ g); 3, EWD₂₉₉ LT (5 μ g); 4, H74-114 LT (4 μ g); 5, 711(F_1 LT) LT, heated; 6, EWD₂₉₉ LT, heated; 7, H74-114 LT, heated; 8, 711(F_1 LT) LT, heated and reduced; 9, EWD₂₉₉ LT, heated and reduced; 10, H74-114 LT, heated and reduced.

the galactose treatment in the isolation procedure used. This does not, however, appear to be true in all instances since the isolation procedure utilized for the H74-114 LT was the same as that used previously for 711(F_1 LT) LT (3) and for EWD₂₉₉ LT. Before and after heating, all three LT preparations showed A-subunit bands at the 28,000-molecular-weight region. After 711(F_1 LT) LT and EWD₂₉₉ LT were heated, each showed a B-subunit band at the 11,500-molecular-weight region. However, the H74-114 LT band migrated at 12,700 molecular weight. Thus, the porcine and human LTs differ in the monomers which comprise their B-subunits. After heating and reduction with 2-mercaptoethanol, the three LTs showed a variable distribution of A and A₁ bands at the 28,000- and 21,000-molecular-weight regions, respectively, indicative of a varying degree of proteolytic cleavage. Interestingly, the 711(F_1 LT) LT preparation was more nicked than it was previously (3), suggesting that some proteolytic effects became manifest during storage, and the H74-114 LT showed little, if any, evidence of nicking.

In immunodiffusion (Fig. 2), the porcine LTs from strains 711(F_1 LT) and EWD₂₉₉ were appar-

ently immunologically identical (e.g., Fig. 2, wells 3, 1, and 4). However, although anti-porcine LT (well 3) clearly recognized the human LT (well 2), it also demonstrated that porcine LT has unique antigenic determinants in comparison with human LT as illustrated by spur formation (wells 3, 1, and 2). The converse was also true: anti-human LT (well 7) recognized both porcine LTs (wells 1 and 6) but gave a spur of the human LT (well 2) over porcine LT (well 1). This is also illustrated in the second plate of Fig. 2 (wells 8 to 12). Further, equine anticholera toxin (well 14) recognized cholera toxin (well 13), the human LT (well 8), and porcine LT (well 9) but produced clear spurs of cholera toxin over human LT (wells 14, 13, and 8) and of human LT over porcine LT (wells 14, 8, and 9). Anti-human LT (well 12) also recognized a unique determinant in human LT as compared with cholera toxin (wells 12, 8, and 13). It had previously been demonstrated (4) that porcine LT and cholera toxin each possessed both shared and unique antigenic determinants in their B-regions.

Amino acid analysis (Table 1) revealed 2.0- and 4.5-fold decreases in the levels of glutamic

FIG. 2. Precipitin analyses of human and porcine LTs and cholera toxin. Well 1, Porcine LT (EWD₂₉₉); well 2, human LT (H74-114); well 3, goat anti-porcine LT [711(F₁LT)]; well 4, porcine LT [711(F₁LT)]; well 5, normal goat serum; well 6, porcine LT [711(F₁LT)]; well 7, goat anti-human LT (H74-114); well 8, human LT (H74-114); well 9, porcine LT (EWD₂₉₉); well 10, goat anti-porcine LT [711(F₁LT)]; well 11, porcine LT (EWD₂₉₉); well 12, goat anti-human LT (H74-114); well 13, cholera toxin; well 14, equine anticholera toxin.

acid and methionine, respectively, and a 3.5-fold increase in the level of tryptophan in H74-114 LT compared with EWD₂₉₉ LT. The analysis for EWD₂₉₉ LT was virtually identical to that reported previously for LT from 711(F₁LT) whole-cell lysates (3).

DISCUSSION

The present study reveals that LTs derived from enteropathogenic strains of *E. coli* other than 711(F₁LT) can also be isolated by the single-step, galactose elution procedure (3), although apparently with different efficiency. LT derived from a human strain of *E. coli*, although sharing many properties, differs from the por-

cine LT in the conformation and size of its B-region and B-subunits, immunologically, and in amino acid composition.

As expected, since the LT gene(s) was derived from the same porcine strain, immunodiffusion experiments showed reactions of identity between porcine LTs isolated from strains 711(F₁LT) and EWD₂₉₉. The porcine LTs, although clearly strongly immunologically related to the human LT, possessed immunologically unique antigenic determinants. This was also true for the human LT. Both human and porcine LTs were also strongly related to cholera toxin but, again, each had unique antigens with respect to the others. It thus appears that there may be a family of related LTs, perhaps of common ancestry but showing evidence of evolutionary drift. Because of the unique antigens and the differences in certain amino acid compositional ratios, it does not appear likely that post-translational modifications could account for the differences observed. From the intensity of the precipitin bands and the degree of spur formation (Fig. 2), the human LT seems to be more closely related to cholera toxin than does the porcine LT. Specific antisera may be useful in fingerprinting LTs from different sources.

The porcine and human LTs differed in the behavior of their B-oligomers as well as in the apparent molecular weights of their B-subunits in SDS-PAGE. As was observed previously (13), the B-oligomers (coligenoid) of our porcine LTs formed diffuse protein-staining bands in a region of 56,000 molecular weight, whereas the B-oligomers of isolated human LT formed a sharp band at 48,000 daltons. The 711(F₁LT) and EWD₂₉₉ porcine LTs both showed a B-subunit band at the 11,500-molecular-weight region, whereas the H74-114 human LT showed a

TABLE 1. Amino acid composition of cholera toxin, EWD₂₉₉ LT, and H74-114 LT

Amino acid	% Conc'n		
	Cholera toxin	EWD ₂₉₉ LT	H74-114 LT
Aspartic acid	11.8	10.4	8.9
Threonine	7.4	8.8	8.2
Serine	4.4	6.1	6.2
Glutamic acid	12.1	11.7	5.8
Proline	3.4	2.8	2.4
Glycine	2.8	2.6	2.4
Alanine	6.3	3.2	3.2
Valine	3.6	3.4	4.0
Methionine	2.6	1.6	0.4
Isoleucine	7.5	9.1	9.2
Leucine	6.3	5.1	5.2
Tyrosine	6.0	6.5	4.1
Phenylalanine	3.8	2.4	3.2
Histidine	4.7	1.9	2.6
Lysine	7.9	8.0	6.8
Arginine	5.8	7.0	6.0
Tryptophan	0.7	2.7	9.4
Cystine/cysteic acid	1.2	0.9	1.6

band at the 12,700-molecular-weight region. The results may also be interpreted to suggest that the human LT species isolated in this study may have only four B-subunits per holotoxin molecule rather than five, as reported for porcine LT previously (13). However, as the B-subunit oligomer is not fully dissociated without heating in SDS, its behavior may be atypical; further studies are needed to resolve this question. Whether that portion of human LT antigen which was complexed with other components or, for other reasons, did not bind to agarose and thus was not purified in this study has a different configuration also remains to be determined.

Amino acid analyses revealed that H74-114 LT had significantly more (3.5-fold) tryptophan than EWD₂₉₉ LT and a lower content of methionine and glutamic acid. De Wolf et al. (7) suggested that tryptophan is involved in the binding of cholera toxin to GM₁ ganglioside. However, despite the differences in amino acid content, the two LTs isolated in the present study had similar specific activities to each other and to cholera toxin in Y-1 adrenal cells. Thus, the process of binding, entry, and enzymic activity was not adversely affected by these compositional differences.

Our results suggest that some of the H74-114 LT may interact more firmly with lipopolysaccharide or other membrane components of the smooth H74-114 (O8:H9) strain than the porcine LT does to its rough K-12 vector. Further studies are needed to resolve the reason(s) for the poorer recovery of the human LT and to define further the extent of structural and antigenic drift in the cholera-coli family.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI 16776 to R.A.F. under the U.S.-Japan Cooperative Medical Science Program, from the National Institute of Allergy and Infectious Diseases.

We thank C. W. Gehrke and L. Wall, Experimental Station Chemical Laboratories, University of Missouri-Columbia, for the automated amino acid analytical data.

ADDENDUM IN PROOF

After submission of this manuscript, Honda et al. (Infect. Immun. 34:337-340), using similar methodology, reported similar antigenic differences between LTs of human and porcine origins.

LITERATURE CITED

- Benson, J. V., Jr., and J. A. Patterson. 1971. Chromatographic advances in amino acids and peptide analysis using spherical resins and their applications in biochemistry and medicine, p. 1-67. In A. Niederweiser and G. Pataki (ed.), New techniques in amino acid, peptide and protein analysis. Ann Arbor Scientific Publishers, Ann Arbor, Mich.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.
- 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.
- Clements, J. D., R. J. Yancey, and R. A. Finkelstein. 1980. Properties of homogeneous heat-labile enterotoxins from *Escherichia coli*. Infect. Immun. 29:91-97.
- Dallas, W. S., and S. Falkow. 1980. Amino acid sequence homology between cholera toxins and *Escherichia coli* heat-labile toxins. Nature (London) 228:499-501.
- Dallas, W. S., D. M. Gill, and S. Falkow. 1979. Cistrons encoding *Escherichia coli* heat-labile toxin. J. Bacteriol. 139:850-858.
- De Wolf, J. J. S., M. Fridkin, M. Epstein, and L. D. Kohn. 1981. Structure-function studies of cholera toxin and its A and B protomers. J. Biol. Chem. 256:5481-5488.
- Donta, S. T., H. W. Moon, and S. C. Whipp. 1974. Detection of heat-labile *Escherichia coli* enterotoxins with the use of adrenal cells in tissue culture. Science 183:334-336.
- Evans, D. J., L. C. Chen, G. T. Curtin, and D. G. Evans. 1972. Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. Nature (London) New Biol. 236:137-138.
- Finkelstein, R. A. 1970. Monospecific equine antiserum against cholera exo-enterotoxin. Infect. Immun. 2:691-697.
- Finkelstein, R. A., K. Fujita, and J. J. LoSpalluto. 1971. Procholeragenoid: an aggregated intermediate in the formation of choleragenoid. J. Immunol. 107:1043-1051.
- Finkelstein, R. A., and J. J. LoSpalluto. 1969. Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. J. Exp. Med. 130:185-202.
- Gill, D. M., J. D. Clements, D. C. Robertson, and R. A. Finkelstein. 1981. Subunit number and arrangement in the heat-labile enterotoxin of *Escherichia coli*. Infect. Immun. 33:677-682.
- Holmes, R. K., M. C. Bramucci, and E. M. Twiddy. 1979. Genetic and biochemical studies of heat-labile enterotoxin of *Escherichia coli*, p. 187-201. In Proceedings of the 13th Joint Conference of the U.S.-Japan Cooperative Medical Science Program Symposium on Cholera, DHEW Publ. 78-1590. Department of Health, Education and Welfare, Washington, D.C.
- Holmgren, J. 1973. Comparison of the tissue receptors for *Vibrio cholerae* and *Escherichia coli* enterotoxins by means of gangliosides and natural cholera toxoid. Infect. Immun. 8:851-859.
- Kunkel, S. L., and D. C. Robertson. 1979. Purification and chemical characterization of the heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. Infect. Immun. 25:586-596.
- Laemmli, J. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature (London) 227:680-685.
- Levner, M., F. P. Wiener, and B. A. Rubin. 1977. Induction of *Escherichia coli* and *Vibrio cholerae* enterotoxins by an inhibitor of protein synthesis. Infect. Immun. 15:132-137.
- Mancini, G., J. P. Vaerman, A. O. Carbonara, and J. F. Heremans. 1964. A single-radial diffusion method for the immunological quantitation of proteins, p. 370. In H. Peeters (ed.), XI Colloquium on protides of the biological fluids. Elsevier, Amsterdam.
- Moss, J. S., P. H. Garrison, and S. H. Richardson. 1979. Gangliosides sensitize unresponsive fibroblasts to *Escherichia coli* heat-labile enterotoxin. J. Clin. Invest. 64:381-384.
- Rappaport, R. S. 1978. Observations on the mechanism of

- release of heat-labile *Escherichia coli* enterotoxin, p. 443–474. In Proceedings of the 13th Joint Conference of the U.S.-Japan Cooperative Medical Science Program Symposium on Cholera. Publ. no. (NIH) 78-1590. Department of Health, Education, and Welfare, Washington, D.C.
22. Richards, K. L., and S. D. Douglas. 1978. Pathophysiological effects of *Vibrio cholerae* and enterotoxigenic *Escherichia coli* and their enterotoxins on eukaryotic cells. Microbiol. Rev. 42:592–613.
23. Sack, D. A., and R. B. Sack. 1975. Test for enterotoxigenic *Escherichia coli* using Y-1 adrenal cells in miniculture. Infect. Immun. 11:334–336.