INFECTION AND IMMUNITY, Nov. 1982, p. 444–448
0019-9567/82/110444-05$02.00/0

Vol. 38, No. 2

Molecular Heterogeneity of Heat-Labile Enterotoxins from Human and Porcine Enterotoxigenic *Escherichia coli*

TAKAO TSUJI,* SEKIKO TAGA, TAKESHI HONDA, YOSHIFUMI TAKEDA, AND TOSHIO MIWATANI

Department of Bacteriology and Serology, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565, Japan

Received 18 December 1981/Accepted 2 August 1982

The heat-labile enterotoxins produced by human enterotoxigenic *Escherichia coli* (LT_h) and porcine enterotoxigenic *E. coli* (LT_p) were purified to homogeneity, and their molecular properties were compared with those of purified cholera enterotoxin (CT). On polyacrylamide gel disk electrophoresis without sodium dodecyl sulfate, LT_h, LT_p, and CT differed in mobility, suggesting differences in their ionic charges. The pI values of LT_h, LT_p, and CT were 7.50, 8.10, and 6.80, respectively. On sodium dodecyl sulfate-polyacrylamide gel slab electrophoresis, the B subunit and A_1 and A_2 fragments of LT_h, LT_p, and CT differed in mobility, suggesting that they differed in molecular size. Their molecular sizes seemed to decrease in the following order: B subunit, $LT_h > LT_p \simeq CT$; A_1 fragment, $LT_p > LT_h \simeq CT$; A_2 fragment, $LT_h \simeq CT > LT_p$. Amino acid compositions of LT_h, LT_p, and CT were also compared.

The immunological similarity of cholera enterotoxin to heat-labile enterotoxin produced by porcine enterotoxigenic *Escherichia coli* (LT_p) is well established (1, 2, 11, 12). The molecular structure of LT_p is also very similar to that of CT. LT_p consists of two subunits, A and B, with molecular weights of 28,000 and 11,500, respectively (3), and these values are very similar to those of the subunits of CT (17, 18).

Recently, the immunological nonidentity of LT_p and heat-labile entertoxin produced by enterotoxigenic *E. coli* isolated from humans (LT_h) was reported (14). LT_p, LT_h, and CT share a common antigenic determinant(s), but each enterotoxin also has a unique antigenic determinant(s) (14). Similar results were also reported independently by several other investigators (10; R. K. Holmes et al., Abstr. 17th Joint Conference of the U.S.-Japan Cooperative Medical Science Program, Cholera Panel, Baltimore, 1981, p. 43; J. D. Clements and D. C. Flint, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B63, p. 28). Thus, it was of interest to compare the molecular natures of these enterotoxins. Results showed that the surface ionic charges of LT_p and LT_h differed and the molecular sizes of the A and B subunits of LT_p and LT_h were also slightly different.

MATERIALS AND METHODS

Preparation of purified LT_h and LT_p. LT_h and LT_p were isolated and purified from whole-cell lysates of human strain *E. coli* 536-5 and porcine strain *E. coli* O-149-26, respectively. Their purifications were carried out essentially as described by Clements and Finkelstein (3). Details of the culture of cells, isolation of LT from the cells, and purification by successive column chromatographies on Bio-Gel A5m and Sephacryl S-200 were described previously (22). For separation of a possible contamination of spontaneous aggregates of B subunits of LT_h and LT_p, Sephacryl S-200 eluates were further chromatographed on a Sephadex G-75 column (superfine, 1.5 by 150 cm) in 0.05 M Tris-hydrochloride buffer (pH 7.4) containing 1 mM EDTA, 3 mM sodium azide, and 0.2 M NaCl (TEAN buffer) (9). Fractions containing biological activities, such as activities to induce morphological changes of Chinese hamster ovary cells (13) and rabbit skin vascular permeability (4), were collected, concentrated by Amicon PM-10 membrane filtration, and used as purified LT_h and LT_p. They were treated with trypsin as described below.

Purified CT. CT purified from the culture filtrate of *Vibrio cholerae* 569B by the method of Ohtomo et al. (19) was purchased from Sanko Junyaku Co., Tokyo.

Treatment of LT_h and LT_p with trypsin. Purified LT_h and LT_p were treated with trypsin as described by Rappaport et al. (20), since these preparations were isolated and purified from whole-cell lysates. About 10 μg of purified preparation in 0.2 ml of 50 mM ammonium bicarbonate was incubated with about 0.5 μg of trypsin (Sigma Chemical Co., St. Louis, Mo.; Type XI, diphenyl carbamyl chloride-treated bovine pancreas trypsin) at 37°C for 60 min.

Isoelectric focusing of purified toxin. Electrofocusing of the purified toxin on ampholine carrier ampholytes was carried out in LKB electrofocusing system 8100-10, using a pH gradient of 3 to 10. A sample of about 2 mg of purified toxin was applied, and electrophoresis was performed at a constant voltage of 300 V for 72 h.

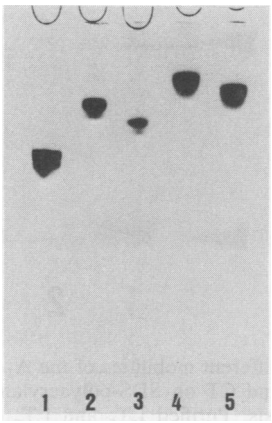

FIG. 1. Polyacrylamide gel disk electrophoresis of CT, LT_h, and LT_p. Polyacrylamide gel disk electrophoresis of 5- to 10-μg samples of purified CT, purified LT_h, and purified LT_p was carried out as described in the text. Lane 1, purified CT; 2, purified LT_h, 3, purified LT_h treated with trypsin; 4, purified LT_p; 5, purified LT_p treated with trypsin.

Then fractions were collected, and absorbance at 280 nm and activity to cause morphological changes of Chinese hamster ovary cells were measured.

Polyacrylamide gel electrophoresis. Polyacrylamide gel disk electrophoresis was carried out as described by Davis (7) on 7% acrylamide. Electrophoresis was performed at a constant current of 2 mA per tube for 3 h at 4°C.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel slab (0.2 by 13.5 by 12.0 cm) electrophoresis in 0.1% SDS was carried out as decribed by Laemmli (16) with 12% acrylamide. Electrophoresis was performed at a constant current of 25 mA for 4.5 h.

Gels were stained with Coomassie brilliant blue and then destained as described previously (8).

Amino acid analysis. The amino acid compositions of the purified toxins were determined in a Hitachi type-835 analyzer with samples hydrolyzed in 4 M methanesulfonic acid at 110°C for 24 h in evacuated sealed tubes.

RESULTS

Polyacrylamide gel disk electrophoresis of CT, LT_h and LT_p. For examination of possible differences in ionic charge of CT, LT_h, and LT_p, the preparations were subjected to polyacrylamide gel disk electrophoresis without SDS. The results in Fig. 1 show that purified CT (lane 1), purified LT_h (lane 2), and purified LT_p (lane 4) migrated to different positions, indicating that they differed in ionic charge. After LT_h and LT_p had been treated with trypsin (lane 3 and lane 5, respectively), they migrated slightly faster than the untreated toxins, but they still differed in mobility.

Isoelectric points of CT, LT_h and LT_p. The isoelectric points of CT, LT_h, and LT_p were determined as 6.80, 7.50, and 8.10, respectively (Fig. 2). These results confirm that the ionic charges on the surface of the toxin molecules differed.

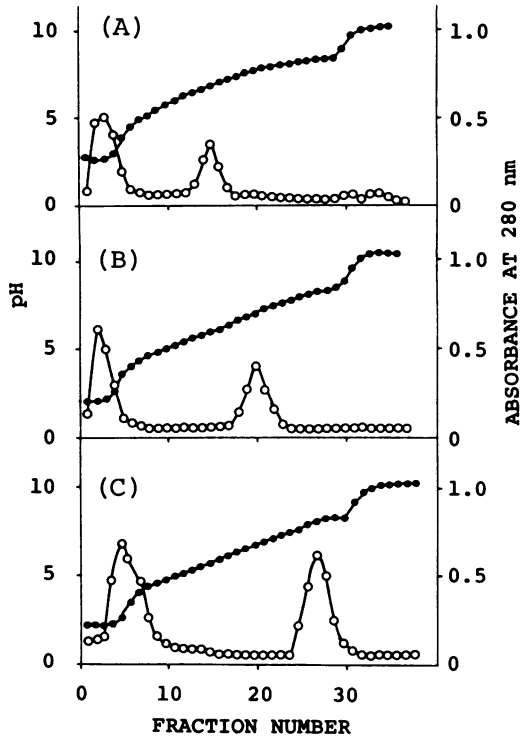

FIG. 2. Isoelectric focusing of CT, LT_h, and LT_p. Isoelectric focusing of purified CT, purified LT_h, and purified LT_p was carried out as described in the text. ●, pH; ○, absorbance at 280 nm. (A) purified CT; (B) purified LT_h; (C) purified LT_p.

Comparison of the sizes of the subunits of LT_h, LT_p and CT. The results of SDS-polyacrylamide gel slab electrophoresis of purified LT_h, LT_p, and CT are shown in lanes 1, 2, and 3, respectively, of Fig. 3a. Both LT_h (lane 1) and LT_p (lane 2) gave two bands corresponding to the A and B subunits of CT (lane 3). The B subunit of LT_h (lane 1) migrated slower than those of LT_p (lane 2) and CT (lane 3), which migrated to almost the same position. When trypsin-treated LT_h, LT_p, and CT were treated with dithiothreitol before analysis, the A_1 fragments of these toxins migrated to almost the same position, whereas the B subunits of LT_h (lane 4) migrated slower than those of LT_p (lane 5) or CT (lane 6).

These findings were confirmed by coelectrophoresis of LT_h, LT_p, and CT on SDS-polyacrylamide slab gel (Fig. 3b). In this experiment, two different samples of purified LT_h and LT_p treated with trypsin and CT were mixed before analysis. It was found that the A subunit of either combination of toxins with and without dithiothreitol treatment gave one band on the gel. Coelectrophoresis of LT_h and LT_p (lane 1

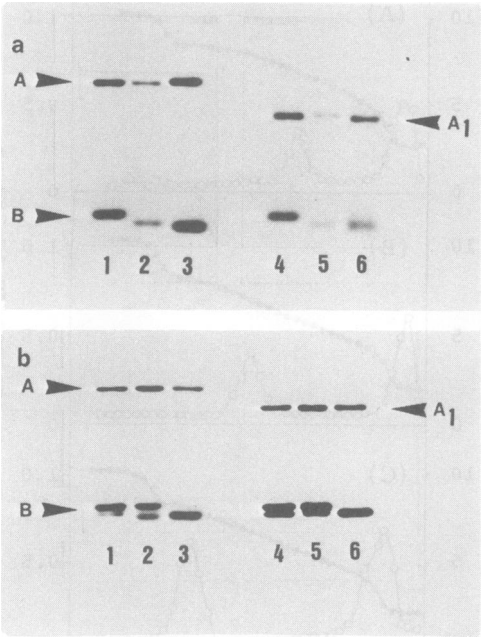

FIG. 3. (a) SDS-polyacrylamide gel slab electrophoresis of LT_h and LT_p treated with trypsin and CT. SDS-polyacrylamide gel slab electrophoresis of approximately 2-μg samples of purified LT_h, LT_p, and CT with and without dithiothreitol treatment was carried out as described in the text. Samples were heated at 100°C for 2 min either in the absence (lanes 1 to 3) or in the presence (lanes 4 to 6) of 10 mM dithiothreitol. Lanes 1 and 4, LT_h; 2 and 5, LT_p; and 3 and 6, CT. Positions of fragments A, A_1, and B are indicated. (b) Coelectrophoresis of LT_h and LT_p treated with trypsin and CT on SDS-polyacrylamide gel slab electrophoresis. Purified LT_h, LT_p, and CT were mixed as indicated and applied to SDS-polyacrylamide slab gel. Samples of about 2 μg were used for each analysis. Samples were heated at 100°C for 2 min either in the absence (lanes 1 to 3) or in the presence (lanes 4 to 6) of 10 mM dithiothreitol. Lanes 1 and 4, a mixture of LT_h and LT_p; 2 and 5, a mixture of LT_h and CT; 3 and 6, a mixture of LT_p and CT. Positions of fragments A, A_1, and B are indicated.

and 4) and of LT_h and CT (lane 2 and 5) resulted in two distinct bands of B subunits. On the other hand, coelectrophoresis of LT_p and CT (lane 3 and 6) gave a single band of B subunits.

These results suggest that the sizes of the B subunits of LT_h, LT_p, and CT have the following relationship: $LT_h > LT_p \simeq CT$.

Although the mobilities of the A subunits and A_1 fragments of LT_h, LT_p, and CT were indistinguishable in the preceding experiments, careful examination showed a slight difference in mobilities of the A_1 and A_2 fragments. When LT_h and LT_p were treated with trypsin and subjected to electrophoresis after dithiothreitol treatment, the A subunit gave two bands attributable to A_1

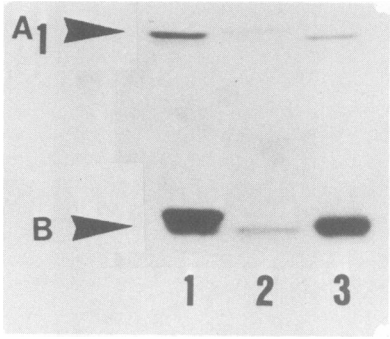

FIG. 4. Different mobilities of the A_1 fragments of LT_h, LT_p, and CT on SDS-polyacrylamide gel slab electrophoresis. Purified LT_h and LT_p were treated with trypsin as described in the text. Samples were treated with dithiothreitol as described in the legend to Fig. 3 and analyzed by SDS-polyacrylamide gel slab electrophoresis as described in the text. Samples of about 2 to 5 μg were applied, and electrophoresis was carried out for 6 h. Lane 1, LT_h; 2, LT_p; 3, CT. Positions of fragments A_1 and B are indicated.

and A_2 fragments. The mobility of fragment A_1 of LT_p (Fig. 4, lane 2) was slightly less than that of LT_h (lane 1) or CT (lane 3), the latter two migrating to almost the same position. The bands of fragments A_2, the fastest bands, also migrated with different mobilities (Fig. 5). Fragment A_2 of LT_p (lane 2) migrated slightly faster than that of LT_h (lane 1) or CT (lane 3), which migrated to almost the same position.

From these results it was concluded that the sizes of the A_1 fragments are in the order $LT_p > LT_h \simeq CT$ and those of the A_2 fragment are in the order $LT_h \simeq CT > LT_p$.

Amino acid compositions of LT_h, LT_p, and CT. Since differences in the ionic charges on the surface of the toxin molecules and the sizes of B and A subunits of LT_h, LT_p, and CT were demonstrated, it was of interest to compare the amino acid composition of each toxin molecule. The results shown in Table 1 indicate that there was a marked difference in the amino acid compositions of LT_h and CT as well as in those of LT_p and CT. Differences between LT_h and LT_p was not significant, but there were appreciable differences in quantities of several amino acids, such as threonine, serine, alanine, methionine, and lysine.

DISCUSSION

Similarity in the molecular structure of LT_p and CT has been reported by several workers. Dallas and Falkow (5) showed that LT synthesized in minicells containing porcine Ent plasmid was composed of two distinct proteins with molecular weights of 25,500 and 11,500. Clements and Finkelstein (3) obtained purified LT_p

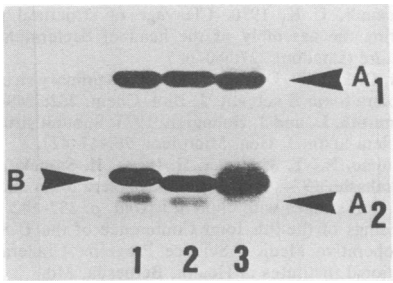

FIG. 5. Different mobilities of the A_2 fragments of LT_h, LT_p, and CT on SDS-polyacrylamide gel slab electrophoresis. Samples of about 8 μg were used for analysis. The other experimental conditions are the same as those described in the legend to Fig. 4. Lane 1, LT_h; 2, LT_p; 3, CT. Positions of fragments A_1, A_2, and B are indicated.

and demonstrated that it consists of A and B subunits, with molecular weights of 28,000 and 11,500, respectively. Similarities in the amino acid sequences of the A and B subunits of LT_p and CT were reported by Spicer et al. (21) and Dallas and Falkow, (6), respectively. However, Kunkel and Robertson (15) examined the physicochemical properties of purified LT_h and found that the amino acid composition of LT_h is quite different from that of CT. In this paper, we present evidence that the ionic charges of the toxin molecules of LT_h, LT_p, and CT are different and that the sizes of the B and A subunits of LT_h and LT_p are different. Moreover, an appreciable difference in the amino acid compositions of LT_h and LT_p is demonstrated. An independent work with similar results was recently reported by Geary et al. (10). Using SDS-polyacrylamide gel disk electrophoresis, Kunkel and Robertson (15) did not observe any difference in the mobilities of LT_h and LT_p, but our results by SDS-polyacrylamide gel slab electrophoresis showed distinct differences in their mobilities, and thus in their molecular sizes. This discrepancy is probably due to a difference(s) in the experimental conditions for electrophoresis. There is also a possibility that the LT preparations used in this work are not natural toxin molecules, since the organisms were cultured in the presence of lincomycin. However, there is evidence that LTs produced in the presence and absence of lincomycin showed the same mobilities on SDS-polyacrylamide gel slab electrophoresis, the same pIs, and the same amino acid compositions (unpublished observations).

The immunological nonidentity of LT_h and LT_p was previously reported from this laboratory (14). This had previously been suggested by Kunkel and Robertson (15) and was recently demonstrated independently by Geary et al. (10) and by Holmes et al. (Abstr. 17th Joint Conference of the U.S.-Japan Cooperative Medical Science Program, Cholera Panel, Baltimore, 1981, p. 43). These findings and the data presented in this paper lead us to conclude that LT_h and LT_p are coded by different Ent plasmids and are distinct protein molecules.

TABLE 1. Amino acid composition of LT_h, LT_p, and CT

Amino acid	Concentration (%)		
	LT_h	LT_p	CT
Aspartic acid/asparagine	11.11	11.12	11.62
Threonine	8.36	9.24	7.70
Serine	8.09	7.54	5.72
Glutamic acid/glutamine	12.45	12.61	11.73
Proline	3.76	3.76	4.32
Glycine	5.16	5.04	5.55
Alanine	5.78	5.23	9.36
Cysteine	1.55	1.52	1.57
Valine	3.65	3.63	4.02
Methionine	2.77	3.02	2.30
Isoleucine	9.14	9.10	7.21
Leucine	5.40	5.41	6.07
Tyrosine	5.64	5.42	4.36
Phenylalanine	2.17	2.23	2.57
Histidine	1.71	1.47	3.78
Lysine	6.61	7.08	6.75
Arginine	5.47	5.53	4.20
Tryptophan	1.17	1.05	1.17

ACKNOWLEDGMENTS

We thank Saburo Hara, Department of Chemistry, Faculty of Science, Osaka University for the amino acid analysis.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

LITERATURE CITED

1. **Clements, J. D., and R. A. Finkelstein.** 1978. Immunological cross-reactivity between a heat-labile enterotoxin(s) of *Escherichia coli* and subunits of *Vibrio cholerae* enterotoxin. Infect. Immun. **21**:1036–1039.
2. **Clements, J. D., and R. A. Finkelstein.** 1978. Demonstration of shared and unique immunological determinants in enterotoxins from *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. **22**:709–713.
3. **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.
4. **Craig, J. P., K. Yamamoto, T. Takeda, Y. Takeda, and T. Miwatani.** 1981. Vascular permeability activity of *Escherichia coli* heat-stable enterotoxin. Infect. Immun. **33**:473–476.
5. **Dallas, W. S., and S. Falkow.** 1979. The molecular nature of heat-labile enterotoxin (LT) of *Escherichia coli*. Nature (London) **277**:406–407.
6. **Dallas, W. S., and S. Falkow.** 1980. Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. Nature (London) **288**:499–501.
7. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. **121**:404–427.
8. **Fairbanks, G., T. L. Steck, and D. F. H. Wallach.** 1971. Electrophoretic analysis of the major polypeptide of the human erythrocyte membrane. Biochemistry **10**:2606–2617.

9. **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.
10. **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.
11. **Gyles, C. L.** 1974. Immunological study of the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. Infect. Immun. **9**:564–570.
12. **Gyles, C. L., and D. A. Barnum.** 1969. A heat-labile enterotoxin from strains of *Escherichia coli* enteropathogenic for pigs. J. Infect. Dis. **120**:419–426.
13. **Honda, T., M. Shimizu, Y. Takeda, and T. Miwatani.** 1976. Isolation of a factor causing morphological changes of Chinese hamster ovary cells from the culture filtrate of *Vibrio parahaemolyticus*. Infect. Immun. **14**:1028–1033.
14. **Honda, T., T. Tsuji, Y. Takeda, and T. Miwatani.** 1981. Immunological nonidentity of heat-labile enterotoxins from human and porcine enterotoxigenic *Escherichia coli*. Infect. Immun. **34**:337–340.
15. **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.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
17. **Lai, C.-Y.** 1977. Determination of the primary structure of cholera toxin B subunit. J. Biol. Chem. **252**:7249–7256.
18. **Lönnroth, I., and J. Holmgren.** 1973. Subunit structure of cholera toxin. J. Gen. Microbiol. **76**:417–427.
19. **Ohtomo, N., T. Muraoka, H. Inoue, H. Sasaoka, and H. Takahashi.** 1974. Preparation of cholera toxin and immunization studies with cholera toxoid, p. 132–142. *In* Proceedings of the 9th Joint Conference of the U.S.-Japan Cooperative Medical Science Program, Cholera Panel. National Institutes of Health, Bethesda, Md.
20. **Rappaport, R. S., J. F. Sagin, W. A. Pierzchala, G. Bonde, B. A. Rubin, and H. Tint.** 1976. Activation of heat-labile *Escherichia coli* enterotoxin by trypsin. J. Infect. Dis. **133**:s41–s54.
21. **Spicer, E. K., W. M. Kavanaugh, W. S. Dallas, S. Falkow, W. H. Konigobe, and D. E. Schafer.** 1981. Sequence homologies between A subunits of *Escherichia coli* and *Vibrio cholerae* enterotoxins. Proc. Natl. Acad. Sci. U.S.A. **78**:50–54.
22. **Takeda, Y., T. Honda, S. Taga, and T. Miwatani.** 1981. In vitro formation of hybrid toxins between subunits of *Escherichia coli* heat-labile enterotoxin and those of cholera enterotoxin. Infect. Immun. **34**:341–346.