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Enterotoxicity of El Tor-Like Hemolysin of Non-O1 *Vibrio cholerae*

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The enterotoxicity of an El Tor-like hemolysin purified from non-O1 *Vibrio cholerae* was investigated. Fluid accumulation was induced by injection of purified hemolysin into the ligated intestinal loops in adult rabbits (De test), intraintestinal administration in infant rabbits (Dutta test), and oral inoculation in suckling mice. The accumulated fluid was invariably mucous and bloody, and a histological change in the mucosa was observed. These results suggest that the hemolysin is an enterotoxic factor that is responsible for non-O1 *V. cholerae* gastroenteritis.

Vibrio cholerae other than serovar O1 (non-O1 *V. cholerae*) has been recognized as a causative agent of diarrheal disease (1). Enterotoxin resembling cholera toxin (CT) has been observed in the culture supernatant of non-O1 *V. cholerae* (3, 15, 25), and the toxin was purified and characterized to be identical (23) or similar (24) to CT.

Many clinical isolates of non-O1 *V. cholerae*, however, produce no CT-like enterotoxin or encode no corresponding gene (9, 13, 14, 17). Non-O1 *V. cholerae* gastroenteritis shows a variety of clinical symptoms that are different from those of cholera. Abdominal cramps, fever, and mucous and bloody stools found in non-O1 *V. cholerae* gastroenteritis (1, 7) are uncommon in typical cholera. These findings suggest that an unknown diarrheic factor(s) other than CT-like enterotoxin is involved in this disease.

The majority of non-O1 *V. cholerae* strains are hemolytic (16). This resembles the hemolytic property of *V. cholerae* O1 biotype El Tor. El Tor-like hemolysin has been purified from non-O1 *V. cholerae* (21). We found no biological, immunological, or physicochemical differences between the hemolysins of non-O1 *V. cholerae* and O1 *V. cholerae* biotype El Tor (22).

The role of hemolysin, however, in the pathogenesis of non-O1 *V. cholerae* gastroenteritis has not been clarified. Results of one study (6) suggest that the hemolysin of O1 *V. cholerae* biotype El Tor is pathogenically irrelevant, and although the isolated hemolysin was shown to be cytotoxic and lethal, its enterotoxicity was not examined in that study (6). In this study the enterotoxicity of the El Tor-like hemolysin of non-O1 *V. cholerae* was investigated.

MATERIALS AND METHODS

Preparation of purified hemolysin. El Tor-like hemolysin was purified as described previously (21) from non-O1 *V. cholerae* S7, which was obtained from Y. Zinnaka, Toho University School of Medicine, Tokyo, Japan.

Protein determination. The protein content was assayed by the method described by Bradford (2), with bovine serum albumin used as a standard.

Antibody. Antibodies against the El Tor-like hemolysin and CT were affinity purified from rabbit antiserum as described previously (22).

Ligated intestinal loop test. The ligated intestinal loop test (De test) was carried out by the method described by De and Chatterjee (4). Japanese White rabbits (weight, 2.0 to 2.5 kg) were starved for 48 h, but they had free access to water. The rabbits were anesthetized with sodium pentobarbital, and a laparotomy was done. The small intestine was withdrawn and ligated at a distance of about 10 cm from the ileocecal region. About 10 intestinal loops of 6 to 10 cm, separated by uninoculated segments of 1 to 2 cm, were made in each animal. Immediately after ligation, each loop was injected with 0.5 ml of purified hemolysin in phosphate-buffered saline containing 0.1% gelatin (PBSG). In one animal, two loops each were injected with 25, 50, and 100 μg of hemolysin, PBSG (as the negative control), and 1 μg of CT (as the positive control). After an appropriate period, the animal was sacrificed with sodium pentobarbital, and the abdomen was reopened. The loops were taken out, and the length of each loop and the volume of the accumulated fluid were measured. The extent of fluid accumulation (FA) was expressed by the FA ratio as the volume (in milliliters) of accumulated fluid per length (in centimeters) of the loop.

Infantile rabbit test. The infantile rabbit test (Dutta test), as described by Dutta and Habbu (5), was done as follows. Infant rabbits (weight, 100 to 150 g) were anesthetized by inhalation of ether, and a laparotomy was done. Hemolysin (0.5 ml) in PBSG was injected intraintestinally. In one series of experiments, 1 μg of CT and PBSG were always used as positive and negative controls, respectively. After an appropriate period of time the animals were sacrificed. The abdomen was reopened, and the entire intestine with its contents was taken out and weighed. The FA ratio was expressed by the weight (in grams) of the entire intestine per the remaining body weight (in grams).

Suckling mouse test. Suckling ICR mice (age, 2 to 3 days) were used for the suckling mouse test (19). Hemolysin (0.1 ml) in PBSG containing 0.001% Evans blue dye was administered perorally via a gastric tube to each mouse. The

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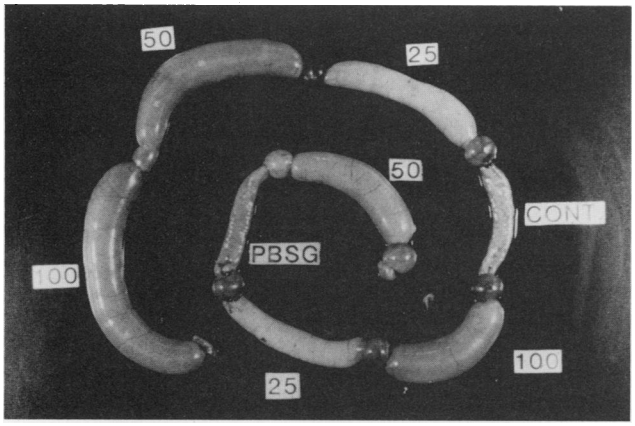

FIG. 1. Visual appearance of FA in the loops injected with El Tor-like hemolysin (8 h). Numbers indicate the amount (in micrograms) of purified hemolysin that was administered. PBSG, Dilution buffer; CONT, no inoculation.

animals were sacrificed at various time intervals by inhalation of chloroform. After confirming the presence of dye in the intestinal lumen, the entire intestine was removed. The FA ratio in each animal was expressed as the ratio of the weight of the entire intestine to that of the remaining body weight.

RESULTS

De test in adult rabbits. In Fig. 1 are shown the swollen intestinal loops with accumulated fluid 8 h after inoculation of El Tor-like hemolysin. In Fig. 2 are shown the serial changes in FA after injection of 100 μg of hemolysin. The fluid began to accumulate after 2 h and reached a maximum level by 8 h. When the dose of hemolysin was 25 or 50 μg, the extent of FA varied with the part of the intestine. In the loops located in the upper region of the small intestine (Fig. 3A), a low dose (25 μg) of the hemolysin induced significant FA. In the lower small intestine (Fig. 3B), however, 25 or 50 μg of the hemolysin did not induce FA. No such regional variation in FA was observed with 100 μg of hemolysin. FA activity of the hemolysin in the De test was specifically neutralized by anti-El Tor-like hemolysin antibody but not by anti-CT antibody.

To determine the neutralization of El Tor-like hemolysin of non-O1 *V. cholerae* by anti-hemolysin and anti-CT antibodies, 50 μg of the hemolysin in 0.25 ml was mixed with an equal volume of antibody or PBSG (buffer) and incubated for 10 min at 37°C. The mixture was inoculated into the intestinal loop. Two pairs of loops were made in the upper and lower intestine in two animals. In one animal, three pairs of loops were made in the upper, middle, and lower portions of the intestine. For the control (PBSG), anti-hemolysin, and anti-CT, the FA ratios were 0.68 ± 0.16, 0.03 ± 0.01, and 0.64 ± 0.07 ml/cm, respectively. These values represent means ± standard errors of seven loops in three animals.

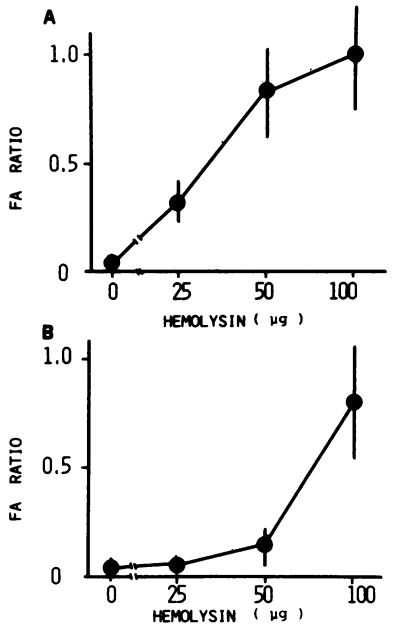

FIG. 3. Regional variation of fluid accumulation in the intestine. (A) Region between 15 and 50 cm from the pylorus. (B) Region between 10 and 50 cm from the ileocecal junction. The incubation period was 8 h. Values represent means ± standard errors of five loops. Five animals were used.

Suckling mouse test. Peroral inoculation of the hemolysin to suckling mice also induced FA. Fluid began to accumulate within 1 h and reached a maximum level at 3 h with an FA ratio of 0.10, followed by a gradual decrease without overt diarrhea (Fig. 4). At 3 h, hemolysin had a dose-dependent effect on FA (Fig. 5), which was clearly inhibited by anti-El Tor-like hemolysin antibody (Fig. 6).

Dutta test. Infant rabbits were challenged with 100 μg of hemolysin, which was administered directly into the small intestine (Table 1). FA occurred 2 h after hemolysin admin-

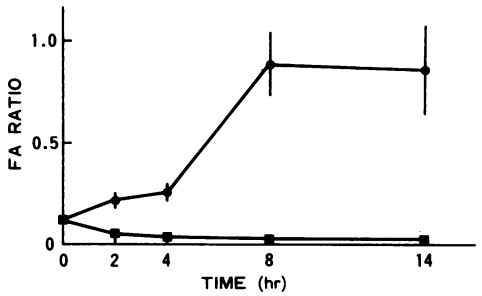

FIG. 2. Time course of fluid accumulation by El Tor-like hemolysin (●; 100 μg) in the De test. PBSG (■) was injected into the control loops. Values represent means ± standard errors of 6 (2 and 4 h), 10 (8 h), and 4 (14 h) loops. Ten animals were used.

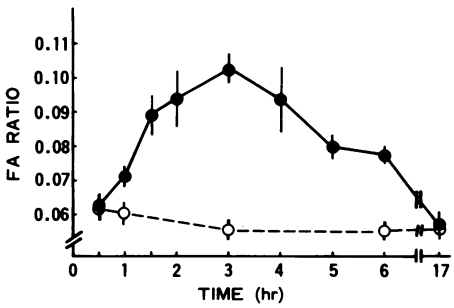

FIG. 4. Time course of fluid accumulation in the suckling mouse test. Two micrograms of hemolysin (●) or PBSG (○) was administered. Values represent means ± standard errors of six animals.

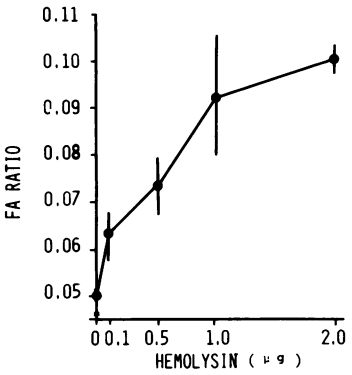

FIG. 5. Dose-response curve of fluid accumulation in the suckling mouse test. The incubation period was 3 h. Values represent means ± standard errors of three animals.

istration. In the cases with an FA ratio of greater than 0.09, 3 to 4 ml of the fluid was accumulated in the cecum, but overt diarrhea was not seen. The accumulated fluid was mucous and darkly colored, which was distinct from the watery fluid caused by CT.

DISCUSSION

The pathogenic mechanism of gastroenteritis due to non-O1 *V. cholerae* was investigated by challenge with CT-negative, non-O1 *V. cholerae* or administration of the culture filtrate; and induction of intestinal FA or diarrhea with the these agents has been demonstrated by the De, Dutta, or suckling mouse tests (11, 14, 17). However, there were many extracellular proteins in the culture filtrate; therefore, the causative agent of FA was not clarified in those studies. In this study we induced FA in these three commonly used models with El Tor-like hemolysin purified from non-O1 *V. cholerae*. This suggests that El Tor-like hemolysin is an enterotoxic factor for non-O1 *V. cholerae* gastroenteritis because (i) most non-O1 *V. cholerae* strains produced the hemolysin (16), (ii) the purified hemolysin induced FA in experimental animals, and (iii) the pathological features caused by the hemolysin were in good agreement with those in the infection model (10, 11, 13, 14) and in patients with gastroenteritis (1, 7).

Bloody and mucous stool has been reported in non-O1 *V. cholerae* gastroenteritis (1, 7). The accumulated fluid in the

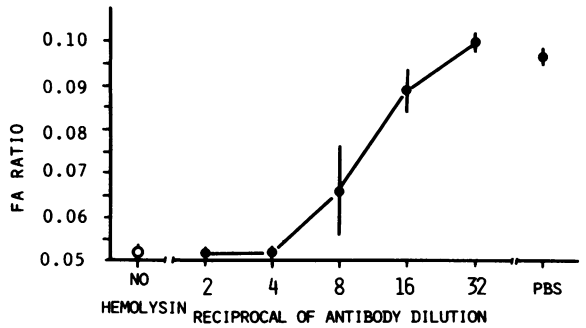

FIG. 6. Neutralization test by anti-El Tor-like hemolysin in suckling mice. Twofold serially diluted antibodies were added to 2 μg of the hemolysin, and the mixture was inoculated. The incubation period was 3 h. Values represent means ± standard errors of three animals.

TABLE 1. Intestinal fluid accumulation induced by El Tor-like hemolysin in infant rabbits

Incubation period (h)	FA ratio (ml/g) for[a]:	
	Hemolysin	Control (PBSG)
2	0.081 ± 0.001	0.072 ± 0.007
4	0.080 ± 0.004	0.059 ± 0.006
8	0.091 ± 0.005	0.071 ± 0.001
12	0.091 ± 0.003	0.061 ± 0.004

[a] Hemolysin (100 μg) was administered into the small intestines of infant rabbits. Values represent means ± standard errors of four hemolysin-treated or two control animals.

De tests was more or less, but invariably, mucous and bloody; and hemorrhage was observed in the mucosa. This indicates that the hemolysin is cytotoxic to mucosal cells. The histological changes in the intestinal mucosa that were found in this study agree with previous observations (11). In our experience, bloody fluid accumulation has been occasionally (but not invariably) seen in the loops challenged with CT or *V. cholerae* O1. This might have been caused by some technical failure or by excessive expansion of the loops. Also, it is possible that the bloody fluid accumulation was caused by hemolysin from El Tor vibrios. Histological findings of intestinal mucosa of ordinary positive loops challenged with CT indicated that they were almost intact.

A large amount (more than 25 μg) of the purified hemolysin was required to induce FA in the De test (Fig. 3). This suggests that a relatively large amount of hemolysin is necessary to induce diarrhea in patients. When live organisms colonize the intestinal epithelium and secrete hemolysin, however, the hemolysin may effectively attack the epithelial cells (18). In such a case, a smaller amount of hemolysin may induce diarrhea. Also, it is possible that the organisms produce a large amount of hemolysin in the infected intestine. Non-O1 *V. cholerae* S7, from which we obtained purified hemolysin, produced CT-like enterotoxin (24). Therefore, this organism is not suitable for studying the enterotoxicity of hemolysin, although it was consistently positive for the loop test and produced bloody mucous fluid with an FA ratio of 0.8:1.3 (data not shown). A study with a strain with no CT-like enterotoxin is required.

In the Dutta test, the amount of FA was not as large as that seen in the animals given 1 μg of CT. The possible reasons for this are that (i) FA activity of hemolysin is much lower than that of CT, (ii) biological activity of hemolysin is readily lost (8, 20), and (iii) the accumulated fluid is reabsorbed in the cecum or small intestine without subsequent damage. In infant rabbits, the darkly colored FA in the cecum suggests that there is corresponding tissue damage in the intestine. In the suckling mouse test, the reduction of FA after the peak at 3 h without overt diarrhea strongly suggests that there is reabsorption.

A patient with severe diarrhea caused by *V. cholerae* O1 lacking the CT gene was reported in 1984 (12), and the symptoms resembled those associated with non-O1 *V. cholerae* gastroenteritis. This case suggests that an unknown diarrheic factor is responsible for the illness. The vibrios isolated from the patient were hemolytic. Therefore, it is speculated that the hemolysin was a diarreic factor in that patient because the El Tor hemolysin is identical to the hemolysin of non-O1 *V. cholerae*.

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