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A NEW ENTEROTOXIN PRODUCED BY VIBRIO CHOLERAE 01

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Abstract

Isolation from diverse environmental sources of *Vibrio cholerae* O1 that lack the cholera toxin (CT) gene has encouraged researchers to use them, or CT gene-deletion mutant strains, as potential candidates for a live oral cholera vaccine. We examined 13 such strains in greater detail, using various enterotoxin assay systems, to determine whether they do completely lack the capability to produce any toxin(s). Live cells of all these strains caused significant accumulation of fluid in ligated adult rabbit ileal loops and diarrhoea in infant rabbits. Similar results were obtained using their culture filtrates. The filtrates also increased capillary permeability of rabbits' skin, and blueing was accompanied by blanching or necrosis. Suckling mice assays were negative for all test materials. The culture filtrates lost their loop and skin toxic activities when held at 56°C for 30 min. However, the toxin could not be neutralized by antisera against CT or its A and B subunits in rabbits' skin and ileal loop assays. The culture supernates did not cause cytotonic effects on Chinese hamster ovarian and mouse adrenal cells; and did not bind to GM_1 ganglioside, in enzyme-linked immunosorbent assay. When tested against anti-CT, no precipitin band was observed in Ouchterlony's gel-diffusion technique with any of the concentrated culture filtrates. The strains did not show any homology when retested with CT or LT probes. Thus, this study indicates that these *V. cholerae* O1 strains produce a toxin not previously recognized. This new toxin seems to differ from the known CT in antigenic nature, receptor site, mode of action and genetic homology. Before embarking upon a direct vaccine development program, this toxin requires further immuno-biologic and genetic studies. The general belief about the non-toxic nature of environmental *V. cholerae* isolates also may be a myth.

Key words : Vibrio cholerae; Cholera toxin; Enterotoxins.

Introduction

Isolation of non-toxigenic *Vibrio cholerae* O1 strains from diverse environmental sources, such as sewage, oysters and brackish water, has been reported from Bangladesh, Brazil, Guam, Great Britain, India, Japan, the United States, the USSR and Australia (1). Such strains also were isolated from human intestinal and extra-intestinal infections. Many of them failed to demonstrate any homology with *Escherichia coli* heat-labile toxin (LT) and cholera toxin (CT) genes (2,3). These strains, therefore, had not been recognized as a cause of diarrhoea—even though, for almost a decade, numerous cases of acute diarrhoea had been attributed to unexplainable contamination of the very types of marine environments where the mutant *V. cholerae* 01 varieties were known to exist. Especially in the United States, Western Europe and Australia, which have been free of cholera epidemics for decades, minor cholera outbreaks were thought to have been caused by bacteria that somehow had been imported to the area. No one believed the bacteria to be indigenous. This was the case with minor outbreaks in Louisiana and Texas that began in 1973, continued each year, and culminated in a total of 21 cases in 1981 alone (4). The first scientific report to the contrary appeared in 1980 (5), concerning a mini-outbreak in Louisiana in 1978. There, the disease was traced to cooked crabs from local marshes. Investigators concluded that the responsible bacteria had been indigenous to the area for at least eight years. Nevertheless, most scientists did not connect this finding with the toxin gene-deficient, mutant cholera strains found in the marine environments. On the other hand, the genetic evidence for the absence of CT or LT genes in these strains has encouraged researchers to use them—or laboratory tailored CT gene-deletion mutant strains of *V. cholerae* O1 possessing other functions, such as the ability to colonize the small intestine—as potential candidates for a live oral cholera vaccine (2,6,7). These developments prompted us to

examine some of these strains in greater detail, using various animal, tissue culture and immunological assays. The aim was to determine whether these strains completely lack the ability to produce any toxin. We observed that these strains elaborate an enterotoxin that has not been previously recognized, and is different from the known CT (8).

Materials and methods

Organisms

Thirteen strains of *V. cholerae* O1, isolated from various sources in different countries (Table) and that failed to demonstrate any homology with CT or LT genes, were supplied by J.B. Kaper of the Centre for Vaccine Development, University of Maryland School of Medicine, and by R.M. Twedt, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio, U.S.A. These strains were isolated from such environmental sources as sewage, oysters and brackish water, as well as from intestinal and other human infections. The toxigenic *V. cholerae* strain 569B came from reference strains stored at the ICDDR,B. All the strains were obtained in freeze-dried form. A non-pathogenic *Escherichia coli* strain 265 was included to serve as a negative control. The strains were grown on gelatin agar, and were maintained in peptone agar stab cultures during the course of the study.

Antisera

Antisera against pure CT and its A and B subunits were supplied by J. Holmgren and Ann-Marie Svennerholm of the Institute of Medical Microbiology, University of Göteborg, Sweden.

Preparation of culture filtrates

Five or six smooth colonies from cultures of the test and control strains, grown overnight on gelatin agar, were inoculated into 10 ml of Richardson's medium (9) contained in 50 ml conical flasks. The flasks were incubated at 37°C in a shaking water bath with 80-120 oscillations per min for 18 h; cultures were centrifuged at 4°C for 30 min at 22,000 x g; supernates were filtered through millipore membranes of 0.22 μ average pore diameter; and finally were stored at –20°C in different aliquots for use in different tests.

Culture filtrates also were prepared in syncase medium (10) using 5 of the strains selected randomly, following the same procedures as above. The filtrates then were slowly saturated with ammonium sulfate in the cold, with constant stirring. The precipitates then were dialysed at 4°C against phosphate-buffered saline (PBS), pH 7.4 with repeated changes, until the precipitates were free of ammonium sulfate. The dialysed materials were reconstituted to the original volumes with PBS.

Ileal-loop test

Live cells and culture filtrates of the 13 CT-negative strains, along with those of the known toxigenic *V. cholerae* strain 569 B and the nontoxic *E. coli* strain 265, were tested in ileal loops of adult albino rabbits (New Zealand strain) weighing 1.5-2.0 kg, following the method described earlier (11). Live cells were administered in doses of about 10^6 colony-forming units (c.f.u.), and for sterile culture filtrates there was 1 ml per loop. The two preparations were tested in different sets of experimental animals. Usually, 6 loops were tied in one rabbit, and the animals were sacrificed after 18 h. Each strain/culture filtrate was tested in at least 2 rabbits. The results were expressed as the range of fluid accumulated in ml/cm in different rabbits.

Passage in ileal loops

Four of the CT gene-negative strains, as well as *V. cholerae* strain 569B and the *E. coli* strain 265, were given serial passages in ileal loops (12). Briefly, the strains causing little or no fluid accumulation were sub-cultured aseptically from the gut loops onto GA plates, were incubated overnight at 37°C; and 5 or 6 smooth colonies of each strain were grown for 3-4 h in trypticase soy broth (BBL), and were diluted 10^{-2} in PBS, before 1 ml amounts were inoculated again into loops. This process was repeated until good positive responses were obtained.

Skin PF of culture filtrates

To test the skin permeability factor (13) activity (PF) of the culture filtrates, the hairs were removed by an electric clipper from the dorsal and lateral surfaces of adult albino rabbits, taking precaution not to damage the skin. Two hours later, culture filtrates in 0.1 ml amounts were inoculated intradermally at premarked sites, in dilutions of 1:2, 1:10 and 1:100, in gel borate buffer (H_3BO_3, 3.09 g; gelatin, 0.198 g; NaCl, 7.01 g; NaOH, 0.8 g; distilled water, 1000 ml; pH, 7.5). The presence of induration and necrosis was examined after 22 h,

and 5% Pontamine sky blue in half normal saline was administered intravenously in a dose of 1.2 ml/kg of body weight. One h after the dye was used, the dorsal and lateral surfaces of the experimental rabbits were smeared with liquid paraffin to measure the blueing zones; and the results were expressed as the mean cross-diameter of the reaction. A blueing zone of 7 mm or more in diameter (BD_7) indicated a positive reaction (13). The inoculated areas also were examined for signs of blanching and necrosis. After the results were noted, the skin flap of the dorsal surface was everted from the shoulder down to the waist, and blueing zones again were measured on that surface. The experiments always were done in duplicate. Culture filtrates of *V. cholerae* strain 569B and gel borate buffer served as positive and negative controls, respectively.

Infant rabbit test

Eight-to-eleven-day-old suckling rabbits were administered about 10^{6-7} c.f.u. of each strain intraduodenally on laparotomy, under a light ether anesthesia (14). Until about 18 h, they were observed for soiling of the perinea and the ventral surface, as well as for dehydration. The infant rabbits then were sacrificed with an excess dose of anesthetic ether, the abdomens were opened, and distention of the intestines with fluid was noted. *V. cholerae* strain 569B and *E. coli* strain 265 served as positive and negative controls, respectively. Each strain was tested in 2 animals.

Suckling mice assay

All the culture filtrates were tested by this assay (15). Two-to-three-day-old Swiss albino mice were separated from their mothers shortly before use, and were divided randomly into groups of four. Culture filtrates in 0.1 ml amounts, mixed with 2 drops of 2 percent Evans blue dye per ml, were inoculated through the body wall directly into the milk-filled stomach of each mouse. Culture filtrates of known positive and negative *E. coli* strains always were included as controls. The animals were kept at 28°C for 4 h, and then were sacrificed with chloroform. The abdomens were opened with forceps, and the intestines were examined for distention before being removed. The intestines from all four mice were weighed together, and the ratio of gut weight to remaining body weight was calculated. Results from mice showing no dye in the intestine at autopsy were discarded, and the test was repeated.

CHO and Y_1 assays

Chinese hamster ovarian (16) and mouse adrenal cell culture (17) assays were done with all the culture filtrates, in original and in 1:10 dilutions in microtiter plates.

GM_1 ELISA assay

Ganglioside GM_1 enzyme-linked immunosorbent assays (18) were performed with cell-free culture supernates of the strains.

Effect of temperature on culture filtrates

The culture filtrates were held at 56°C for 30 min, 65°C for 15 min and 100°C for 10 min; and then were tested for ligated gut loop and skin PF assays, as described earlier.

Neutralization tests

Attempts were made to neutralize enterotoxic activity of the culture filtrates with anti-CT in ligated ileal loops (19, 20), and also with anti-A and anti-B subunits in rabbits' skin (21, 22). Two-fold dilutions of the culture filtrates of *V. cholerae* strain 569B were titrated, to obtain the dose that would cause fluid accumulation of about 1 ml/cm of gut loop. That dose in a 0.5 ml amount was mixed with equal volumes of two-fold dilutions of anti-CT in PBS, and was incubated at 37°C in a water bath for 30 min. These mixtures then were tested in ileal loops, to obtain the highest dilution of antitoxin that would cause complete neutralization of toxin activity. Normal rabbit serum, inactivated at 56°C for 30 min, mixed with toxin and treated similarly, served as a positive control. In a similar way, four times the highest dilution of the anti-CT that caused complete inactivation of the *V. cholerae* strain 569B culture filtrate was used to neutralize the enterotoxic activity of the CT gene-negative strain X-392.

The dilutions of anti-CT, anti-A and anti-B that completely neutralized the BD_7 of the culture filtrates of *V. cholerae* strain 569B were determined. Four times these dilutions were treated similarly to the procedure used in the loop neutralization test with the BD_7 of the culture filtrates of all the test and control strains. These mixtures were tested in rabbits in duplicate. Culture filtrates in similar dilutions also were mixed with normal rabbit serum, to serve as controls. The *V. cholerae* strain 569B culture filtrate and the gel borate buffer unmixed

with any serum were included in each rabbit, to check skin reactivity.

Gel-diffusion test

The culture filtrates of the CT probe-negative strains were concentrated 20 times, using polyethylene glycol 4000 (J.T. Baker, U.S.A.); and were tested against serially diluted (1:2, 1:8, 1:16 and 1:32) anti-CT, in Ouchterlony's gel-diffusion test.

Results

Ileal loop test

Both live cells, with inocula of about 10^6 c.f.u., and culture filtrates of a majority of the strains tested caused accumulation of fluid in rabbit gut loop, comparable to that of the toxigenic *V. cholerae* strain 569B (Table, Fig. 1). Some of the strains, however, induced relatively smaller amounts of fluid accumulation, compared to those of *V. cholerae* 569B. Strain-to-strain and loop-to-loop variations were observed in the amounts of fluid accumulated. The dialysed ammonium sulfate precipitates of the five strains' culture filtrates grown in syncase medium also caused fluid accumulation. The nontoxigenic *E. coli* 265, the isotonic saline and Richardson's medium never elicited a fluid outpouring response.

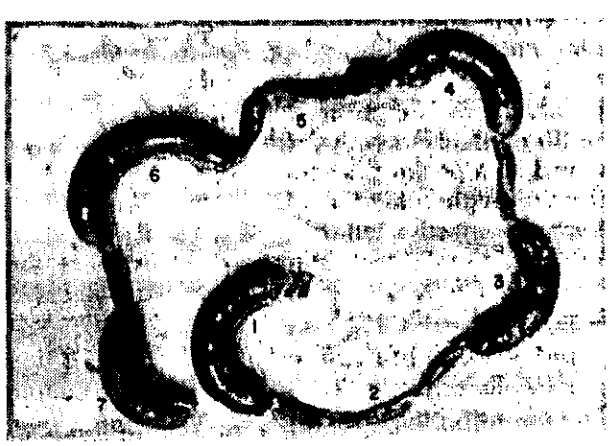

Fig. 1—Fluid accumulation in rabbit ileal loops after 18 h of inoculation with culture filtrates of CT gene-negative Vibrio cholerae O1 strains. Loops 3,4,6 and 7 were inoculated with culture filtrates of test strains, loop 1 with CT-positive V. cholerae 569B, loop 5 with nontoxic E. coli 265, and loop 2 with Richardson's medium.

D.A. Sack of Baltimore City Hospital, U.S.A., retested in his laboratory five of the strains selected randomly, and confirmed our results in this assay system.

Passage in ileal loops

Accumulation of fluid was enhanced on consecutive passages in ileal loops. For the strains tested, fluid almost doubled after each passage; and, on two passages, the amounts either exceeded or reached 2 ml/cm of gut (Fig. 2).

The passaged strains, along with the originals, were retested with CT and LT gene probes, by J.B. Kaper, Center for Vaccine Development, Uni-

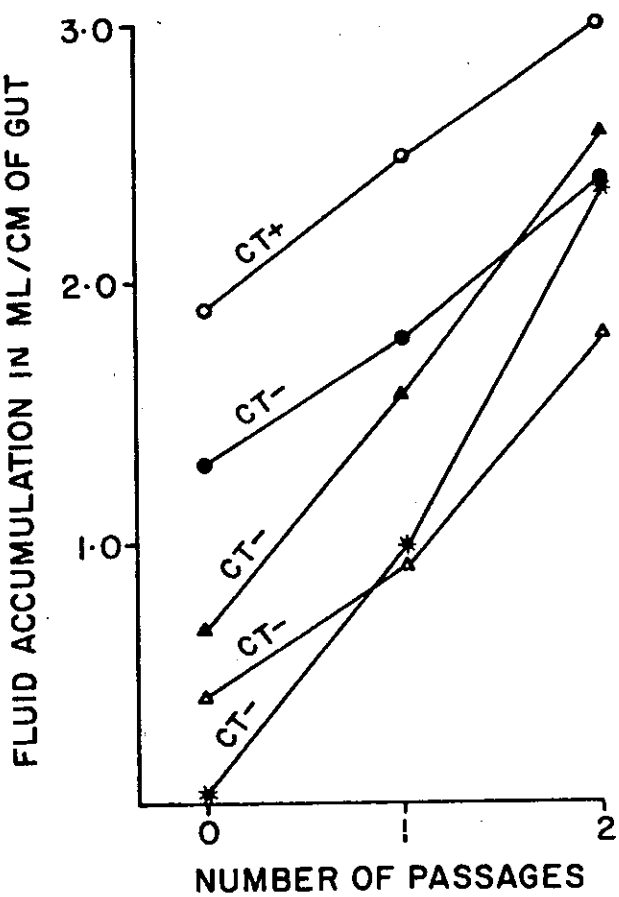

Fig. 2—Enhancement of fluid accumulation by CT gene-negative Vibrio cholerae O1 strains on consecutive passages through rabbit ileal-loops. Four of the CT gene-negative strains, which caused little or no accumulation of fluid during the initial tests. CT gene-positive V. cholerae strain 569B and nontoxic E. coli strain 265 were given serial passages in ileal loops. The strain E. coli 265 did not cause any fluid accumulation.

ENTEROTOXICITY TESTS WITH CHOLERA TOXIN OR HEAT-LABILE TOXIN GENE PROBE-NEGATIVE STRAINS OF *VIBRIO CHOLERAE O1*

Strain designation and source of isolation	Rabbit ileal loop tests: Live cells — No. positive /No. of tests	Rabbit ileal loop tests: Live cells — Range of fluid accumulation (ml/cm of gut)	Rabbit ileal loop tests: Culture filtrates — No. positive /No. of tests	Rabbit ileal loop tests: Culture filtrates — Range of fluid accumulation (ml/cm of gut)	Suckling mice assay and heated culture filtrates in loops	Infant rabbit test	PF*	Y-1 and CHO	GM_1 ELISA
VL 6007 (water, England)	2/2	1.0 – 2.0	5/7	1.8 – 1.1	0	+	+	0	0
1196-74 (sewage, Brazil)	2/3	0.8 – 1.5	2/3	0.6 – 1.2	0	+	+	0	0
X 392 (environment, Guam)	3/3	1.0 – 2.6	4/4	0.8 – 2.7	0	+	+	0	0
1727-79 (oyster, Louisiana)	2/3	0.6 – 1.5	2/3	0.5 – 2.2	0	+	+	0	0
V-69 (water, Maryland)	2/2	0.5 – 1.2	2/2	0.9 – 1.4	0	+	+	0	0
VL 6085 (water, England)	2/2	0.6 – 1.2	2/2	1.0 – 1.5	0	+	+	0	0
1528-79 (oyster, Louisiana)	3/4	0.5 – 1.0	2/2	0.9 – 1.1	0	+	+	0	0
1074-78 (sewage, Brazil)	2/5	0.5 – 0.9	2/2	0.4 – 0.8	0	+	+	0	0
X 725 (environment, Guam)	2/5	0.6 – 0.8	2/2	0.4 – 1.0	0	+	+	0	0
1077-79 (leg ulcer, Louisiana)	2/3	1.0 – 2.4	2/2	1.2 – 1.6	0	+	+	0	0
1165-77 (gall bladder, Alabama)	2/5	0.4 – 0.8	2/2	0.5 – 0.9	0	+	+	0	0
165G7 (oyster, Louisiana)	4/4	1.3 – 2.4	4/4	0.9 – 1.7	0	+	+	0	0
2740-80	2/2	1.3 – 1.5	3/4	0.5 – 1.7	0	+	+	0	0
569B (positive control)	10/10	1.2 – 2.5	10/10	1.3 – 2.5	0	+	+	+	+
Escherichia coli 265, Physiologic saline and Richardson's medium (negative controls)	0/10	0.0	10/10	0.0	0	0	0	0	0

*Blanching or necrosis, along with blueing, was noted for most of the culture supernates.

versity of Maryland School of Medicine, Maryland, U.S.A., and by P. Echeverria, AFIRMS, Bangkok, Thailand. None of the strains showed any homology with the CT or LT gene.

Infant rabbit assay

A majority of the strains caused diarrhoea in the infant rabbits, leading to both soiling of the perinea and ventral surface, as well as to dehydration. Four of the strains, however, only caused fluid accumulation in the large gut.

Skin PF assay

All 13 culture filtrates showed 7 mm or more blueing diameter (Fig. 3). At a dilution of 1:100, none of the test filtrates showed a blueing diameter of 7 mm or more. In subsequent experiments, therefore, only 1:2 and 1:10 dilutions were used. With the majority of test materials, blueing was accompanied by central blanching or necrotic changes. The zones of blanching and necrosis were surrounded by clearly visible intense blue skin. No blanching or necrosis was noted with the culture filtrate of *V. cholerae* strain 569B, and no blueing was noted with the gel-borate buffer. When the skin flap was everted, the only change noted in the areas where the culture filtrates, including that of *V. cholerae* strain 569B, were inoculated, was intense blueing.

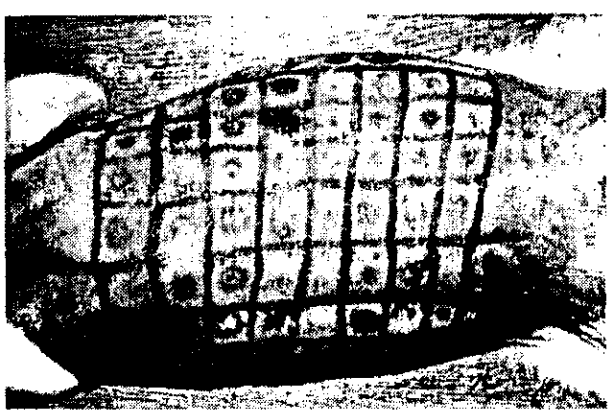

Fig. 3—Skin permeability assay with culture filtrates of CT gene-negative Vibrio cholerae O1 strains. Blueing was accompanied by central blanching or necrotic changes, with the majority of the test materials. The zones of blanching and necrosis were surrounded by clearly visible intense blue skin. No blanching or necrosis was noted with the culture filtrate of V. cholerae strain 569B, and no blueing was noted with gel-borate buffer.

Suckling mice assay and heat treatment

The ratios of gut weight and remaining body weight with all the culture filtrates were less than 0.085, whereas the positive control test always showed a ratio of more than 0.09.

The heat-treated culture filtrates did not cause any fluid accumulation in rabbit gut loop; and no increase was noted in capillary permeability, when tested in rabbits' skin.

CHO and Y-1 assays

A majority of the culture filtrates did not cause any change in the morphology of Chinese hamster ovarian and Y-1 mouse adrenal cells. However, four of the culture filtrates caused granulation and dislodgement of the cells from the surface of the plates.

GM_1 ELISA

All 13 culture filtrates were negative in GM_1 ganglioside immunosorbent assay.

Neutralization of loop and skin toxic factors

The titration of culture filtrates prepared with *V. cholerae* strain 569B and CT gene-negative strain X-392 indicated that dilutions of 1:128 and 1:16, respectively, caused fluid accumulations nearest to 1 ml/cm (Fig. 4). The titration of anti-CT showed that a dilution of 1:512 neutralized the minimal loop reacting dose (1:128) of the *V. cholerae* strain 569B culture filtrate (Fig. 5). Four times the dilution of antitoxin (1:512) that completely neutralized the minimal loop reacting dose of *V. cholerae* 569B, was unable to neutralize the minimal loop reacting dose (1:16) of X-392. The antisera against CT and its A and B subunits also failed to neutralize the skin PF activities of the 13 test culture filtrates, whereas that of the *V. cholerae* strain 569B was completely neutralized.

Gel-diffusion test

None of the 13 culture filtrates concentrated 20 times gave any precipitin band in Ouchterlony's gel-diffusion technique when tested against anti-CT.

Discussion

Different methods, depending upon availability, are used to measure the enterotoxic activity of

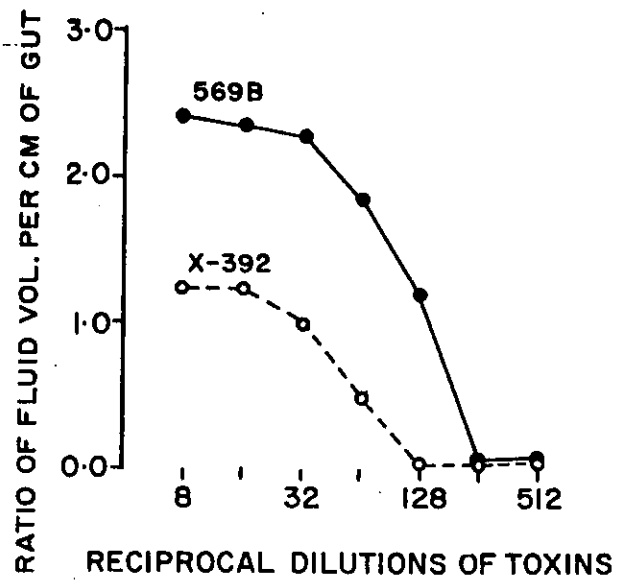

Fig.4—Titration of enterotoxic activity in culture filtrates by ileal-loop assay. The culture filtrates of the CT gene-negative V. cholerae strain X-392 and the CT gene-positive 569B, were titrated in ileal-loops in two-fold dilutions in PBS, to obtain the minimal reacting doses that would cause fluid accumulation of about 1 ml per cm of gut. Dilutions of 1:16 and 1:128 of the culture filtrates of X-392 and 569B, respectively, caused fluid accumulations nearest to 1 ml per cm (about 1.2 ml).

bacteria; and there is no internationally accepted and standardized technique for the purpose. Such factors as inoculum size, time of incubation, medium used for preparation of culture filtrates, cell-associated toxin, sources of laboratory animals, and definition of a positive response which can profoundly affect the observed results, may vary from laboratory to laboratory. Thus, exact comparison of results obtained from such experiments should be avoided.

Genetic techniques now are being evolved that can recognize specific genes governing toxin production. Such genetic techniques, uninfluenced by physiological, cultural and other conditions, would be an unequivocal indicator for toxigenicity. These discoveries generated high expectations amongst scientists who, seeking to discover potential candidates for a live oral cholera vaccine, went all out to find strains of *V. cholerae* 01 that would fail to demonstrate any genetic homology with the CT or LT gene, or who tried to engineer strains that would lack that gene (2, 6, 7). Implicit in these expectations was the assumption that only one important enterotoxin existed in *V. cholerae*, an enterotoxin governed by one genome. However, enterotoxins, by definition, are toxins that produce a secretory response when introduced into the lumen of the small intestines of man and experimental animals (23). Since fluid accumulation in the ligated loops of adult rabbits (24) and diarrhoea in infant rabbits (14) are the classical bioassays for detection of LT or CT, the genetic techniques developed must corroborate with the results obtained in these assays. If a discrepancy is found then the new toxin must be purified, and its genome identified.

The live cells and culture filtrates of the 13 CT gene-negative strains caused accumulation of fluid in rabbit ileal loop, indicating that the organisms elaborate an enterotoxic substance. This observation was further substantiated by the fact that all the strains induced either diarrhoea or fluid accumulation in the large gut of infant rabbits. Moreover, the culture supernates increased the capillary permeability of rabbits' skin. The results of these three biological assays confirm one another. It is known that skin PF activity in the rabbit is antigenically similar to fluid accumulation activity in rabbit ileal loops (25). A number of strains caused fluid accumulation in rabbit ileal loops, com-

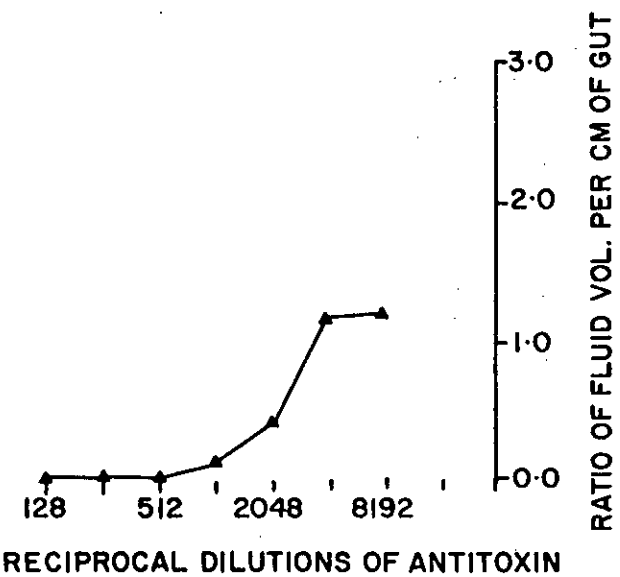

Fig. 5—Neutralization of loop reacting activity by anti-CT. A dilution of 1:512 of the anti-CT caused complete inactivation of the minimal loop reacting dose of V. cholerae 569B toxin (a dilution of 1:128). In a similar way, but without any effect, four times this dilution of anti-CT (1:64) was used to neutralize the minimal loop reacting dose (a dilution of 1:16 of the culture filtrate) of the CT gene-negative V. cholerae strain X-392. The curve is not shown here.

parable to that caused by the CT-positive *V. cholerae* strain 569B. Some of the strains, however, induced relatively small amounts of fluid accumulation, as compared to that induced by the *V. cholerae* strain 569B.

The strain-to-strain and loop-to-loop variations observed in the amounts of fluid accumulated might have been due to both differences in the quantitative release of toxin by individual strains, and to biological variations in rabbits (7). A majority of the strains caused diarrhoea in the infant rabbits, leading to soiling of the perinea and ventral surface, and to dehydration. Four of the strains, however, only caused fluid accumulation in the large gut. The nontoxic *E. coli* strain 265 did not cause any diarrhoea or intestinal fluid accumulation. Although not all infant rabbits developed frank diarrhoea with the *V. cholerae* 01 strains, the fluid accumulation in the large gut noted with 4 strains indicates these strains' ability to produce diarrhoea. The blanching and necrotic changes observed at the inoculation site suggest the possession of these activities by the culture filtrates, in addition to skin PF. Similar blanching and necrotic effects were, however, described in culture filtrates of LT-producing *E. coli* and *V. cholerae* non-01 strains, thus raising the issue of other activities associated with these organisms (26, 27). Any confusion arising out of the presence of blanching or necrosis in the skin PF assays could be avoided by taking the PF readings in the everted skin flaps.

The titrated minimal loop reacting dose of the culture filtrate of the CT-negative strain X-392 could not be neutralized by four times the dilution of anti-CT that completely inactivated the minimal loop reacting dose of the CT-positive *V. cholerae* strain 569B, in rabbit ileal loop assay. The antisera against CT and its A and B subunits, in dilutions of 1:64 and 1:10, respectively, also failed to neutralize the skin PF activity of the 13 test culture filtrates diluted 1:2 and 1:10; whereas the skin PF activity of *V. cholerae* strain 569B was completely neutralized with four times higher dilutions of the antitoxins. None of the twenty times concentrated culture filtrates gave any precipitin band, when tested against serial two-fold dilutions (up to 1:32) of anti-CT in Ouchterlony's gel-diffusion test. These observations strongly suggest that the toxin is immunologically different from the known CT.

The culture filtrates did not cause, in the morphology of CHO and Y-1 cells, any alterations that would indicate cytotonic changes (16, 17). These observations may suggest either that the culture filtrates did not stimulate the adenylate cyclase system, as is done by CT or LT (16, 28), or that binding does not occur to GM_1 ganglioside receptors. The ganglioside GM_1 enzyme-linked immunosorbent assays performed with the cell-free culture supernates yielded negative results, indicating their receptor site to be different from that of the known CT or LT (29).

None of the culture filtrates gave a positive result in suckling mice assay, indicating the absence of a heat-stable toxin (30). This observation was confirmed by ileal loop tests, using both heat-treated culture filtrates without any fluid accumulation, and negative skin PF assays.

The dialysed ammonium sulfate precipitates of the culture filtrates of strains grown in syncase medium caused fluid accumulation in rabbit gut loops. The heat lability and precipitation with ammonium sulfate suggests the protein nature of the enterotoxin (10).

The enterotoxic activity of the strains was greatly enhanced after two animal passages through rabbits' guts. The strains produced quantitatively more toxin of the same kind after passage; and did not show any homology with the CT or LT genes, indicating that they did not convert to making the typical toxin. This enhancement of enterotoxicity may suggest that, if such an organism is fed to humans for prophylaxis and is excreted in nature, it may be ingested by others in the community and may circulate there, leading to a further increase in virulence (31)

The results of this study indicate that CT gene-negative *V. cholerae* 01 strains, isolated from sewage, brackish water, oysters, and intestinal and extraintestinal infections in man, do possess enterotoxic activity. The diarrhoeal illness of the patient, from whom the CT gene-negative strain 2740-80 used in this study was isolated in association with seafood contamination in Florida, U.S.A., might have been due to this toxin. The deletion mutant of the human isolate of *V. cholerae* 01, Texas Star-SR, which lacks the gene for CT's toxic A subunit (32), caused diarrhoea in a number of volunteers who were fed an inoculum of 10^5 bacteria (33). The possibility that the mutant strain elaborated this toxin, which then was responsible for the diarrhoeal episodes, cannot be excluded. The strain 1074-78, isolated from sewage in Brazil, was administered orally, in a dose of 10^6 c.f.u., to seven volunteers, after neutralization of stomach acidity (6). None of the volunteers excreted the organism, indicating that it did not colonize in their guts. However, in the present study, this strain caused fluid accumulation after

only one passage in rabbit gut loop, suggesting enhancement of its virulence factors. The strain also multiplied by 3-4 logs in the loops upon passage, thus indicating that it regained its colonizing capability. The result might have been similar if the strain could have been passed through a human gut, as expected of an oral vaccine strain fed to members of a community. Therefore, before a direct vaccine development program is embarked upon, the immunobiologic and genetic aspects of this toxin should be defined.

It is suggested that such *V. cholerae* 01 strains are much more common in the aquatic environments in different parts of the world than people realize (34); and that this, rather than serial dissemination from tropical areas, may account for such strains' presence in temperate zones (35). In other words, such strains appear to occur naturally in aquatic environments in many places (34). The present study further suggests that the isolates from diverse environmental sources, such as water, sewage and oysters in different countries, including the U.K. and the U.S.A., produce an enterotoxin. Therefore, the general belief about the nontoxic nature of environmental *V. cholerae* isolates may not be true. Such a conclusion would point to the futility of implementing occasional trade embargoes against certain countries' marine food products that are contaminated with vibrios.

Thus, the present study demonstrates that the CT or LT gene-negative strains produce a toxin that has not been previously recognized. This new toxin is a heat-labile protein, and differs from the known CT in (i) antigenicity, (ii) receptor site, (iii) mode of action, and (iv) genetic homology. It is indicated that until this toxin is well understood, it is premature to use such gene-negative strains as potential candidates for a live oral vaccine. Furthermore, environmental isolates from different countries are equally enterotoxic.

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