Evidence That a Non-O1 *Vibrio cholerae* Produces Enterotoxin That Is Similar but Not Identical to Cholera Enterotoxin

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Cholera-like enterotoxin produced by a non-O1 strain of *Vibrio cholerae*, S7 (S7 enterotoxin), isolated from human diarrheal stool, was purified, and its physicochemical, biological, and immunological properties were compared with those of cholera enterotoxin from *V. cholerae* O1 569B (CT) and an enterotoxin produced by another non-O1 *V. cholerae* (E8498 enterotoxin) reported previously (Yamamoto et al., Infect. Immun. **39**:1128–1135, 1983). The purified S7 enterotoxin had physicochemical properties different from those of CT and E8498 enterotoxin. S7 enterotoxin had greater relative mobility in conventional polyacrylamide gel disc electrophoresis and a lower isoelectric point, and its B subunit was smaller than those of CT and E8498 enterotoxin. The results of sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis suggested that the size of the aggregate of the B subunits of S7 enterotoxin is larger than that of CT and E8498 enterotoxin. The biological and immunological properties of S7 enterotoxin were indistinguishable from those of CT and E8498 enterotoxin. These results indicate that non-O1 vibrios may produce more than one kind of cholera-like enterotoxin: one which is identical to CT (E8498 enterotoxin type) and another which is not identical to CT (S7 enterotoxin type).

It has been reported that some non-O1 strains of *Vibrio cholerae* (non-O1 vibrios) produce an enterotoxin similar to cholera toxin (CT) (4, 19, 29, 30, 32). We have purified and characterized an enterotoxin (E8498 toxin) from a non-O1 vibrio strain, E8498, which was isolated from the environment, and concluded that the E8498 toxin is indistinguishable from CT biologically, immunologically, and in molecular composition (4, 29).

In the present paper, we report the purification and characterization of an enterotoxin (S7 enterotoxin) produced by a non-O1 vibrio, strain S7, which was isolated from a diarrhea patient (30–32). We found that S7 toxin was biologically and immunologically indistinguishable from CT and E8498 toxin, but was not identical physicochemically.

MATERIALS AND METHODS

Strain. *V. cholerae* S7, non-O1, was isolated from the feces of a diarrhea patient during an epidemic in Sudan in 1968 (30–32) and provided by Y. Zinnaka, Defense Medical College, Tokorozawa, Saitama, Japan. A lincomycin-resistant mutant of S7 was isolated by plating on agar containing 300 µg of lincomycin (Lincocin Injection; Japan Upjohn, Tokyo, Japan) per ml (28).

Preparation of partially purified toxin. Culture for toxin production by the lincomycin-resistant mutant of S7 was carried out as previously described (29). An overnight culture was inoculated into Roux flasks containing 150 ml of Casamino Acids-yeast extract medium supplemented with 0.2% glucose (4) and 100 µg of lincomycin per ml (28) and incubated in the resting state for 24 h at 30°C.

The cultures were pooled and centrifuged at 25,000 × g for 30 min. The pH of the culture supernatant was adjusted to 5.0 with hydrochloric acid. Aluminum hydroxide powder was added to a final concentration of 0.1%, mixed for 6 h with stirring, and allowed to stand for an additional 18 h. After centrifugation, the aluminum hydroxide precipitate was washed several times with 0.01 M ammonium formate, after which the toxin was eluted with a buffer containing 50 mM Tris-hydrochloride, 1 mM EDTA, 3 mM NaN₃, and 0.2 M NaCl (TEAN buffer) (pH 8.5). Elution was repeated six times, and the eluates were pooled. The pooled eluate was concentrated by ultrafiltration through an Amicon PM10 membrane (Amicon Corp., Lexington, Mass).

Purified CT and E8498 toxin. CT purified from *V. cholerae* 569B, serovar O1, by the method of Ohtomo et al. (20) was obtained from Sanko Junyaku Co., Tokyo, Japan. E8498 enterotoxin was purified from *V. cholerae* E8498, serovar O344, as previously reported (29).

Rabbit skin PF test. Vascular permeability factor

(PF) tests were carried out essentially as described previously (3, 4, 29). Serial threefold dilutions (0.1 ml) were injected intradermally into clipped rabbit skin. Twenty-three hours later, 1.2 ml of 5% diphenyl brilliant blue FF-Supra (CIBA-GEIGY, Greensboro, N.C.) per kg of body weight was injected intravenously. One hour later, the diameters of the blue lesions in the skin were measured to the nearest 0.5 mm. Mean diameters were plotted against the logarithm of the dilution of the test sample, and the toxic potency of each preparation was expressed in 4-mm blueing doses. One 4-mm blueing dose was defined as the amount of toxin producing a mean lesion diameter of 4 mm. The intensity of blue lesions was read in seven grades. The limit of blueing dose of toxin was determined as previously described (4). In brief, serial 0.15-log dilutions of toxin were each mixed with an equal volume of cholera antitoxin (U.S. standard cholera antitoxin, lot 001) containing 1 antitoxin unit per ml. After incubation at 37°C for 1 h, the mixtures were injected in quadruplicate in 0.1-ml volumes, and PF activity was measured as described above. The limit of blueing dose was defined as that amount of toxin which, when mixed with 1 antitoxin unit and injected in a 0.1-ml volume, yielded a mean blueing diameter of 4 mm.

Polyacrylamide gel electrophoresis. Conventional polyacrylamide disc gel electrophoresis was carried out as described by Davis (6) on 7% acrylamide (pH 9.5) with tray buffer of pH 8.3. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel (0.2 by 13.5 by 12.0 cm) electrophoresis in 0.1% SDS was carried out as described by Laemmli (16) with 12 or 15% acrylamide. Before electrophoresis, samples were boiled for 3 min in the presence or absence of 40 mM dithiothreitol. Gels were stained with Coomassie brilliant blue R250 (Serva Blue; Serva Feinbiochemica, Heidelberg, Germany) and then destained.

Polyacrylamide gel isoelectrofocusing was carried out essentially as described previously (27) with a 5% polyacrylamide gel column. Horse cytochrome c and its acetylated derivatives (Oriental Yeast Co., Tokyo, Japan) were used as a standard pH marker. Gels were stained with 0.0001% Coomassie brilliant blue G250 in
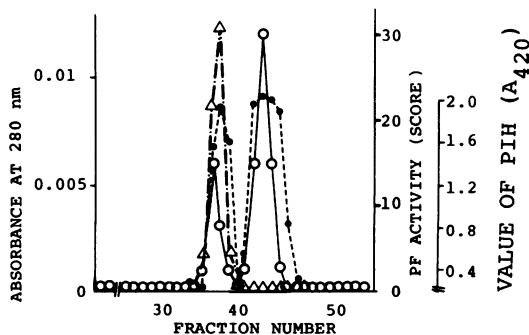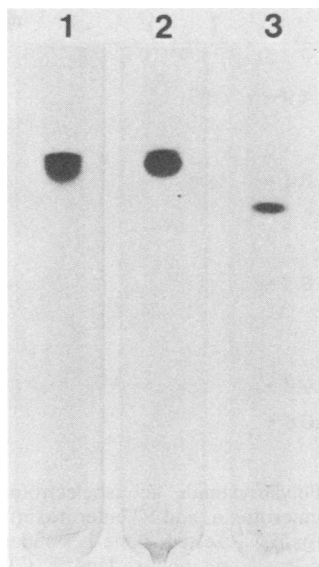

FIG. 2. Polyacrylamide disc gel electrophoresis of CT, E8498 enterotoxin, and S7 enterotoxin. About 100 µg of each toxin was tested. Lane 1, CT; lane 2, E8498 enterotoxin; lane 3, S7 enterotoxin.

3.5% perchloric acid for 2 h as described previously (22).

Ouchterlony immunodiffusion test. Double diffusion tests were carried out by the method of Ouchterlony (21) with 1.2% Noble agar (Difco Laboratories, Detroit, Mich.) in phosphate-buffered saline (pH 7.0) containing 0.01% NaN_3. After the samples were applied, the agar plates were incubated in a humidified incubator for 18 h at 37°C.

PIH. Passive immune hemolysis (PIH) was carried out to detect CT antigen as previously described (29). Samples were mixed with washed sheep erythrocytes, anti-CT antibody, and guinea pig complement, with incubation at 37°C. Hemolysis was determined by measuring the absorbance of the supernatant at 420 nm.

Antiserum. Antiserum was obtained by subcutaneous injection of four doses (15 µg each) of purified toxin preparations in 2 ml of phosphate-buffered saline (pH 7.0) into rabbit foot pads as described previously (29). The first injection was made with an equal volume of Freund complete adjuvant (Difco), and the other three were made with Freund incomplete adjuvant (Difco). Blood was taken 7 days after the fourth injection.

Determination of protein. Protein content was determined by the method of Lowry et al. (17), with bovine serum albumin as a standard.

FIG. 1. Sephadex G-75 gel filtration of partially purified S7 enterotoxin. O, Absorbance at 280 nm; ●, enterotoxin antigen by PIH (absorbance at 420 nm); △, PF activity. The PF score was calculated by multiplying mean lesion diameter (millimeters) by grade of intensity evoked by undiluted fractions. A_{420}, Absorbance at 420 nm.

RESULTS

Purification of S7 enterotoxin. Procedures for the purification of the enterotoxin from S7 were essentially those previously described for the environmental strain E8498. Partially purified toxin was chromatographed successively on Sephadex G-100, Bio-Gel A-5m and Sephadex G-
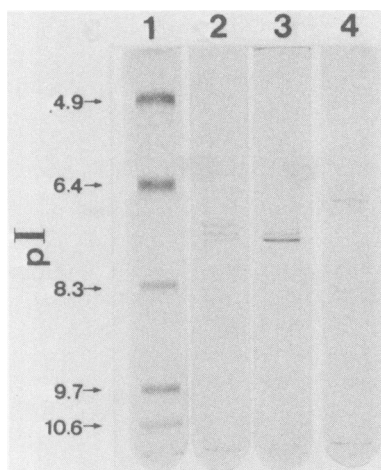
FIG. 3. Polyacrylamide gel isoelectrofocusing of CT, E8498 enterotoxin, and S7 enterotoxin. About 10 μg of each toxin was tested. Lane 1, standard marker proteins for isoelectric points; lane 2, CT; lane 3, E8498 enterotoxin; lane 4, S7 enterotoxin.

75 columns, and enterotoxin was monitored immunologically by PIH. A typical pattern of Sephadex G-75 column chromatography is shown in Fig. 1. Two protein peaks were observed. The first peak was positive in both the PIH and PF tests, whereas the second peak was positive only in the PIH test. The first peak was pooled and used as purified S7 toxin for further study. The second peak was considered to be a spontaneous aggregate of B subunits which corresponded to the choleragenoid found in culture supernatants of *V. cholerae* 569B (8).

Conventional polyacrylamide disc gel electrophoresis. The purity of the purified toxin was examined by conventional polyacrylamide disc gel electrophoresis. The purified S7 toxin gave a single band stained as shown in column 3 of Fig. 2. This band coincided with biological activity in the PF test (data not shown). However, S7 toxin migrated faster than 569B CT (column 1) and E8498 toxin (column 2), suggesting that the electrical charge of the surface of S7 toxin was different from that of 569B CT and E8498 toxin.

Isoelectric points. The isoelectric point of S7 toxin was determined by polyacrylamide gel electrofocusing, as shown in Fig. 3. The pIs of both 569B CT and E8498 toxin were between 6.8 and 7.3, whereas that of S7 toxin was about 6.6.

SDS slab gel electrophoresis. SDS slab gel electrophoresis was carried out to compare the structures of the toxin molecules. As shown in lane 3 of Fig. 4, S7 toxin gave two bands corresponding to subunits A and B of 569B CT (lane 1). The mobility of subunit A of CT (lane 1), E8498 toxin (lane 2), and S7 toxin (lane 3) was almost identical, indicating that the molecu-

lar sizes of these three molecules are similar. When subunit A of S7 toxin was reduced, it was separated into A_1 and A_2 fragments, as is the case for subunit A of CT and E8498 toxin. The mobility of subunit B of S7 toxin (Fig. 4, lanes 3 and 6) was slightly faster than that of CT (Fig. 4, lanes 1 and 4) and E8498 toxin (Fig. 4, lanes 2 and 5), indicating that subunit B of S7 was smaller than that of CT and E8498 toxin.

Experimental data shown in Fig. 5 demonstrate another difference in the molecular structure of S7 toxin from that of CT and E8498 toxin. In this experiment, toxins were incubated with SDS without heating before electrophoresis was carried out. Under these experimental conditions, B subunits migrate as a pentamer, rather than as a monomer. As shown in Fig. 5, CT (lane 1) and E8498 toxin (lane 2) gave bands corresponding to a pentamer (B_5) and a monomer. On the other hand, S7 toxin gave a band corresponding to a multi-B subunit aggregate (B_x), which migrated much more slowly than that of B_5 of CT and E8498 toxin. Spontaneous aggregates of the B subunit obtained by Sephadex G-75 chromatography as the last step in the purification of S7 toxin (peak 2 of Fig. 1) also migrated to the same position as the multi-B subunit aggregate of S7 toxin (Fig. 1, lane 4). Higher concentrations of SDS up to 1.0% did not affect the results in Fig. 5 (data not shown). These results indicate that the molecular structure of subunit B of S7 toxin is different from that of CT and E8498 toxin.

Biological (PF) activity. The dose-response curves for the PF test are shown in the left part of Fig. 6. S7 toxin produced a curve almost identical to those of 569B CT and E8498 toxin.
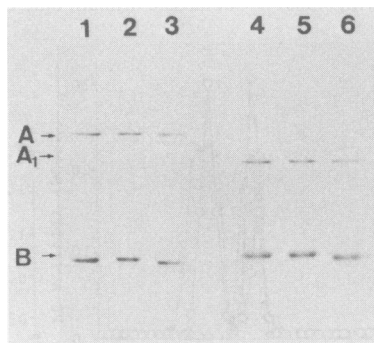

FIG. 4. SDS-polyacrylamide slab gel electrophoresis of CT, E8498 enterotoxin, and S7 enterotoxin. About 10 μg of each toxin was used for each analysis. The concentration of acrylamide was 15%. Samples were treated at 100°C for 3 min in the absence (lanes 1 to 3) or presence (lanes 4 to 6) of 40 mM dithiothreitol. Lanes 1 and 4, CT; lanes 2 and 5, E8498 enterotoxin; lanes 3 and 6, S7 enterotoxin.

One blueing dose was 14.0 pg for S7 toxin, 10.2 pg for 569B CT, and 10.4 pg for E8498 toxin.

Neutralization curves of the PF of each toxin with anti-569B CT are shown on the right side of Fig. 6. Limit of blueing doses of 569B CT, E8498 toxin, and S7 toxin were 56.8, 57.8, and 66.2 ng, respectively. These results indicate that the three toxins share almost the same amount of CT antigenic determinants.

Immunological identity. Ouchterlony immunodiffusion tests were carried out with 569B CT, E8498 toxin, and S7 toxin against antisera prepared by immunization of rabbits with each purified toxin as antigen. As shown in Fig. 7, each antiserum gave single and common precipitation lines against 569B CT, E8498 toxin, and S7 toxin, and the lines were fused with each other without spur formations. These results indicate that S7 toxin is immunologically identical to 569B CT and E8498 toxin.

DISCUSSION

Previously we purified and characterized an enterotoxin from an environmental non-O1 vibrio, strain E8498 (29). The E8498 toxin was indistinguishable from 569B CT biologically, immunologically, and in molecular construction. In
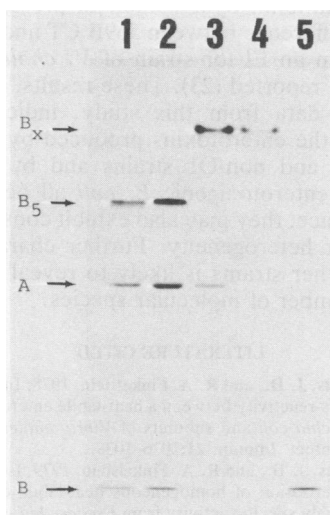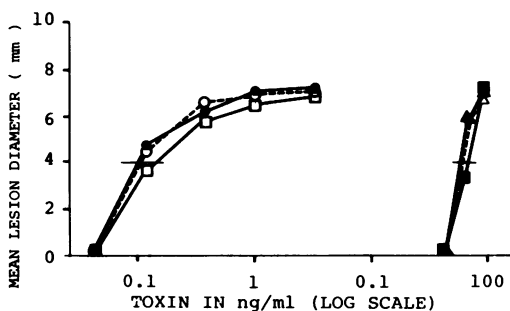

FIG. 6. Rabbit skin vascular permeability activities of CT, E8498 enterotoxin, and S7 enterotoxin. The curves on the left are blueing dose titrations of CT (●), E8498 enterotoxin (○), and S7 enterotoxin (□). The curves on the right are limit of blueing titrations of CT (▲), E8498 enterotoxin (△), and S7 enterotoxin (■) in which each toxin was preincubated with an equal volume of cholera antitoxin containing 1 antitoxin unit/ml (29). Each value represents the mean of four determinations. The horizontal bars represent the 4-mm intersects.

FIG. 5. SDS-polyacrylamide slab gel electrophoresis of CT, E8498 enterotoxin, and S7 enterotoxin without heat treatment. About 10 μg of CT and S7 enterotoxin and about 20 μg of E8498 enterotoxin were used. The concentration of acrylamide was 12%. Lane 1, CT; lane 2, E8498 enterotoxin; lane 3, S7 enterotoxin; lanes 4 and 5, aggregates of B subunit of S7 enterotoxin (peak 2 of Fig. 1). Each sample except lane 5 was incubated for 30 min at 37°C in the presence of 0.1% SDS. The sample in lane 5 was heated at 100°C for 3 min before electrophoresis. B_x, Multi-B subunit aggregate.

this paper we report the purification and properties of an enterotoxin from a human isolate, strain S7. Purified S7 toxin showed greater relative mobility in conventional polyacrylamide disc gel electrophoresis (Fig. 2), more acidic pI (Fig. 3), smaller molecular size of subunit B (Fig. 4), and larger molecular size of the subunit B aggregate (Fig. 5), as compared with 569B CT and E8498 toxin. These differences in physicochemical properties indicate that S7 toxin may not be identical to 569B CT and E8498 toxin, which appeared to be identical to one another (29).

Several previous reports suggested that non-O1 enterotoxin may not be completely identical to CT. Ohashi et al. (19) reported that CT-like non-O1 enterotoxin might differ from CT because PF activity in the culture filtrate of a non-O1 strain was not completely neutralized by cholera antitoxin. Zinnaka and Carpenter (30) reported similar results, but later they showed that the unneutralizable nonspecific PF activity could be removed from the toxin preparation (32). Nevertheless, Zinnaka and Fukuyoshi (31) found other evidence that suggested that non-O1 toxin and CT might be different; that is, the PF activity of a non-O1 strain, S2, which was isolated from a patient during the same epidemic in Sudan from which S7 was isolated, migrated more rapidly than that of 569B CT in conventional polyacrylamide gel electrophoresis. Our observations on the electrophoresis of S7 (Fig. 2) support their findings with the S2 strain. Kaper et al. (14) recently reported from a genetic study that the DNA sequence encoding enterotoxin in non-O1 strains is not identical to that
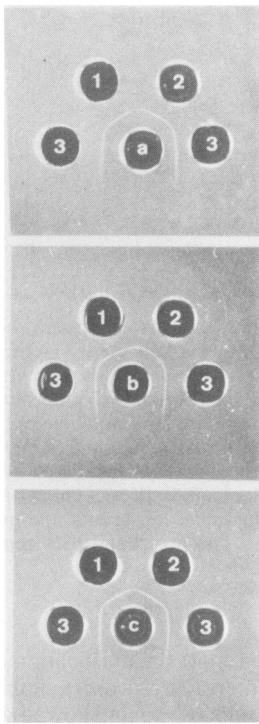
FIG. 7. Ouchterlony immunodiffusion test of CT, E8498 enterotoxin, and S7 enterotoxin. Well 1, CT; well 2, E8498 enterotoxin; well 3, S7 enterotoxin; well a, cholera antitoxin; well b, anti-purified E8498 enterotoxin; well c, anti-purified S7 enterotoxin.

encoding CT in O1 strains. This also provides evidence that some *V. cholerae* non-O1 strains produce an enterotoxin that is similar to but not identical to CT from *V. cholerae* 569B.

It has been reported that CT consists of one molecule of A subunit and four to six molecules of B subunits (10, 11). The results shown in Fig. 5 demonstrate that the B subunits of S7 enterotoxin did not migrate as one would expect of a pentamer similar to that found in CT and E8498 enterotoxin. This may be due to the presence of larger aggregates of B subunits in S7 toxin than in CT and E8498 enterotoxin. Alternatively, it might indicate that the number of B subunits of S7 toxin is greater than that in 569B CT and E8498 toxin. This problem remains to be elucidated.

The E8498 and S7 enterotoxins used in this work were produced in the presence of lincomycin. Thus, it might be asked whether toxin produced in the presence of lincomycin is identical to that produced in its absence. Direct comparison of E8498 and S7 enterotoxins produced in the presence and absence of lincomycin is difficult because the amounts of toxin produced in the absence of lincomycin are too small to carry out the required physicochemical charac-

terizations. However, we have compared several physicochemical properties of a heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* grown in the presence and absence of lincomycin. The results (unpublished observations) showed that both toxins gave the same mobilities on SDS-polyacrylamide gel slab electrophoresis, the same pIs, and the same amino acid composition. Moreover, E8498 toxin produced in the presence of various concentrations of lincomycin (100, 200, and 300 μg/ml) gave the same mobility on SDS-polyacrylamide slab gel electrophoresis and the same pIs. Thus, we assume that the toxins produced in the presence of lincomycin have the same physicochemical properties as those produced in the absence of lincomycin.

LT produced by enterotoxigenic *E. coli* is similar to 569B CT biologically, enzymatically, immunologically, and in molecular construction (1, 7, 12, 15, 18, 25), although some heterogeneity in antigenicity and amino acid sequence has been demonstrated (1, 2, 5, 15, 24). Recently, it was found that there are two kinds of LT, human (LT_h) and porcine (LT_p), and that LT_h and LT_p differ in antigenicity, amino acid composition, and size of subunits (9, 13, 26; R. K. Holmes, E. M. Twiddy, and R. J. Neill, *in* Y. Takeda and T. Miwatani, ed., *Bacterial Diarrheal Diseases: An International Symposium,* in press). Moreover, a difference between 569B CT and enterotoxin from an El tor strain of *V. cholerae* has also been reported (23). These results, together with the data from this study, indicate that although the enterotoxins produced by *V. cholerae* O1 and non-O1 strains and by various kinds of enterotoxigenic *E. coli* all bear close resemblance, they may also exhibit considerable molecular heterogeneity. Further characterization of other strains is likely to reveal an even larger number of molecular species.

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