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Purification and Some Properties of a Non-O1 *Vibrio cholerae* Enterotoxin That Is Identical to Cholera Enterotoxin

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Cholera-like enterotoxin was isolated and purified from the culture supernatant of a non-O1 strain of *Vibrio cholerae*, E8498, isolated from the environment. Enterotoxin was purified by aluminum hydroxide absorption and elution and successive gel filtrations on Sephadex G-100, Bio-Gel A-5m, and Sephadex G-75. Purified enterotoxin gave a single stained band on polyacrylamide gel disc electrophoresis, and the mobility was the same as that of cholera enterotoxin. The specific biological activity of the purified enterotoxin was almost the same as that of cholera enterotoxin in the Chinese hamster ovary cell assay, fluid accumulation in mouse ligated intestine, increase in vascular permeability in rabbit skin, and passive immune hemolysis. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis showed that the purified enterotoxin consisted of subunits A and B, identical to those of cholera enterotoxin, and Ouchterlony double gel diffusion tests indicated that the two toxins were immunologically identical. Enterotoxins prepared from several non-O1 strains isolated from human patients were also immunologically identical to cholera enterotoxin.

Cholera enterotoxin (CT) produced by *Vibrio cholerae*, serovar O1, has been recognized as the mediator of cholera diarrhea (11, 32). CT has been purified by several workers, and the biological and biochemical properties of the toxin have been extensively studied (13, 18, 30). In addition, a number of cases of cholera-like diarrhea in humans associated with non-O1 *V. cholerae* have been reported, and the production of CT-like enterotoxin by non-O1 vibrios has been suggested (1, 2, 15, 22, 27, 29, 33, 40). Recently, we reported that a non-O1 strain of *V. cholerae* isolated from fresh water in Louisiana produced an enterotoxin which is immunologically and biologically indistinguishable from CT (7). To further confirm this, we purified this enterotoxin and compared its physicochemical, biological, and immunological properties with those of CT.

MATERIALS AND METHODS

Bacterial strains. Strains of non-O1 *V. cholerae* used in this study were: E8498, serovar O344, isolated from surface water in the Dewitt Canal in Louisiana in 1978 and provided by P. Blake, Centers for Disease Control, Atlanta, Ga. (3); S7, isolated from a diarrheal patient in Sudan and provided by Y. Zinnaka, Defence Medical College, Tokorozawa, Saitama, Japan; 62058, isolated from a diarrheal patient and provided by M. I. Huq, International Center for Diarrhoeal Disease Research, Bangladesh; and WBDV-101E_1, isolated from a human patient and provided by P. Echeverria, Armed Forces Research Institute for Medical Sciences, Bangkok, Thailand. A lincomycin-resistant mutant of E8498 was isolated by plating the cells on an agar plate containing 300 μg of lincomycin (Lincocin injection; Japan Upjohn, Tokyo, Japan) per ml. All strains were maintained at room temperature on slants containing 1% Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1% NaCl, and 1.5% agar (T_1N_1 agar) covered with mineral oil.

Culture of cells. Procedures for culture of the lincomycin-resistant strain of E8498 for toxin production were as described previously (7). A loopful of organisms was inoculated into 5 ml of a broth containing 1% Trypticase (BBL) and 1% NaCl (T_1N_1 broth). A 1.5-ml portion of a 6-h culture at 37°C was transferred to a 2-liter Erlenmeyer flask containing 150 ml of a medium containing 3% Casamino acids (Difco Laboratories, Detroit, Mich.), 0.3% yeast extract (Difco), and 0.05% K_2HPO_4 (pH 7.0) (Casamino acids-yeast extract medium) and incubated without shaking at 37°C for 18 h. Then 1.5 ml of this culture was inoculated into 1-liter Roux flasks containing 150 ml of the Casamino acids-yeast extract medium supplemented with 0.2% glucose and 300 μg of lincomycin per ml; this yielded a surface/volume ratio of 2 cm^2/ml. Lincomycin was added to stimulate toxin production (39). Cultures were incubated without shaking at 30°C for 48 h. These cultural conditions were found to be optimal for the production of maximal amounts of toxin (7). Cultures were then centrifuged at 25,000 × *g* for 30 min at 4°C. Sodium azide and sodium EDTA were added to culture supernatants at final concentrations of 0.02 and 0.05%, respectively.

Purified CT. CT from *V. cholerae* 569B, serovar O1, purified by the method described by Ohtomo et al.

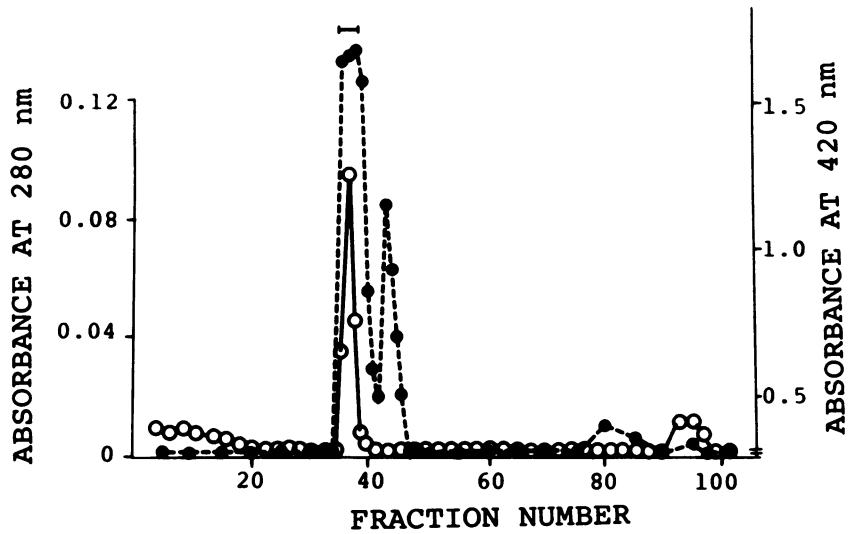

FIG. 1. Sephadex G-75 gel filtration of the pooled Bio-Gel A-5m column eluate of E8498 enterotoxin. ○, Absorbance at 280 nm; ●, enterotoxin antigen content by passive immune hemolysis (absorbance at 420 nm). Bar, fractions collected for further purification.

(30), was purchased from Sanko Junyaku Co., Tokyo, Japan. Separation of subunits A and B was carried out as previously described (35).

Cholera antitoxin. Rabbit antitoxin against the purified CT was prepared by subcutaneous injections of four doses of 15 μg of CT contained in 1 ml of phosphate-buffered saline (pH 7.0) at 3-week intervals. The first dose was administered with an equal volume of complete Freund adjuvant (Difco), and the other three were administered with incomplete Freund adjuvant (Difco). Blood was taken 7 days after the fourth injection.

Ganglioside. G_{M1} ganglioside was purchased from Supelco, Inc., Bellefonte, Pa.

Determination of protein content. Protein content was determined by the method of Lowry et al. (26), with crystalline bovine serum albumin as the standard.

Chromatography. Gel filtrations were carried out at 4°C with columns of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden), Bio-Gel A-5m (200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.), and Sephadex G-75 (superfine; Pharmacia Fine Chemicals). The columns were equilibrated with TEAN buffer (12), composed of 50 mM Tris, 1 mM EDTA, 3 mM NaN_3, and 0.2 M NaCl (pH 7.8), and elutions were made with the same buffer. The toxin was monitored by passive immune hemolysis.

Passive immune hemolysis. The procedure for passive immune hemolysis was as previously described (38). A 1-ml sample was mixed with 0.1 ml of a 2% sheep erythrocyte suspension in 0.02 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 6.7) containing 0.9% NaCl and incubated at 37°C for 30 min. Anti-CT rabbit serum (0.1 ml), purified by an immunoaffinity column coupled with purified CT as previously described (20), was added, and incubation was continued at 37°C for 30 min. Then 0.1 ml of guinea pig complement solution was added, and incubation at 37°C was continued for 60 min. The mixture was diluted with 1.6 ml of saline and centrifuged at 1,500 × *g* for 5 min. Hemolysis was measured by absorbance of the supernatant at 420 nm.

PF assay. Vascular permeability factor (PF) assays were carried out essentially as described previously (6, 7). The hair was clipped from the backs of Japanese white rabbits, and 0.1-ml volumes of threefold dilutions of samples in borate-gelatin buffer (50 mM H_3BO_3, 120 mM NaCl, and 0.1% gelatin [pH 7.5]) were injected intracutaneously into partially randomized sites in duplicate on the backs of two rabbits. Twenty-four hours later, the animals were inoculated intravenously with 1 ml of 5% diphenyl brilliant blue FF supra (Ciba Geigy, Greensboro, N.C.) in 0.45% NaCl solution per kg of body weight. 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 dose (BD_4). One BD_4 was defined as the amount of toxin producing a mean lesion diameter of 4 mm. The limit of blueing (Lb) dose of toxin was determined as previously described (7). The antitoxin units of the cholera antitoxin prepared as described above were determined by simultaneous Lb titration against U.S. standard cholera antitoxin (NIH lot 001).

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

Intestinal loop test in mice. The intestinal loop test in mice was carried out essentially as described previously (36). Male ddY mice weighing 20 to 25 g were used without starvation. The mice were anesthetized by intravenous injection (0.1 mg/g of body weight) of sodium pentobarbital, and one loop (7 to 14 cm long) of the jejunum was ligated. Then 0.2 ml of the sample was injected into the loop, the abdominal wall was sutured,

TABLE 1. Purification of E8498 enterotoxin

Fraction	Volume (ml)	Total protein (mg)	Total PF activity (BD_4)	Sp act (BD_4/mg)	Relative activity	Yield (%)
Crude toxin	26	385	5.6×10^8	1.5×10^6	1	100
Sephadex G-100 column eluate	9.3	47	1.2×10^8	2.6×10^6	1.7	21
Bio-Gel A-5m column eluate	2.0	4.0	1.0×10^8	2.5×10^7	17	18
First Sephadex G-75 column eluate	1.0	2.8	9.9×10^7	3.5×10^7	23	18
Second Sephadex G-75 column eluate	1.2	0.68	6.4×10^7	9.4×10^7	63	11

and 6 h later the loop was removed. The weights of the loop with (*A*) and without (*B*) fluid were measured, and the weight of fluid ($A - B$) was calculated. Toxic activity was expressed as the ratio of the weight (*W*) of fluid in milligrams to the length (*L*) of the loop in centimeters (*W*/*L* ratio).

Polyacrylamide gel electrophoresis. Polyacrylamide gel disc electrophoresis was carried out as described by Davis (9) with a 7% polyacrylamide gel (pH 9.5) and a tray buffer of pH 8.3. Sodium dodecyl sulfate-polyacrylamide gel slab (0.2 by 13.5 by 12.0 cm) electrophoresis in 0.1% sodium dodecyl sulfate was carried out as described by Laemmli (25) with 15% acrylamide. Before electrophoresis, samples with and without 40 mM dithiothreitol were treated at 100°C for 3 min. Gels were stained with Coomassie brilliant blue and destained.

Double gel diffusion test. The double gel diffusion test was carried out as described by Ouchterlony (31) with 1% Noble agar (Difco) in phosphate-buffered saline containing 0.01% NaN_3. After the samples were applied, the plates were placed in a humidified incubator at 37°C for 16 to 24 h and then washed extensively with a solution containing 0.4% NaCl and 0.4% $Na_2B_4O_7$. Then they were dried, stained with 0.5% Coomassie brilliant blue in 50% methanol–10% acetic acid, and destained in a solution containing 50% methanol and 10% acetic acid.

RESULTS

Preparation of crude toxin from culture supernatant of *V. cholerae* E8498. The culture supernatant of *V. cholerae* E8498 (about 13,800 ml) was adjusted to pH 5.0 with 12 N HCl. Aluminum hydroxide powder (0.1% [wt/vol]) was added and stirred for 6 h at 4°C. After standing for 18 h, the aluminum hydroxide was collected by centrifugation at 8,000 × *g* for 5 min. It was estimated by passive immune hemolysis that about 98% of the toxin in the culture supernatant was adsorbed to the aluminum hydroxide. The precipitate was then washed with about 400 ml of 10 mM ammonium formate solution, stirred for 20 min, and centrifuged at 8,000 × *g* for 5 min. This procedure was repeated five times. The enterotoxin was eluted from the aluminum hydroxide with 50 ml of TEAN buffer (pH 8.5) by stirring the suspension for 1 h at 4°C. The suspension was centrifuged at 8,000 × *g* for 5 min, and the supernatant was collected. This elution procedure was repeated six times, and the supernatants were pooled. The pooled eluate (335 ml) was concentrated by adding solid ammonium sulfate to 50% saturation. After standing for 10 h at 4°C, the resulting precipitate was collected by centrifugation at 25,000 × *g* for 20 min. The precipitate was dissolved in about 26 ml of TEAN buffer (pH 7.8). Undissolved material was sedimented by centrifugation at 25,000 × *g* for 20 min, and the supernatant was used as crude toxin.

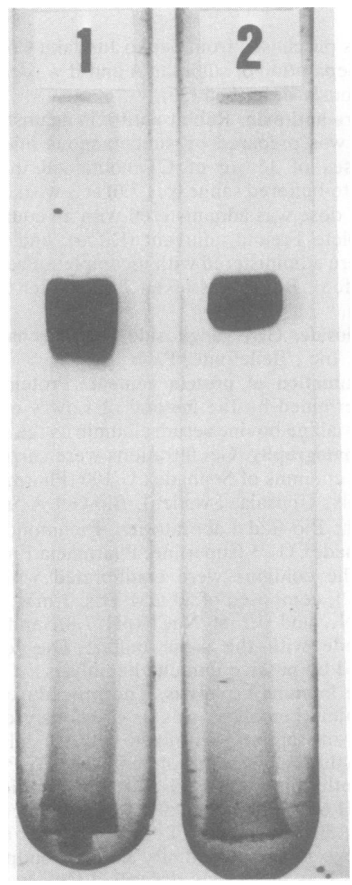

FIG. 2. Polyacrylamide gel disc electrophoresis of CT from strain 569B and purified E8498 enterotoxin. A 100-μg amount of each toxin was applied. 1, CT; 2, E8498 enterotoxin.

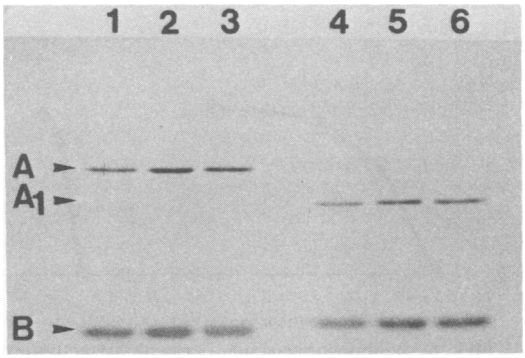

FIG. 3. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of CT and purified E8498 enterotoxin. About 10 μg of toxin was used for each analysis. 1, CT; 2, E8498 enterotoxin; 3, CT plus E8498 enterotoxin; 4, CT treated with dithiothreitol; 5, E8498 enterotoxin treated with dithiothreitol; 6, CT plus E8498 enterotoxin treated with dithiothreitol.

Column chromatography of crude toxin. Crude toxin was applied to a Sephadex G-100 gel column (4 by 144 cm) equilibrated with TEAN buffer (pH 7.8), and elution was made with the same buffer. The fractions of enterotoxin as detected by passive immune hemolysis were pooled and concentrated by ultrafiltration with an Amicon PM 10 membrane (Amicon Corp., Lexington, Mass.). The concentrated material was applied to a Bio-Gel A-5m column (2.3 by 96 cm) equilibrated with TEAN buffer (pH 7.8), and elution was made with the same buffer. The fractions of enterotoxin were collected and concentrated by ultrafiltration with an Amicon PM 10 membrane.

The concentrated material was then applied to a Sephadex G-75 column (2 by 150 cm) equilibrated with TEAN buffer (pH 7.8), and elution was made with the same buffer. As shown in Fig. 1, one major protein peak was obtained, whereas enterotoxin was found in two peaks, one identical to the major protein peak and a second immediately following. PF tests were carried out with these two peaks, and it was found that only the first peak had PF activity (data not shown). Thus, it is likely that the second peak is an aggregate of B subunits similar to the choleragenoid found in culture supernatants of *V. cholerae* 569B (13). Fractions (indicated by a bar in Fig. 1) were collected, concentrated by ultrafiltration on an Amicon PM 10 membrane, and applied to the same column for rechromatography. The results indicated that the protein peak corresponded to the peak of enterotoxin as measured by the passive immune hemolysis test. Fractions of enterotoxin were collected and concentrated by ultrafiltration on an Amicon PM 10 membrane and were considered to be purified E8498 enterotoxin.

The steps in the purification of E8498 enterotoxin are summarized in Table 1. About 63-fold purification was achieved, with a recovery of about 11% of the PF activity present in the aluminum hydroxide-concentrated material.

Homogeneity of purified E8498 enterotoxin. The homogeneity of the purified E8498 enterotoxin was examined by polyacrylamide gel disc electrophoresis. As shown in Fig. 2, the E8498 enterotoxin (lane 2) gave a single stained band on the gel when 100 μg was applied. To demonstrate that this band corresponded to the enterotoxin activity, the polyacrylamide gel was cut into sections 2 mm in width after electrophoresis, and these were suspended in phosphate-buffered saline (pH 7.0). Assay of the extracts for morphological changes in CHO cells showed that the toxin was in the same position as the stained band (data not shown). These results suggested that the E8498 enterotoxin was purified almost to homogeneity.

It was also found that the mobility of the purified E8498 enterotoxin (Fig. 2, lane 2) was almost the same as that of purified CT (Fig. 2, lane 1).

Molecular structure of E8498 enterotoxin. To compare the molecular structure of E8498 enterotoxin with that of CT, sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of the purified E8498 enterotoxin and CT was carried out (Fig. 3). The E8498 enterotoxin (Fig. 3, lane 2) migrated as two stained bands corresponding to A and B subunits of CT (Fig. 3, lane 1). The mobilities of these two bands of E8498 enterotoxin were almost the same as those of CT. When the E8498 enterotoxin was treated with dithiothreitol, the stained band corresponding to A subunit disappeared, and a band corre-

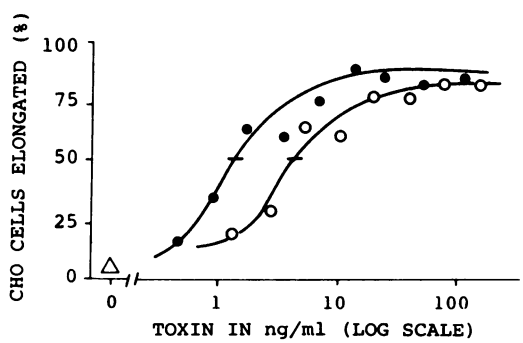

FIG. 4. Morphological changes in CHO cells caused by CT and purified E8498 enterotoxin. Toxins were added to the culture medium of CHO cells at the final concentration indicated; the percentage of elongated cells was determined by observing 200 cells after 18 h of incubation. Each value represents a mean of eight determinations. ○, CT; ●, E8498 enterotoxin; △, control without toxin. Bars, 50% elongation.

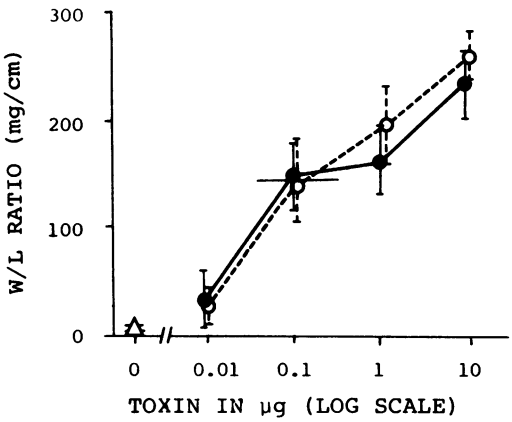

FIG. 5. Fluid accumulation in mouse intestinal loops by CT and purified E8498 enterotoxin. Each value represents the mean ± 1 standard error of 12 determinations. ○, CT; ●, E8498 enterotoxin; △, control without toxin.

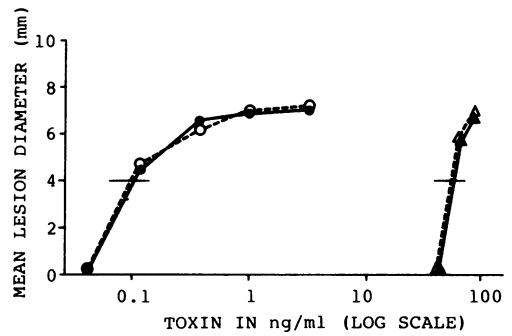

FIG. 6. Rabbit skin vascular permeability activities of CT and purified E8498 enterotoxin. The curves on the left are blueing dose titrations of CT (○) and E8498 enterotoxin (●). The curves on the right are Lb titrations of CT (△) and E8498 enterotoxin (▲) in which each toxin was preincubated with an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

sponding to the A_1 fragment was observed (Fig. 3, lane 5). The mobility of the A_1 fragment of E8498 enterotoxin was almost the same as that of the A_1 fragment of CT (Fig. 3, lane 4).

The identities of the mobilities of A and B subunits and of A_1 fragments of CT and E8498 enterotoxin were demonstrated by coelectrophoresis of the two toxins. In this experiment, CT and E8498 enterotoxin were mixed before analysis. As shown in lanes 3 and 6 of Fig. 3, coelectrophoresis of CT and E8498 enterotoxin with and without dithiothreitol treatment, respectively, resulted in a single band of A and B subunits and of the A_1 fragment.

These data indicate that the molecular structures of the two toxins are identical.

Biological activity of E8498 enterotoxin. Biological activity of the purified E8498 enterotoxin was compared with that of CT in several assay systems. Figure 4 compares the activities of the two toxins in the CHO cell assay. Elongation of 50% of CHO cells was produced by about 1.3 ng of E8498 enterotoxin per ml and by 4.2 ng of CT per ml.

Fluid accumulation in mouse ligated intestinal loops evoked by the two toxins is shown in Fig. 5. About 100 ng of both toxins produced 140 mg/cm in *W/L* ratio, which is about half of the mean maximal response.

Figure 6 shows that in the PF test 1 BD_4 of the E8498 enterotoxin was 10.6 pg, and 1 BD_4 of CT was 10.2 pg. The Lb doses of E8498 enterotoxin and CT were 57.8 and 56.8 ng, respectively.

The inhibitory effects of G_{M1} ganglioside of PF from CT and E8498 enterotoxin were also compared. As shown in Fig. 7, PF activity of both CT and E8498 enterotoxin was similarly inhibited by G_{M1} ganglioside.

These results all indicated that the specific activities of both toxins in the biological assays examined were very similar. The differences observed were considered to be due to the experimental errors inherent in these assay systems. Moreover, the nearly identical Lb values in the PF tests showed that both toxins were neutralized to the same degree by cholera antitoxin, suggesting a very close immunological relationship.

Immunological specificity of E8498 enterotoxin. Immunological properties of the two toxins were further compared in in vitro assays. Passive

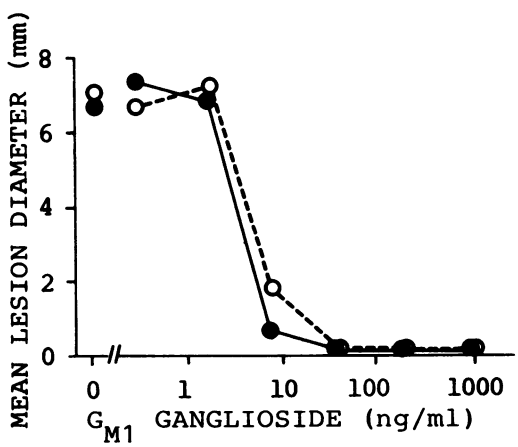

FIG. 7. Inhibition of PF activities of CT and E8498 enterotoxin by G_{M1} ganglioside. Toxin (15 ng/ml of each) was mixed with an equal volume of graded doses (nanograms per milliliter of 0.89% saline) of G_{M1} ganglioside as indicated, and PF activity of 0.1 ml of each mixture was tested as described in the text. ○, CT; ●, E8498 enterotoxin.

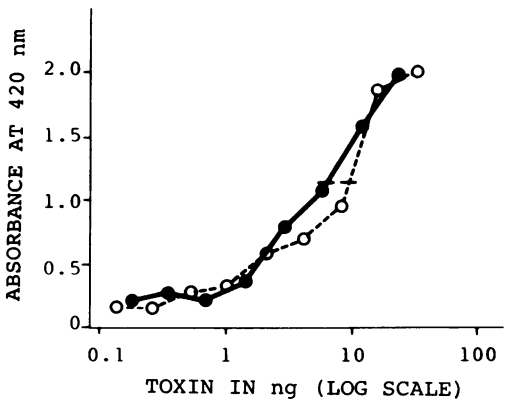

FIG. 8. Activity of CT and purified E8498 enterotoxin in passive immune hemolysis. O, CT; ●, E8498 enterotoxin. Bar, 50% hemolysis.

immune hemolysis of purified E8498 enterotoxin and CT were carried out with cholera antitoxin. As shown in Fig. 8, both toxins gave very similar dose-response curves. The amounts of toxins producing 50% hemolysis were 6.6 ng for E8498 enterotoxin and 9.5 ng for CT. Thus, the immunological specificities of the two toxins were also very similar.

Ouchterlony double gel diffusion tests were carried out with cholera antitoxin and serum prepared against purified E8498 enterotoxin. As shown in Fig. 9, the culture supernatant of E8498 grown in a medium without lincomycin, purified E8498 enterotoxin prepared in the presence of lincomycin, and CT all gave a line of identity against both cholera antitoxin (Fig. 9A) and anti-E8498 enterotoxin prepared in the presence of lincomycin (Fig. 9B). These results indicate that the E8498 enterotoxin is immunologically identical to CT and that the toxin produced by E8498 cultured in the presence of lincomycin is immunologically identical to that produced in the absence of lincomycin.

Immunological identity of enterotoxins produced by human isolates of non-O1 *V. cholerae* and by the environmental strain, E8498. The results described above demonstrated that the enterotoxin produced by E8498, an environmental strain of non-O1 *V. cholerae*, possessed biological, molecular, and immunological properties in common with CT. Since there are several reports of isolation of non-O1 *V. cholerae* from patients with diarrhea, it was of interest to compare their enterotoxins with the enterotoxin produced by the environmental strain. Crude enterotoxins were prepared from non-O1 *V. cholerae* strains S7, 62058, and WBDV-101E_1, all of which were isolated from human stool specimens. The results of Ouchterlony double gel diffusion tests with antiserum against purified CT and purified E8498 enterotoxin showed that all of these enterotoxins shared a common antigenic determinant(s) (Fig. 10).

DISCUSSION

We obtained highly purified cholera-like enterotoxin from a strain of *V. cholerae* O344, E8498. This toxin exhibited specific activities identical to those of CT in several biological and immunological systems. The subunits of the purified E8498 enterotoxin were the same A and B subunits as those found in CT. Ouchterlony double gel diffusion tests also showed that E8498 enterotoxin and CT had the same antigenic specificities. From these results, we concluded that the cholera-like enterotoxin produced by this strain of *V. cholerae* O344 is indistinguishable from CT produced by the O1 strain, 569B.

Until now it has not been possible to determine whether cholera-like enterotoxins produced by non-O1 strains are identical to CT produced by O1 strains because highly purified non-O1 enterotoxin has not heretofore been prepared. The non-O1 vibrios so far studied

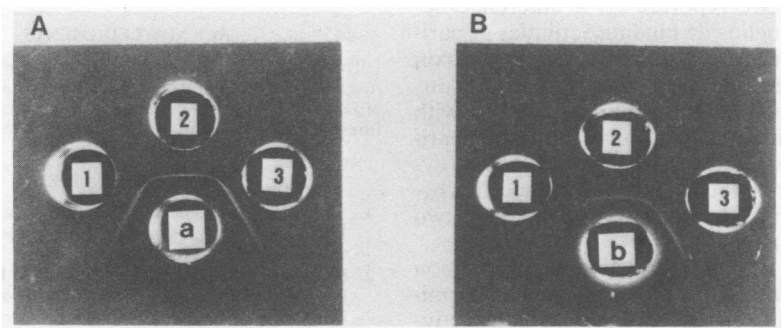

FIG. 9. Ouchterlony double gel diffusion test of CT and E8498 enterotoxin. 1, Crude E8498 toxin prepared in the absence of lincomycin; 2, CT; 3, purified E8498 enterotoxin; a, cholera antitoxin prepared in the presence of lincomycin; b, anti-purified E8498 enterotoxin prepared in the presence of lincomycin.

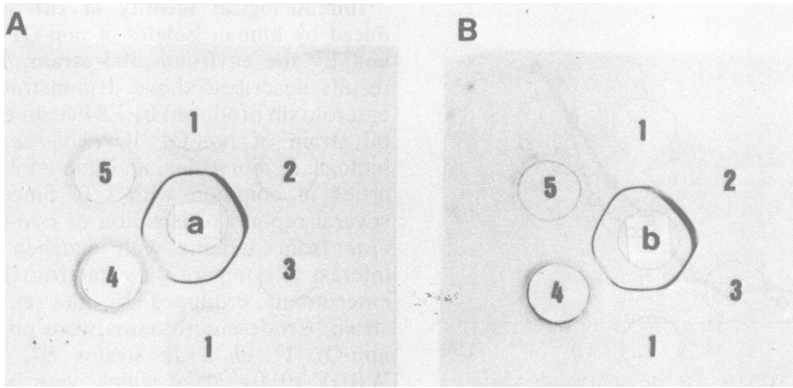

FIG. 10. Ouchterlony double gel diffusion test of crude filtrates from various strains of *V. cholerae* non-O1. 1, CT; 2, purified E8498 enterotoxin; 3, crude toxin from S7; 4, crude toxin from WBDV-101E_1; 5, crude toxin from 62058; a, cholera antitoxin; b, anti-purified E8498 enterotoxin.

have produced relatively small amounts of enterotoxin in vitro. Moreover, they often produce several biologically active substances (7, 29, 40, 41) which interfere with or mask the activity of cholera-like enterotoxin in test systems. Zinnaka et al. (41) prepared partially purified toxin from one non-O1 strain and showed that when nonspecific substances were removed, the activity of the non-O1 enterotoxin was completely neutralized by cholera antitoxin. Their report suggested that the non-O1 enterotoxin was very similar to CT. In the present work, we were able to confirm their findings and to further purify and analyze the non-O1 enterotoxin directly, leading us to the conclusion that the enterotoxins from E8498 and 569B are identical.

E8498 enterotoxin was composed of the same subunits as O1 cholera enterotoxin. This is consistent with the findings of Kaper et al. (23) that some strains of non-O1 vibrios possess genes which hybridize with DNA coding for the heat-labile enterotoxin (LT) from *Escherichia coli*. However, our findings indicate that E8498 enterotoxin is much more similar to CT than is LT.

Previously, we reported (7) a difference between G_{M1} ganglioside binding activities of purified CT and partially purified E8498 enterotoxin and postulated that all cholera enterotoxins might not be alike in their binding ratios with G_{M1} ganglioside. The present study with purified CT and purified E8498 enterotoxin, however, showed that there is no difference in G_{M1} ganglioside binding activities between these two toxins (Fig. 7).

Although LT and CT share some common biological activities, immunological determinants, molecular structure, and enzymatic activities (4, 10, 17, 24, 28, 35), their antigenic and amino acid compositions are clearly distinguishable (4, 5, 8, 34). Moreover, immunological and molecular heterogeneity of LTs of human and porcine origin have recently been reported (14, 21, 37). Since we have examined only a few non-O1 strains and their enterotoxins have been carefully compared with only one CT from an O1 strain (569B), it is quite possible that differences in molecular structure will be found to exist between the enterotoxins produced by different serovars of the species *V. cholerae*.

The fact that the enterotoxin produced by E8498, an environmental strain, has the same specific biological activity as CT provides evidence that this organism is a potential causative agent of diarrhea. The amount of toxin produced in vitro by E8498 is relatively small, but it is no less than that synthesized in vitro by many known virulent O1 strains of *V. cholerae* of the El Tor biotype (unpublished observations). Since other factors of virulence in addition to the enterotoxin are almost certainly required to produce diarrhea in humans, the actual enteropathogenic potential of this non-O1 strain can be demonstrated only by oral challenge in volunteers.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Aldová, E., K. Laznickova, E. Stepankova, and J. Lietava.** 1968. Isolation of non-agglutinable vibrios from an enteritis outbreak in Czechoslovakia. J. Infect. Dis. **118**:25–31.
2. **Bhattacharya, S., A. K. Bose, and A. K. Gosh.** 1971. Permeability and enterotoxic factors of non-agglutinable vibrios. *Vibrio alkaligenes* and *Vibrio parahaemolyticus*. Appl. Microbiol. **22**:1159–1161.
3. **Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barret, L. McFarland, C. T. Caraway, J. C. Feeley, J. P. Craig, J. V. Lee, N. D. Pur, and R. A. Feldman.** 1980. Cholera—

a possible endemic focus in the United States. N. Engl. J. Med. 302:305–309.
4. **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.
5. **Clements, J. D., and R. A. Finkelstein.** 1979. Isolation and characterization of homogeneous heat-labile enterotoxin with highly specific activity from *Escherichia coli* culture. Infect. Immun. 24:760–769.
6. **Craig, J. P.** 1965. A permeability factor (toxin) found in cholera stool and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) 207:614–616.
7. **Craig, J. P., K. Yamamoto, Y. Takeda, and T. Miwatani.** 1981. Production of cholera-like enterotoxin by a strain of *Vibrio cholerae* non-O1, isolated from the environment. Infect. Immun. 34:90–97.
8. **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.
9. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404–427.
10. **Evans, D. J., L. C. Chin, G. T. Curlin, and D. G. Evans.** 1972. Stimulation of adenyl cyclase by *Escherichia coli* enterotoxin. Nature (London) New Biol. 236:137–138.
11. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. 2:553–623.
12. **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.
13. **Finkelstein, R. A., and J. J. LoSpalluto.** 1972. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. 121:S63–S72.
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. **Goodner, K., H. L. Smith, Jr., K. A. Monsur, and I. Huq.** 1966. Non-cholera vibrios in diarrheal diseases of East Pakistan. East Pakistan Med. J. 10:1–9.
16. **Guerrant, R. L., L. L. Brinton, T. S. Schnaitman, L. I. Rebhun, and A. G. Gilman.** 1974. Cyclic adenosine monophosphate and alteration of Chinese hamster ovary cell morphology: a rapid, sensitive in vitro assay for the enterotoxins of *Vibrio cholerae* and *Escherichia coli*. Infect. Immun. 10:320–327.
17. **Gyles, G. L.** 1974. Immunological study of the heat-labile enterotoxins of *Escherichia coli* and *Vibrio cholerae*. Infect. Immun. 9:564–570.
18. **Holmgren, J.** 1981. Actions of cholera toxin and the prevention and treatment of cholera. Nature (London) 292:413–417.
19. **Honda, T., M. Shimizu, Y. Takeda, and T. Miwatani.** 1976. Isolation of a factor causing morphological changes of Chinese hamster ovary cells from *Vibrio parahaemolyticus*. Infect. Immun. 14:1028–1033.
20. **Honda, T., Y. Takeda, and T. Miwatani.** 1981. Isolation of special antibodies which react only with homologous enterotoxins from *Vibrio cholerae* and enterotoxigenic *Escherichia coli*. Infect. Immun. 34:333–336.
21. **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.
22. **Hughes, J. M., D. G. Hollis, E. J. Gangarosa, and R. E. Weiner.** 1978. Non-cholera vibrio infections in the United States. Ann. Intern. Med. 88:602–606.
23. **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.
24. **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.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
26. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. F. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. 193:265–275.
27. **McIntyre, O. R., J. C. Feeley, W. B. Greenough III, A. S. Benenson, S. I. Hassan, and A. Saad.** 1966. Diarrhea caused by non-cholera vibrios. Am. J. Trop. Med. Hyg. 14:412–418.
28. **Moss, J., and S. H. Richardson.** 1978. Activation of adenylate cyclase by heat-labile *Escherichia coli* enterotoxin. Evidence for ADP-ribosyltransferase activity similar to that of choleragen. J. Clin. Invest. 62:281–285.
29. **Ohashi, M., T. Shimada, and H. Fukumi.** 1972. In vitro production of enterotoxin and hemorrhagic principle by *Vibrio cholerae*, NAG. Jpn. J. Med. Sci. Biol. 25:179–194.
30. **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 Ninth Joint Cholera Conference, US-Japan Cooperative Medical Science Program, Grand Canyon, Arizona, Oct. 1–3, 1973. Department of State Publication no. 8762, U.S. Department of State, Washington, D.C.
31. **Ouchterlony, Ö.** 1969. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. 26:507–515.
32. **Ouchterlony, Ö., and J. Holmgren.** (ed.). 1980. Cholera and related diarrheas. 43rd Nobel Symposium, Stockholm, Sweden, 1978. S. Karger, Basel.
33. **Singh, S. J., and S. C. Sanyal.** 1978. Enterotoxicity of the so-called NAG vibrios. Ann. Soc. Belg. Med. Trop. 58:133–140.
34. **Spicer, E. K., W. M. Kavanaugh, W. S. Dallas, S. Falkow, W. H. Konigsberg, and D. E. Schafer.** 1981. Sequence homologies between A subunits of *Escherichia coli* and *Vibrio cholerae* enterotoxin. Proc. Natl. Acad. Sci. U.S.A. 78:50–54.
35. **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.
36. **Takeda, Y., T. Takeda, T. Yano, K. Yamamoto, and T. Miwatani.** 1979. Purification and partial characterization of heat-stable enterotoxin of enterotoxigenic *Escherichia coli*. Infect. Immun. 25:978–985.
37. **Tsuji, T., S. Taga, T. Honda, Y. Takeda, and T. Miwatani.** 1982. Molecular heterogeneity of heat-labile enterotoxins from human and porcine enterotoxigenic *Escherichia coli*. Infect. Immun. 38:444–448.
38. **Tsukamoto, T., Y. Kinoshita, S. Taga, Y. Takeda, and T. Miwatani.** 1980. Value of passive immune hemolysis for detection of heat-labile enterotoxin produced by enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. 12:768–771.
39. **Yamamoto, K., Y. Takeda, T. Miwatani, and J. P. Craig.** 1981. Stimulation by lincomycin of production of cholera-like enterotoxin in *Vibrio cholerae* non-O1. FEMS Microbiol. Lett. 12:245–248.
40. **Zinnaka, Y., and C. C. J. Carpenter.** 1972. An enterotoxin produced by non-cholera vibrios. Johns Hopkins Med. J. 131:403–411.
41. **Zinnaka, Y., S. Fukuyoshi, and Y. Okamura.** 1973. Some observations on the NAG vibrio toxin, p. 116–123. *In* Proceedings of the Eighth Joint Conference on Cholera, US-Japan Cooperative Medical Science Program, Tokyo, Japan, August, 1972. National Institute of Health, Tokyo.