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Production of Cholera-Like Enterotoxin by a *Vibrio cholerae* non-O1 Strain Isolated from the Environment

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Vibrio cholerae non-O1 strain E8498, isolated in 1978 from fresh water in Louisiana, produced a vascular permeability factor when cultured in shallow resting cultures of Casamino Acids-yeast extract-glucose medium for 24 h at 30°C. Undiluted resting culture filtrates contained heat-labile permeability factor activity which was only partially neutralized by cholera antitoxin and G_{M1} ganglioside. Supernatants concentrated with PM-10 membranes caused hemorrhage and necrosis in rabbits within 1 h after intracutaneous injection, whereas appropriate dilutions of both filtrates and concentrates demonstrated delayed permeability factor activity, without hemorrhage or necrosis, which was indistinguishable in appearance from that caused by purified cholera enterotoxin produced by *V. cholerae* O1 Inaba strain 569B. Crude E8498 filtrates contained the biological equivalent of about 5 ng/ml of purified enterotoxin. Permeability factor activity in the fraction obtained by 20 to 50% saturation of filtrate concentrate with ammonium sulfate could be completely neutralized by reference standard cholera antitoxin prepared against purified 569 B enterotoxin. Hemorrhagic activity was unaffected by cholera antitoxin. A 5,000-fold concentrate of the culture supernatant yielded a line of identity with purified cholera enterotoxin in an agar gel double-diffusion test against cholera antitoxin purified by affinity column chromatography with BrCN-activated Sepharose 4B-linked purified cholera enterotoxin as the adsorbent. These findings indicate that *V. cholerae* non-O1 E8498 produces a permeability factor which is immunologically and biologically indistinguishable from that produced by a strain of *V. cholerae* O1 classical biotype.

Diarrheal disease associated with non-O1 *Vibrio cholerae* strains has been recognized for a number of years. Cases have characteristically occurred in small localized outbreaks without the dispersiveness and tendency for pandemic spread seen with O1 strains of either the classical or El Tor biotype. The disease associated with *V. cholerae* non-O1 strains has been milder and of shorter duration on the average than that associated with *V. cholerae* O1 strains, although there is marked overlap in the spectrum of severity (1, 10, 12, 16, 20, 22, 23). A number of investigators have shown that some *V. cholerae* non-O1 strains cause accumulation of fluid when live organisms or culture filtrates are injected into the ligated ileal loops of rabbits (2, 8, 17, 20, 23). Some of these investigators have also found that filtrates of some non-O1 strains contain heat-labile permeability factor (PF), which is immunologically related to cholera enterotoxin produced by O1 cholera strains (17, 24-26). On the other hand, Draškovičová et al. (8) and Čižnár et al. (4) have described a PF from non-O1 strains which is more heat resistant than cholera enterotoxin. Ohashi et al. (17) and Zinnaka et al. (26) have also reported that culture filtrates of some non-O1 strains contain a hemorrhagic (HF) factor which causes localized hemorrhage after intracutaneous injection (17). Recently Kaper et al. (13) described a large group of non-O1 *V. cholerae* strains isolated from the Chesapeake Bay and stated that 87% of these produce a substance in vitro which is toxic to Y-1 mouse adrenal cells, but these workers did not demonstrate that this material could be neutralized by cholera antitoxin.

All strains of non-O1 *V. cholerae* which produce enterotoxin-like material or PF which could be neutralized by cholera antitoxin have been isolated from samples of human diarrhea (17, 24). Culture filtrates of a few strains isolated from the environment have been shown to possess enterotoxic activity, but neutralization of this activity with cholera antitoxin was not attempted (20). In this paper, we report the production of vascular PF indistinguishable from

cholera enterotoxin by a strain of *V. cholerae* non-O1 isolated from environmental surface water in association with the 1978 outbreak of cholera in Louisiana (3).

MATERIALS AND METHODS

Bacterial strains. The following strains of *V. cholerae* were used. (i) 569B (O1; Inaba; classical biotype), originally isolated from a cholera patient, was a stock strain maintained for many years in the Department of Microbiology and Immunology, Downstate Medical Center, State University of New York, Brooklyn. (ii) Q4718 (O1; Inaba; El Tor), isolated in 1978 from a cholera patient in Dacca, Bangladesh, was kindly provided by Imdadul Huq, International Centre for Diarrheal Disease Research, Dacca, Bangladesh. (iii) 1196-78 (O1; Ogawa; El Tor), isolated in 1978 from sewage in Santos, Brazil, was received from Gil Vital Alvares Pessoa of the Adolfo Lutz Institute, São Paulo, Brazil through Betty Davis, Centers for Disease Control, Atlanta, Georgia. (iv) E8499 (non-O1; H. L. Smith, personal communication), isolated from shrimp collected in southern Louisiana in 1978, was received from Paul Blake, Centers for Disease Control. (v) E8498 (Smith type O344 [21]), isolated from surface water in the DeWitt Canal near the sites of isolation of two *V. cholerae* O1 strains associated with the 1978 outbreak of cholera in Louisiana (3), was also received from Paul Blake. Organisms were maintained on agar slants containing 1% Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and 1% NaCl (T_1N_1 medium) at room temperature under sterile paraffin oil.

Cultivation for toxin production. The medium used for toxin production was a modification of that described by Kusama and Craig (14) and had the following composition: Casamino Acids (Difco Laboratories, Detroit, Mich.), 3%; yeast extract (Difco), 0.3%; K_2HPO_4, 0.05%; and glucose, 0.2% (pH 7.0) (CAYE-glucose medium). When *V. cholerae* 569B is grown in shaking cultures of this medium for 24 h at 30°C, the enterotoxin concentration in cell-free filtrates is 12 to 18 μg/ml. Organisms were transferred from oil slants to T_1N_1 broth and incubated at 37°C for approximately 6 h, transferred to 5 ml of CAYE-glucose medium, and incubated overnight at 37°C. For toxin production, 1/100 volume of this overnight seed culture was inoculated into either Erlenmeyer or Roux flasks containing sufficient CAYE-glucose medium to yield a surface/volume ratio of 2 cm^2/ml. Cultures were incubated at 30°C for 24 h either as stationary cultures or on a reciprocal shaker at approximately 180 cycles per min. Whole cultures were centrifuged at 10,000 rpm at 4°C for 30 min, and the supernatant fluid was either treated with sodium azide (0.2 g/liter) or filtered through a 0.22-μ membrane (Millipore Corp., Bedford, Mass.).

Procedures of concentration. Supernatant fluids or filtrates were concentrated 20- to 50-fold with a PM-10 membrane (Amicon Corp., Lexington, Mass.). Filtrates were shown to contain negligible amounts of permeability factor. The retentates were rinsed and suspended in a buffer containing 0.05 M tris(hydroxymethyl)aminomethane, 1 mM ethylenediaminetetraacetic acid (EDTA) disodium salt, 3 mM NaN_3, and 0.2 M NaCl (pH 7.5) (TEAN buffer) (10). For further concentration, PM-10 retentates were treated with ammonium sulfate at 20, 50, and 65% saturation. The respective ammonium sulfate precipitates were washed and resuspended in TEAN buffer and dialyzed against approximately 50 volumes of the same buffer for 24 h at 4°C to remove the ammonium sulfate. The materials were maintained at −70°C or lyophilized and kept at 4°C until used.

Determination of protein. Protein content was determined by the method of Lowry et al. (15), employing bovine serum albumin as a standard.

Measurement of PF activity. The method for measuring PF activity was essentially that described by Craig (5, 6), with minor modifications. Materials to be assayed were diluted in borate-buffered saline of the following composition: H_3BO_3, 0.05 M; NaCl, 0.12 M; and gelatin 0.1% (pH 7.5) (BG buffer). Serial threefold dilutions were injected intracutaneously in a partially randomized pattern on the backs of albino rabbits which had been clipped with electric clippers and marked off in a grid of 100 squares with a black felt tip pen. Materials were injected in duplicate or quadruplicate in each of two rabbits for each test. After (24 h) the intracutaneous injections, the rabbits were injected intravenously with Diphenyl Brilliant Blue FF Supra (Ciba Geigy, Greensboro, N. C.), 1.2 ml of a 5% solution in 0.45% NaCl per kg. One h later, the intensity and diameter of the blue lesions in the skin were read and measured to the nearest 0.5 mm. Lesion diameters were plotted against the logarithm of the dilutions of the test material, and the toxic potency of each preparation was expressed as the number of 4-mm blueing doses (BD_4) per ml. One BD_4 was defined as the amount of toxin which produced a mean lesion diameter of 4 mm under the conditions described above.

Reference cholera enterotoxins. For routine assays, a dialyzed, freeze-dried, crude cholera culture filtrate (lot 569B-C-615-3-5) prepared from *V. cholerae* 569B, with assigned values of 1.29×10^6 BD_4/ml and 380 limit-of-blueing doses (Lb)/ml, (see below), was used as a reference toxin standard. All values of test materials were adjusted in accordance with titers of this reference toxin in each assay. One of two lots of purified cholera enterotoxin was used where indicated. (i) Purified cholera enterotoxin from *V. cholerae* 569B (lot B013) (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), containing 26 Lb/μg, was kindly supplied by N. Ohtomo (18). (ii) Purified cholera enterotoxin from *V. cholerae* 569B (lot K) (Becton-Dickinson Immunodiagnostics, Orangeburg, N.Y.), 25.8 Lb/μg was also used. One milligram of either of the purified toxins was rehydrated in 1 ml of sterile distilled water and brought to a concentration of 40 μg per ml in phosphate-buffered saline (pH 7.2) containing 1:10,000 thimerosal as the preservative and 0.1% human serum albumin as the stabilizer. These stocks were maintained at −70°C.

Standard cholera antitoxins. The following cholera antitoxins were used. (i) Purified cholera antitoxin (lot EC3(A-2/67)-B) (Swiss Serum and Vaccine Institute, Berne, Switzerland), 4,470 antitoxin units (AU)/ml, was kindly provided by John Seal, National Institute of Allergy and Infectious Diseases, Bethesda, Md.

(ii) Provisional U.S. standard cholera antitoxin (NIH lot 001 [goat]), 4,400 AU/ml, was kindly supplied by E. Seligmann, Bureau of Biologics, Food and Drug Administration, Rockville, Md. (7). (iii) Monospecific cholera antitoxin was prepared by immunoaffinity chromatography. Purified cholera enterotoxin (lot B013) was used as the immunizing antigen. A 3-kg rabbit was immunized over a period of 136 days with a series of 10 doses of 100 μg each as follows. For the first four doses, 100 μg of the toxin in 0.5 ml of phosphate-buffered saline (pH 7.0) was emulsified in an equal volume of Freund complete adjuvant (Difco). The emulsion was given in divided doses: 0.5 ml subcutaneously in the nape and 0.5 ml intramuscularly in the hind leg on days 1, 9, 16, and 23. The toxin (100 μg) was dissolved in 1 ml of TEAN buffer and given subcutaneously on days 37 and 41. The toxin (100 μg) in 1 ml of TEAN buffer was given intravenously on days 47, 78, 106, and 120. Serum was collected on day 136. Immunoaffinity chromatography of the sera was carried out as described previously (11). In brief, BrCN-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was coupled with purified cholera enterotoxin (lot B013). The immunoglobulin against purified cholera enterotoxin was eluted with 0.2 M glycine-hydrochloride buffer containing 0.5 M NaCl (pH 2.7). The monospecific antibody formed a single precipitin line against both crude and purified cholera enterotoxin. It contained 1,100 AU/ml based on 3-Lb titrations in rabbit skin; the provisional U.S. standard cholera antitoxin was used as a reference.

Neutralization of PF activity by cholera antitoxin. Materials containing PF in the appropriate dilutions in BG buffer were mixed with an equal volume of U.S. standard cholera antitoxin at a concentration of 40 AU/ml in BG buffer, incubated for 1 h at 37°C, and injected as described above.

Inhibition of PF activity by G_{M1} ganglioside. G_{M1} ganglioside was a gift of W. E. van Heyningen, Sir William Dunn School of Pathology, University of Oxford, Oxford, England. Materials containing PF in the appropriate dilutions in BG buffer were mixed with equal volume of solution of G_{M1} ganglioside containing 25 μg/ml in 0.89% NaCl. The mixtures were injected as described above without prior incubation.

Agar gel double-diffusion test. Agar gel double-diffusion tests were carried out essentially as described by Ouchterlony (19). After the precipitin lines developed, agar plates were dried and stained with Coomassie brilliant blue. The photograph was taken after nonspecific staining was eluted and decolorized.

Lb assay. A constant concentration of cholera antitoxin or G_{M1} ganglioside was mixed with varying concentrations of PF or reference toxin in 0.15-log increments and incubated, injected, and read as described above.

Estimation of HF activity. After intracutaneous injection, some crude culture filtrates evoked local hemorrhage and necrosis which appeared between 1 and 6 h after injection. Hemorrhage usually appeared as a sharply defined purpuric spot, usually surrounded by marked erythma. The center of the purpuric area sometimes exhibited a pale, nonhemorrhagic area of central necrosis. Hemorrhage and necrosis occurred both with and without an associated surrounding area of increased vascular permeability. When hemorrhage and necrosis were very marked, they often masked or suppressed the PF effect.

RESULTS

To distinguish the increased PF caused by cholera enterotoxin from that caused by nonspecific PF found in culture filtrates of some nontoxinogenic and hypotoxinogenic strains of *V. cholerae*, we devised a screening test which utilized heat, G_{M1} ganglioside, and specific cholera antitoxin as inhibitors of cholera enterotoxin activity. After preliminary titration was carried out to estimate BD_4/ml, crude culture filtrates diluted to contain approximately 800 BD_4/ml (40 BD_4, or the equivalent of ca. 0.4 ng per injection site) were incubated with either G_{M1} ganglioside or cholera antitoxin as described above or boiled for 15 min. Filtrates containing less then 800 BD_4/ml were tested undiluted. The mixtures were tested in rabbit skin as described above, and blueing scores for each mixture were calculated by multiplying the mean blueing diameter by the mean blueing intensity, as estimated on an arbitrary scale of 1 to 8. The results of screening of some representative strains are given in Table 1. Data from *V. cholerae* 569B (O1, Inaba) showed that blueing was almost totally abolished by the three modifiers. Similarly, the blueing score produced by *V. cholerae* Q4178, isolated from a typical case of cholera, was reduced 7- to 15-fold by heat, G_{M1} ganglioside, and cholera antitoxin, although more background PF activity remained than in the case of the 569B resting filtrate. In contrast, PF activities in the filtrates of the environmental isolates *V. cholerae* 1196-78 and E8499 were not significantly or consistently reduced by any of these treatments. *V. cholerae* E8498 exhibited still a different pattern. Although the blueing score was markedly reduced by G_{M1} ganglioside and cholera antitoxin, considerable residual PF activity remained, especially in the filtrates of the shaken culture. The data were therefore consistent with the hypothesis that *V. cholerae* E8498 produced both a cholera enterotoxin-like PF as well as another PF which was not affected by G_{M1} ganglioside or cholera antitoxin. It is possible that the other noncholera PF was also responsible for the minor non-neutralizable blueing caused by filtrates of *V. cholerae* Q4178, 1196-78, and E8499.

On the basis of similar observations of a number of strains, we have tentatively and arbitrarily postulated that a strain is probably toxinogenic (i.e., capable of producing a cholera-like enterotoxin) if the control score in this screening assay is fourfold or greater than the scores produced

TABLE 1. *PF blueing scores of representative strains of V. cholerae*

Strain	Serovar[a]	Culture condition[b]	Final dilution	Blueing score[c]				
				Control	Boiled	G_{M1}[d]	CAT[e]	CT[f]
569B	O1/Inaba (Classical)	R	1:2	36	0.3	0.2	0.1	+
		S	1:3,200	64	0.6	1.0	0.3	+
Q4178	O1/Inaba (El Tor)	R	1:2	52	3.9	6.8	3.1	+
1196-78	O1/Ogawa (El Tor)	R	1:2	3.9	1.8	3.8	5.9	−
		S	1:2	0.4	0.5	0.5	2.8	−
E8499	Non-O1	R	1:2	6.4	12	5.3	5.9	−
		S	1:2	9.6	11	6.3	6.4	−
E8498	O344	R	1:2	51	3.3	12	10	+
		S	1:2	44	13	21	18	±[f]

[a] Biotype indicated in parentheses.
[b] R, Resting culture; S, shaken culture.
[c] Product of mean lesion diameter (mm) and intensity.
[d] With G_{M1} ganglioside, 25 μg/ml.
[e] CAT, Cholera antitoxin; NIH lot 001; 40 AU/ml.
[f] CT, Cholera toxin; +, present; −, absent; ±, equivocal.

by a heat-, G_{M1} ganglioside- or cholera antitoxin-treated filtrate. Other strains were considered to be non-toxinogenic.

Thus, *V. cholerae* 1196-78 and E8499 were considered to be non-toxinogenic, whereas *V. cholerae* E8498 was considered to be toxinogenic. These designations are shown in the CT column in Table 1. Because of the fact that *V. cholerae* E8498 is a non-O1 strain isolated from surface water, it was of interest to test this hypothesis by attempting to characterize further the PF which *V. cholerae* E8498 produces.

Crude filtrates of a series of resting cultures of *V. cholerae* E8498 in CAYE-glucose medium contained an average of about 500 BD_4 of PF/ml. PF activity was demonstrable at higher dilutions than HF activity. Therefore, at higher dilutions, HF caused no masking or inhibition of the permeability effect. Since PF in crude filtrates occurred in low titers, however, the crude supernatants of both resting and shaken cultures were concentrated on PM-10 membranes. Filtrates contained no detectable PF or HF, whereas retentates contained both. Resting cultures contained greater amounts of both PF and HF than did shaken cultures.

To ascertain the optimal cultural conditions for PF production, we grew *V. cholerae* E8498 in both deep and shallow resting cultures with surface/volume ratios of 0.11 and 2.0 cm^2/ml, respectively. Shallow cultures produced more than 10 times as much PF, as well as proportionately more preblueing erythema. The PF in a 20-fold concentrate of shallow culture filtrate containing approximately 10,000 BD_4/ml was totally inactivated when heated at 56°C for 30 min. Basing our determination on the specific activity of purified cholera enterotoxin, we found that this concentrate contained the activity equivalent to 100 ng of PF/ml. It could therefore be estimated that the original shallow culture supernatant contained about 5 ng/ml of cholera enterotoxin-like equivalent.

Standard cholera antitoxin at a final concentration of 10 AU/ml neutralized all PF activity in the concentrate, which contained the equivalent of 100 ng/ml. As a point of reference, 1 AU of cholera antitoxin neutralizes 40 ng of purified cholera enterotoxin. Standard cholera antitoxin had no significant effect upon HF activity but did markedly reduce the preblueing erythema, suggesting that the erythema was largely associated with PF activity. HF activity was reduced 85 to 90% when the 20-fold concentrate was heated at 56°C for 30 min.

To characterize further the relationship between the PF produced by *V. cholerae* E8498 and cholera enterotoxin, we compared them in an agar gel double-diffusion plate against the monospecific immunoaffinity-purified rabbit cholera antitoxin described above. A 50-fold PM-10 concentrate of culture supernatant of *V. cholerae* E8498 was further concentrated to about 5,000-fold by vacuum dialysis in a collodion bag. Purified cholera enterotoxin derived from *V. cholerae* 569B (lot B013) was applied at a concentration of 25 μg/ml. The antitoxin contained 1,100 AU/ml. The results are shown in Fig. 1. A single line of identity developed. From the identity and symmetry of the precipitin lines, we estimated that the original crude supernatant of *V. cholerae* E8498 contained about 5 ng of

cholera-like enterotoxin per ml, which was the same amount as that estimated by comparing PF produced by *V. cholerae* E8498 filtrates and purified cholera enterotoxin.

For comparing further the PF from *V. cholerae* E8498 with cholera enterotoxin, a large batch (35 liters) of shallow resting culture of *V. cholerae* E8498 in 4-liter Erlenmeyer flasks containing CAYE-glucose medium was prepared and concentrated approximately 25-fold with PM-10 membranes. The concentrate was fractionated by ammonium sulfate precipitation at 20, 50 and 65% saturation, and the precipitates were dialyzed against TEAN buffer. The fractions were maintained at 4°C during preliminary testing and then lyophilized for confirmatory assays. Just before being tested, they were rehydrated in distilled water to their original volume and then diluted in BG buffer for rabbit skin testing. The fractions were tested for PF activity against reference standard cholera enterotoxin. The recovery of PF activity in one of three such sequential assays is shown in Table 2. Nearly 90% of the PF activity was recovered in the PM-10 retentate, and 77% of the PF activity was recovered in the four ammonium sulfate fractions. The bulk of the PF activity was concentrated in the 20 to 50% ammonium sulfate fraction. HF in the PF-rich, 20 to 50% ammonium sulfate fraction was markedly reduced below that expected if HF had retained its potency relative to PF content. It is not known whether HF is a relatively unstable material as compared with PF or whether it was distributed into other ammonium sulfate fractions.

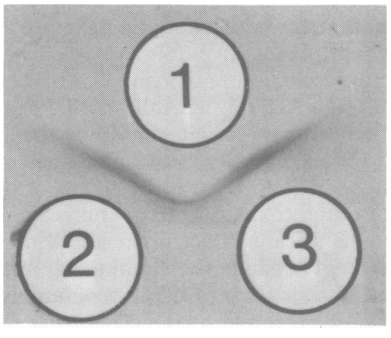

FIG. 1. *Agar gel double-diffusion test of the concentrated supernatant of V. cholerae E8498 and purified cholera enterotoxin. (1) Monospecific immunoaffinity-purified rabbit cholera antitoxin. (2) 5,000-fold concentrate of the supernatant of V. cholerae E8498. (3) Purified cholera enterotoxin (lot B013).*

Studies to compare biological and immunological activities of PF from *V. cholerae* E8498 and cholera enterotoxin were done by using the 20 to 50% ammonium sulfate fraction. Lb titrations in rabbit skin were done to compare the quantitative neutralizations of both toxins by monospecific cholera antitoxin. For purposes of comparison, the concentrate from *V. cholerae* E8498 was assigned a specific activity based on simultaneous BD_4 titrations of purified cholera enterotoxin. Serial 0.15-log dilutions of both toxins were mixed with a constant amount of cholera antitoxin containing 1 AU/ml. The mixtures were injected in rabbit skin, and the PF activity was measured as described above. Each injection site thus received 1/20 AU, and the titration is therefore referred to as an Lb/20 titration. The results are shown in Fig. 2. The neutralization curves were nearly identical when the toxins were adjusted to identity on the basis of BD_4 titrations. The results show that the ratios between direct toxic activity and neutralizability by cholera antitoxin are the same for the two toxins. This would suggest that the ratio between the toxic moiety of subunit A and the antitoxin-binding components of subunit B is the same in both toxins. In theory, molecules containing different proportions of antitoxin-binding subunit should not yield identical BD_4/Lb ratios.

The effects of G_{M1} ganglioside on PF from *V. cholerae* E8498 and purified cholera enterotoxin were also compared. Experiments with graded fivefold doses of G_{M1} ganglioside against a constant dose of both toxins showed that a concentration of 150 ng/ml of G_{M1} ganglioside was required to neutralize the test dose of 10 ng of

TABLE 2. *Recovery of PF activity in ammonium sulfate fractions of culture supernatant of V. cholerae E8498*

Fraction	Volume (ml)	PF (BD_4/ml)	Total PF (BD_4)	Percent recovery of PF
Original supernatant	35,625	2,040	72,700,000	100
PM-10 retentate	1,425	45,400	64,700,000	89
Ammonium sulfate				
0–20%	11.8	11,300	130,000	0.2[a]
20–50%	75	600,000	45,300,000	70.0[a]
50–65%	25.7	3,740	96,000	0.2[a]
>65%	2,948	1,490	4,390,000	6.8[a]

[a] Based on PF in PM-10 retentate.

purified cholera toxin, whereas only 15 ng of G_{M1} ganglioside per ml was required to achieve the same degree of neutralization of a biologically and immunologically equivalent amount of the PF from *V. cholerae* E8498. This relationship was examined in reverse by mixing graded 0.15-log doses of the two toxins against a constant dose of G_{M1} ganglioside in the expected neutralizing concentrations. The results are shown in Fig. 3. The titration of toxic activity alone is shown on the right. The BD_4 for purified cholera enterotoxin was 9.8 pg. All curves for PF from *V. cholerae* E8498 in Fig. 3 were therefore adjusted to the assigned PF value of 9.8 pg/BD_4. The cholera antitoxin neutralization curves against the standard U.S. cholera antitoxin (lot 001) again were nearly identical. Lb values for cholera enterotoxin and PF from *V. cholerae* E8498 were 40 and 35.5 ng, respectively. The G_{M1} ganglioside curves were nearly identical when G_{M1}, at a concentration of 150 ng/ml, was mixed with purified cholera enterotoxin and when G_{M1}, at 15 ng/ml, was mixed with PF from *V. cholerae* E8498, thus confirming the earlier observation.

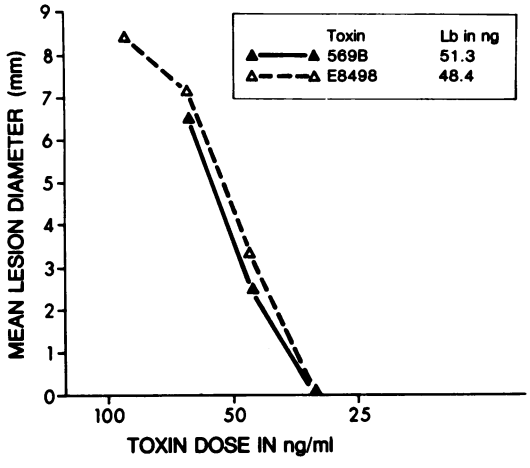

FIG. 2. *Lb/20 titrations of the ammonium sulfate-concentrated supernatant of V. cholerae E8498 (20 to 50% ammonium sulfate fraction) and purified cholera enterotoxin (569B; lot K) against monospecific immunoaffinity-purified cholera antitoxin at 1 AU/ml. The values for E8498 toxin were plotted in ng/ml based on the biological equivalence derived from simultaneous titration with purified cholera enterotoxin.*

DISCUSSION

Our data suggest that non-O1 *V. cholerae* strain E8498 produces a PF which is biologically and immunologically indistinguishable from that produced by O1 classical strain 569B, except for a quantitative difference in G_{M1} ganglioside binding activity. Other investigators (17, 24) have shown that culture filtrates of non-O1 strains isolated from patients with diarrhea may contain PFs which are immunologically related to cholera enterotoxin. We believe, however, that this is the first report of the production of a PF identical or closely related to cholera enterotoxin by a strain of non-O1 *V. cholerae* which was isolated from the environment.

Zinnaka and Carpenter (24) and Zinnaka et al. (25, 26) found that several strains of non-O1

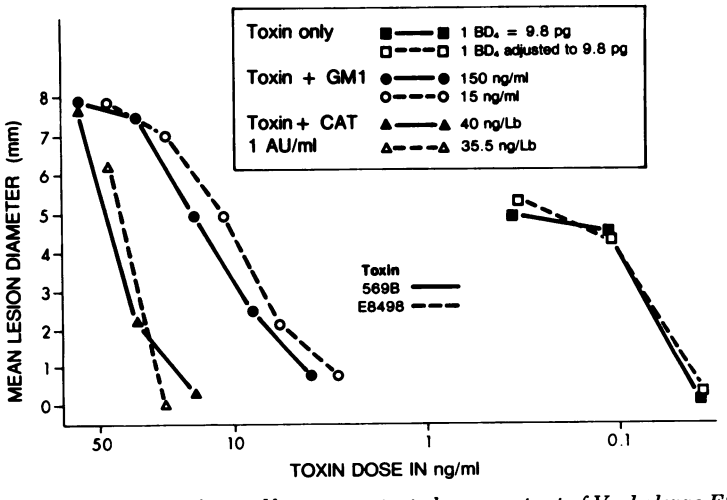

FIG. 3. *PF activities of the ammonium sulfate-concentrated supernatant of V. cholerae E8498 and purified cholera enterotoxin and their neutralization by cholera antitoxin (CAT) and G_{M1} ganglioside. The values for E8498 toxin were plotted in ng/ml based on the biological equivalence derived from simultaneous titration with purified cholera enterotoxin.*

V. cholerae isolated from human diarrhea cases in the Sudan produced small amounts of PF in vitro. Crude filtrates of one of these strains, S2, contained a PF which was not completely neutralized by antitoxin prepared against purified 569B enterotoxin, whereas purified S2 PF was completely neutralized by the same antitoxin. This finding is consistent with our results with E8498 PF and suggests that both S2 and E8498 filtrates contain a PF which is identical to cholera enterotoxin (enterotoxin PF) together with one or more other nonspecific PFs which are antigenically distinct from cholera enterotoxin. These nonspecific PFs are present in small amounts and can be detected only at low dilutions of filtrates. They are therefore more apt to be recognized in filtrates of relatively hypotoxinogenic strains of *V. cholerae* which must be tested at low dilutions to detect the true enterotoxin PF present.

In spite of the cross-neutralization studies which indicated a close immunological relationship between the PFs of S2 and 569B, Zinnaka et al. (25, 26) were not able to consistently show evidence of identity in agar gel double-diffusion studies. On the other hand, our tests with E8498, using a monospecific immunoaffinity-purified cholera antitoxin, indicated identity between purified 569B toxin and one component of the E8498 filtrate. This difference may suggest that E8498 and S2 contain related but different PFs.

Ohashi et al. (17) also demonstrated PF in crude culture filtrates of several strains of non-O1 *V. cholerae* isolated from human diarrheal cases in India, the Philippines, and Sudan. PF from one of the strains isolated in the Philippines, P-4, was studied in detail. It was completely neutralized by antitoxin prepared against crude 569B toxin but was only partially neutralized by antitoxin prepared against 569B choleragenoid. This led them to conclude that P-4 and 569B toxins are closely related but not identical.

Both Zinnaka and Carpenter (24) and Ohashi et al. (17) have also described an HF in culture filtrates of a number of the non-O1 *V. cholerae* strains which they studied. Although no detailed studies of this HF have yet been made, it seems likely that HF produced by strain E8498 is similar or identical to the one which they have described.

V. cholerae E8498 is typical of a number of hypotoxinogenic O1 and non-O1 strains in that undiluted filtrates evoke confusing skin lesions, often with hemorrhage and necrosis. The fact that this strain caused blueing which was not totally neutralizable by cholera antitoxin or G_{M1} ganglioside in the original screening test but proved to be capable of producing cholera PF suggests that screening of strains by this method may be useful in recognizing other strains which produce small amounts of enterotoxin, and in distinguishing them from non-toxinogenic strains.

Variability in HF production from batch to batch may occur with HF-producing cholera vibrios. Since HF may often mask or inhibit the effect of PF in skin, this kind of variability may add to the difficulty and lack of agreement in determining whether a given strain produces cholera-like enterotoxin or not. This is no problem in strains which produce $\geqq$10 ng of enterotoxin per ml in crude filtrates, since HF can usually be diluted out for skin testing. Since HF may be related to the cytotoxic factors which frequently cause difficulty in interpreting the results of toxin testing in cell cultures, the same variability in HF production could lead to confusion in those assays as well. Therefore, G_{M1} ganglioside and cholera antitoxin neutralization may also be helpful in increasing the specificity of cell culture assays.

The difference between G_{M1} ganglioside binding activities of 569B and E8498 PF remains unexplained. Since no comparisons have yet been made concerning G_{M1} ganglioside binding activity of PFs from other strains of cholera vibrios, it would be premature to suggest that this difference is related to serovar. The observation does suggest, however, that all cholera enterotoxins may not be alike in their binding ratios with G_{M1} ganglioside. It is possible that E8498 and 569B toxins differ in either the number or affinity of G_{M1} ganglioside binding determinants. It is also possible that crude toxin contains factors which alter enterotoxin-G_{M1} interaction. The purification of enterotoxin from strain E8498 is now in progress. Future studies with this purified material should help clarify the relationship between the enterotoxins produced by these two strains, including the difference in enterotoxin-G_{M1} interaction which we have observed when we used crude material.

The question arises concerning the significance of the production of such seemingly small amounts of PF, even if it is identical to cholera enterotoxin. One may ask whether strains such as *V. cholerae* E8498 should be considered potentially pathogenic. It is likely that many such strains will be found in the environment. One can only cite the fact that a number of *V. cholerae* El Tor strains which produce no more PF in vitro than *V. cholerae* E8498 does have recently been isolated from cholera cases associated with severe diarrhea. Preliminary results with the screening test indicate that these strains are hypotoxinogenic: the slight PF activity is abolished by heat, cholera antitoxin, and G_{M1} ganglioside. On the other hand, undiluted fil-

trates of some El Tor strains isolated from the environment have produced marked PF as well as HF in vitro, but none of this activity was affected by cholera antitoxin or G_{M1} ganglioside and was only slightly affected by heat. These are tentatively considered to be non-toxinogenic (unpublished data). In general, these results, together with those we have reported concerning *V. cholerae* E8498, suggest that strains which are hypotoxinogenic in vitro may very well produce enough toxin in vivo to cause disease and that although toxin is probably required to produce disease, very little may be needed to evoke severe diarrhea. Other factors associated with colonization in the gut may be much more important in determining whether a strain is ultimately pathogenic.

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LITERATURE CITED

1. **Aldová, E., K. Lázničková, E. Štěpánková, 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. Ghosh.** 1971. Permeability and enterotoxic factors of nonagglutinable vibrios *Vibrio alcaligenes* and *Vibrio parahaemolyticus*. Appl. Microbiol. **22**:1159–1161.
3. **Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barrett, 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. New Engl. J. Med. **302**:305–309.
4. **Čižnár, I., M. Draškovičová, A. Hoštacká, and J. Karolček.** 1977. Partial purification and characterization of the NAG vibrio enterotoxin. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **239**:493–503.
5. **Craig, J. P.** 1965. A permeability factor (toxin) found in cholera stool and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) **20**:614–616.
6. **Craig, J. P.** 1971. Cholera toxins, p. 189–254. *In* S. Kadis, T. Montie, and S. J. Ajl (ed.), Microbial toxins, vol. 2A. Academic Press, Inc., New York.
7. **Craig, J. P.** 1978. Toward the development of a standard reference cholera antitoxin. Report of the Ad Hoc Committee of the U.S. Cholera Panel. Dev. Biol. Stand. **41**: 415–422.
8. **Draškovičová, M., J. L. Karolček, and D. Winkler.** 1977. Experimental toxigenicity of NAG vibrios. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg., Abt. 1 Orig. Reihe A **237**:65–71.
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. **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.
11. **Honda, T., S. Taga, Y. Takeda, and T. Miwatani.** 1981. Modified Elek test for detection of heat-labile enterotoxin of enterotoxigenic *Escherichia coli*. J. Clin. Microbiol. **13**:1–5.
12. **Hughes, J. M., D. G. Hollis, E. J. Gangarosa, and R. E. Weiner.** 1978. Non-cholera vibrio infections in the United States. Ann. Int. Med. **88**:602–606.
13. **Kaper, J., H. Lockman, R. R. Colwell, and S. W. Joseph.** 1979. Ecology, serology, and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. Appl. Environ. Microbiol. **37**:91–103.
14. **Kusama, H., and J. P. Craig.** 1970. Production of biologically active substances by two strains of *Vibrio cholerae*. Infect. Immun. **1**:80–87.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **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.
17. **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.
18. **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, U.S.-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.
19. **Ouchterlony, O.** 1969. Antigen-antibody reactions in gels. Acta Pathol. Microbiol. Scand. **26**:507–515.
20. **Singh, S. J., and S. C. Sanyal.** 1978. Enterotoxicity of the so-called NAG vibrios. Ann. Soc. Belge Med. Trop. **58**:133–140.
21. **Smith, H. L., Jr.** 1979. Serotyping of non-cholera vibrios. J. Clin. Microbiol. **10**:85–90.
22. **Spira, W. M., R. R. Daniel, Q. S. Ahmed, A. Huq, A. Yusuf, and D. A. Sack.** 1979. Clinical features and pathogenicity of O group 1 non-agglutinating *Vibrio cholerae* and other vibrios isolated from cases of diarrhea in Dacca, Bangladesh, p. 137–154. *In* K. Takeya and Y. Zinnaka (ed.), Proceedings of the Fourteenth Joint Conference on Cholera, U.S.-Japan Cooperative Medical Science Program, Karatsu, Japan, Sept. 27–29, 1978. The Japanese Cholera Panel, Toho University, Tokyo.
23. **Zakhariev, Z., T. Tyufekchiev, V. Valkov, and M. Todeva.** 1976. Food poisoning caused by parahaemolytic and NAG vibrios after eating meat products. J. Hyg. Epidemiol. Microbiol. Immunol.(Praha) **21**:150–156.
24. **Zinnaka, Y., and C. C. J. Carpenter.** 1972. An enterotoxin produced by non-cholera vibrios. Johns Hopkins Med. J. **131**:403–411.
25. **Zinnaka, Y., and S. Fukuyoshi.** 1974. Further observations on the NAG vibrio toxin, p. 61–81. *In* Proceedings of the Ninth Joint Cholera Conference, U.S.-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.
26. **Zinnaka, Y., S. Fukuyoshi, and Y. Okamura.** 1973. Some observations on the NAG vibrio toxin, p. 116–123. *In* H. Fukumi and M. Ohashi (ed.), Proceedings of the Eighth Joint Conference on Cholera, U.S.-Japan Cooperative Medical Science Program, Tokyo, Japan, August, 1972. The Japanese Cholera Panel, National Institute of Health, Tokyo.