A Study on the Enterotoxic Factors in *Vibrio cholerae* Infection

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Abstract: The relationships among protease/hemagglutinin activity, the productivity of cholera toxin and hemolysin and fluid accumulation (FA ratio) in the ligated ileal loop test were investigated in 35 strains of *Vibrio cholerae* (biotype classical, El Tor and non-O1 *V. cholerae,* 10, 15, and 10 respectively). Some cholera toxin was detected in the accumulated fluid challenged with *V. cholerae* O1 classical biotype but no hemolysin was detected. In *V. cholerae* O1, biotype El Tor, both cholera toxin and hemolysin were detected. Cholera toxin was detected only from one out of 10 strains of *V. cholerae* non-O1 and hemolysin was detected from 6 strains. *Escherichia coli* heat-stable enterotoxin-like toxin was not detected in the accumulated fluid and *V. parahaemolyticus* thermo-stable direct hemolysin-like toxin was detected only from one strain of *V. cholerae* non-O1. The FA ratio was correlated with the cholera toxin titers (Spearman's rank correlation $p<0.01$) but not with the hemolysin titer or protease activity. The enterotoxicity of *V. cholerae* O1 biotype classical is suggested to be responsible for cholera toxin and those of *V. cholerae* O1 biotype El Tor is responsible for cholera toxin and hemolysin. However, the enterotoxicity of *V. cholerae* non-O1 may not be mainly responsible for cholera toxin but for other toxic substances.

Key words: *Vibrio cholerae,* Enterotoxicity, Cholera toxin, Hemolysin

INTRODUCTION

Non-O1 *Vibrio cholerae* has been recognized as a causative agent of diarrheal diseases as is *V. cholerae* O1. The entire mechanism of pathogenesis of choleraic diarrhea has not been clarified yet, although several diarrheagenic factors other than cholera toxin, hemolysin (Yamamoto, *et al.* 1984), *Escherichia coli* heat-stable enterotoxin-like toxin (Honda, *et al.* 1985) and *Vibrio parahaemolyticus* thermo-stable direct hemolysin-like toxin (Honda, *et al.* 1985), have been reported. Little is known about which toxin is mainly responsible for the enterotoxicity or to what degree toxin substances other than cholera enterotoxin are involved. Cholera toxin is the only toxic substance associated with illness due to *V.*

Receiced for Publication, October 31, 1987.
Contribution No. 1991 from the Institute of Tropical Medicine, Nagasaki University

cholerae O1 classical biotype. On the other hand, Booth and Finkelstein (1986) reported that hemagglutinin/protease activity, which has been considered to be involved in the adherence of the vibrio cells to the small intestinal epithelium, as well as cell-associated hemagglutinin, is widely distributed in *V. cholerae* O1 and non-O1 and concluded that neither factor is a discriminating pathogenic characteristic, although they may contribute to virulence. However, we found that proteases purified from *V. cholerae* increase the FA ratio when applied to the intestinal wall before the challenge with cholera vibrios (unpublished data). In the present study, we investigated the relationships among protease/hemagglutinin activities, the productivity of toxins (cholera toxin, hemolysin) and FA ratio to clarify the role of toxins and proteolytic enzymes in the enterotoxicity.

Materials and Methods

a) Bacterial strains

A total of 35 strains, 10 of *Vibrio cholerae* O1, classical biotype, 15 of *V. cholerae*

Table 1. *Vibrio cholerae* strains

Biotype	Strain	Serotype	Sources
Classical	Hikojima	Hikojima	Japan, 1963 (1)
	NIH35A 3	Inaba	India, 1941 (1)
	Vc 4	Ogawa	India, unknown (1)
	31	Ogawa	unknown, unknown(1)
	86B 1	Ogawa	Bangladesh, 1986 (2)
	86B 3	Ogawa	Bangladesh, 1986 (2)
	86B 6	Ogawa	Bangladesh, 1986 (2)
	86B 7	Ogawa	Bangladesh, 1986 (2)
	86B10	Ogawa	Bangladesh, 1986 (2)
	H218	Inaba	Thailand, 1954 (1)
El Tor	82P 4	Ogawa	The Philippines, 1982
	82P 5	Ogawa	The Philippines, 1982
	82P12	Ogawa	The Philippines, 1982
	England6009	Ogawa	England, 1977 (1)
	England6085	Ogawa	England, 1977 (1)
	1074−78	Ogawa	Brazil, 1978 (1)
	HB57	Ogawa	Kenya, 1983
	HB58	Ogawa	Kenya, 1983
	HB59	Ogawa	Kenya, 1983
	HB67	Ogawa	Kenya, 1983
	HB136	Ogawa	Kenya, 1983
	HB140	Ogawa	Kenya, 1983
	HB147	Ogawa	Kenya, 1983
	2741−80	Inaba	USA, 1980
	England5962	Ogawa	England, 1977 (1)
non-O1	NAG27		Japan, 1985 (2)
	NAG30		Japan, 1985 (2)
	NAG31		Japan, 1985 (2)
	NAG41		Japan, 1985 (2)
	NAG44		Japan, 1985 (2)
	NAG47		Japan, 1985 (2)
	S 7		Sudan, 1969
	9614VcO6		The Philippines, 1970
	106VcO17		The Philippines, 1970
	6880VcO5		The Philippines, 1970

O1, biotype El Tor and 10 of *V. cholerae* non-O1 were used (Table 1). The strains indicated as (1) and (2) were provided by S. Shimotori, School of Health Science, Kyushu University, Fukuoka, Japan and M. Iwanaga, Department of Bacteriology, University of the Ryukyus, School of Medicine, Okinawa, Japan, respectively. The strains of S7 and 2741-80 were obtained from Y. Zinnaka, Toho University School of Medicine, Tokyo, Japan and J. Glenn Morris, Jr., Center for Vaccine Development, University of Maryland, Baltimore, Md., respectively.

b) Biochemical properties tested

The strains stored in nutrient agar were again isolated onto TCBS agar (Eiken) and Brom-thymol-blue media (Eiken) to check for purity. A suspicious colony from each stock was examined with the following media for identification, Kligler, SIM, Voges-Proskauer (VP), lysine, ornithine, arginine, Simmon citrate and 0% NaCl peptone water and was simultaneously checked for the level of cytochrome oxidase.

c) Serological properties tested

Suspected strains were tested for agglutination with monospecific Ogawa and Inaba antisera (Denka Seiken, Japan). In the cases of *V. cholerae* non-O1, boiled cells were also tested with these antisera.

d) Biotyping

All strains of *V. cholerae* O1 were checked for sensitivity against phage IV (Mukerjee, 1963) and polymyxin B (50 IU) (Gangarosa *et al.*, 1967), chicken red blood cell agglutination (Finkelstein and Mukerjee, 1963) and sheep red blood cell hemolysis (Zinnaka, 1981). The hemolysis test was also done with heart infusion broth containing 1% glycerol. The degree of hemolysis was classified as follows: complete hemolysis (++), marked hemolysis with RBC sediments (+), weak hemolysis (±), and no hemolysis (−).

e) Rabbit ileal loop test (De test)

Bacteria were inoculated in 2ml of heart infusion broth and incubated at 37℃ overnight with resting. One tenth ml of the culture was used as the inoculum. The inoculum bulk was about 2×10^7 bacteria. All rabbits each weighing 2-3kg were starved but provided water for 48h prior to challenge. Rabbits were anesthetized intramuscularly with ketamine hydrochloride and intravenously with pentobarbital. The abdomen was opened, and the small intestine was tied into 6cm segments with 1cm spacer loops between the experiment loops. The large loops were injected with 0.1ml of bacterial culture. Control loops were injected with 5μg of purified cholera toxin (0.5ml) for positive control and 0.5ml of N.S.S. for negative control, respectively. The intestine was replaced and the incision was closed. The rabbits were killed and the intestine was removed after 13h. The length of the loop was measured and the volume, color and character of fluid accumulated in the loop were recorded.

f) Analysis of the intestinal fluid in the loops

The accumulated fluid in the loop was centrifuged at 16,000xg for 20min. Cholera toxin and hemolysin in the supernatants were titrated with reversed passive latex agglutination method (RPLA), which gave a maximal sensitivity of 1-2 ng/ml (Ichinose *et al.*, 1987).

Vibrio parahaemolyticus thermo-stable direct hemolysin-like toxin was titrated with KAP-RPLA (DENKA SEIKEN CO., LTD.) and *Escherichia coli* heat-stable enterotoxin-like toxin was assayed by the suckling mouse test (Takeda *et al.*, 1979).

g) Hemagglutinin activity (HA)

Bacteria were innoculated in 50ml of tryptic soy broth (Difco Laboratories) in 300ml Erlenmeyer flasks and cultured with shaking at 30℃ for 20h. Culture supernatants were obtained by centrifugation at 40,000xg for 20min. Microtiter quantitation of hemagglutinin activity was performed as previously described by Hanne & Finkelstein (1982). The titer is defined as the reciprocal of the highest dilution in which hemagglutination was visible to the naked eye. HA preparetions were diluted in two-fold series in round-bottomed microtiter plates in 25μl of Krebs-Ringer buffer (KRT). RBCs suspended to 1.5% concentration (vol/vol) were added in 25μl, incubated for 30min at room temperature and HA reactions were examined after 30min. For determination of cell-associated HA, cells were suspended in saline to a concentration of 1×10^9 to 2×10^9/ml before assay.

h) Protease activity

Protease activity was detected by using a single-diffusion technique in agar gel (0.75%) containing skim milk (1.5%) as a substrate (Honda *et al.*, 1987). Sample solution (20μl) was added to wells 3.5mm in diameter, and plates were incubated for 12h at 37℃. Zone of clearing was measured.

RESULTS

Table 2 shows the hemolytic property of 35 strains of *V. cholerae* determined by the method of Zinnaka (HIB) and heart infusion broth containing 1% of glycerol (HIBG). One of the 10 classical strains of *V. cholerae* were hemolytic, although classical strains are considered to be non hemolytic. Ten of the 15 strains of *V. cholerae* El Tor were hemolytic. All the strains of *V. cholerae* non-O1 were hemolytic. The rate of hemolytic strains in HIBG was higher than that in HIB.

Table 2. Hemolytic property of the cholera strains

media / Strains / degree of hemolysis	HIB				HIBG				Total
	++	+	±	−	++	+	±	−	
V. cholerae O1 classical	0	1	3	6	1	1	4	4	10
V. cholerae O1 El Tor	9	1	2	3	15	0	0	0	15
V. cholerae non-O1	10	0	0	0	10	0	0	0	10

The enterotoxicity of these strains of *V. cholerae* was checked by the ligated intestinal loop test. The average FA ratio was 1.17 in classical strains, 0.99 in El Tor strains and 0.47 in non-O1 strains. The FA ratio in classical and El Tor was higher than that in non-O1 strains (statistically significant, T-test $p<0.01$, $p<0.05$, respectively) and there was no significant difference between classical and El Tor strains. The average FA ratio in control loops, cholera toxin and N.S.S. was 1.65 and 0, respectively (Table 3).

Table 4 shows the character of accumulated intestinal fluid in the ligated intestinal loop test. Watery or watery and bloody fluid was observed in the loops challenged with the classical strains of *V. cholerae*. Mucous or mucous and bloody intestinal fluid was observed only in the loops challenged with El Tor and non-O1. This coincides with the hemolytic property of the strains challenged. Table 5 shows the results of titration of cholera toxin and hemolysin in accumulated intestinal fluid by RPLA. Some cholera toxin was detected in the accumulated fluid challenged with classical *V. cholerae* strains except for two strains but no hemolysin was detected. In the accumulated fluid challenged with *V. cholerae* El Tor, both cholera toxin and hemolysin were detected. No cholera toxin was detected from non-toxigenic (CT negative) strains, 1074-78 and 2741-80. Cholera toxin alone was detected in the accumulated fluid from 4 of the 15 strains of *V.*

Table 3. Results of ileal loop test

Strains	F. A. ratio (ml/cm)			Average
	<0.5	0.5−1.0	1.0<	
V. cholerae O1 classical	1	3	6	1.17±0.53
V. cholerae O1 El Tor	6	1	8	0.99±0.60
V. cholerae non-O1	5	4	1	0.47±0.45

Values represent means ± standard deviations of FA ratio

Table 4. Character of accumulated intestinal fluid

Strains/character	W	W+B	M	M+B	Others
V. cholerae O1 classical	7	3	0	0	0
V. cholerae O1 El Tor	2	9	3	1	0
V. cholerae non-O1	0	3	3	2	2
Total	9	15	6	3	2

W: watery, W+B: watery and bloody, M:mocous,
M+B: mucous and bloody

cholerae O1, biotype El Tor. The average FA ratio in these strains was 1.53. On the other hand, hemolysin alone was detected from 6 of the 15 strains of *V. cholerae* O1 biotype El Tor. The average FA ratio was 0.56. However, cholera toxin was detected only from one

Table 5. Titration of cholera toxin and hemolysin in accumulated intestinal fluid by RPLA

Titer 2^n (μg/ml)		ND	−8	−7	−6	−5	−4	−3	−2	−1	0	1	2	3	4	5
Classical	CT	2	1	0	0	1	0	1	0	1	2	0	2	0	0	0
	Hly	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
El Tor	CT	7	1	1	1	1	2	1	1	0	0	0	0	0	0	0
	Hly	5	0	0	0	0	0	1	0	3	1	4	0	0	0	1
Non-01	CT	9	0	1	0	0	0	0	0	0	0	0	0	0	0	0
	Hly	4	0	0	0	0	0	0	2	1	1	2	0	0	0	0

CT: cholera toxin, Hly: hemolysin, ND: not detected
RPLA: reversed passive latex agglutination

Table 6. Titration of toxins and protease and hemagglutinin activities

strain No.	FA	PA	CT	Hly	SH	CH
1	0.56	3.30	0.0	0.0	−	128
2	1.70	3.83	0.0	0.0	−	4
3	1.68	3.83	1000.0	0.0	−	−
4	0.54	7.67	4000.0	0.0	−	4
5	1.34	8.11	4000.0	0.0	−	4
6	1.61	8.54	31.3	0.0	−	2
7	1.53	3.91	5000.0	0.0	−	−
8	0.32	7.09	1000.0	0.0	−	4
9	1.68	5.57	125.0	0.0	−	8
10	0.74	3.40	3.9	0.0	−	32
11	1.56	11.44	250.0	0.0	32	128
12	1.43	7.17	500.0	0.0	8	32
13	1.62	12.16	125.0	0.0	32	512
14	0.36	6.37	0.0	500.0	−	64
15	0.30	11.66	0.0	500.0	16	512
16	0.23	4.20	0.0	43.4	−	128
17	0.36	5.79	10.1	160.1	16	64
18	1.65	5.79	22.5	2000.0	−	128
19	1.65	5.79	125.0	2000.0	−	32
20	1.27	9.80	31.3	2000.0	−	64
21	1.69	5.10	0.0	2000.0	−	64
22	1.50	5.10	3.9	0.0	−	16
23	0.40	11.20	0.0	455.2	8	16
24	0.36	11.80	0.0	1010.0	−	128
25	0.53	8.20	0.0	0.0	−	64
26	0.89	8.20	0.0	0.0	−	128
27	0.14	8.70	0.0	2750.0	16	128
28	0.60	3.70	7.8	0.0	−	16
29	0.11	5.50	0.0	2300.0	−	64
30	0.09	5.70	0.0	0.0	16	512
31	1.55	6.30	0.0	0.0	16	8
32	0.56	7.20	0.0	250.0	4	32
33	0.12	11.08	0.0	175.0	−	128
34	0.59	6.20	0.0	500.0	16	32
35	0.05	3.70	0.0	1080.0	−	−

FA: fluid accumulation ratio (ml/cm), PA: protease activity (diameter of translucent zone), CT: cholera toxin (ng/ml), Hly: hemolysin (ng/ml), SH: soluble hemagglutinin titer, CH: cell associated hemagglutinin titer

of the *V. cholerae* non-O1 strains. The correlation between the cholera toxin titer and FA ratio was statistically significant (Spearman's rank correlation $p<0.01$) but no correlation was seen between the hemolysin titer and FA ratio. Correlations were observed between protease activity and soluble HA ($p<0.01$), between protease activity and cell-associated HA ($p<0.05$) and between soluble HA and cell-associated HA ($p<0.01$) but not between protease activity and FA ratio (Table 6). No fluid accumulation was induced by oral inoculation of supernatants in suckling mice and no *V. parahaemolyticus* thermo-stable direct hemolysin-like toxin was detected by KAP-RPLA except for one strain of *V. choleral* non-O1.

Discussion

The enterotoxicity of *V. cholerae* non-O1 was lower than that of *V. cholerae* O1, although whether this reflects directly virulence is still unknown. Mucous or mucous and bloody intestinal fluid was observed only in the loops inoculated with *V. cholerae* O1, biotype El Tor and non-O1. This finding corresponds to the hemolytic property of the strains challenged. *V. cholerae* O1 biotype El Tor and *V. cholerae* non-O1 produce immunologically, physicochemically and biochemically identical heat labile hemolysin as previously reported by Yamamoto *et al.* (1986). Furthermore, this hemolysin could induce significant fluid accumulation in the ligated adult rabbit intestinal loop test, intra-intestinal administration in infant rabbit and oral inoculation in suckling mice (Ichinose *et al.*, 1987) and its character was in good agreement with those in patients with gastroenteritis (Hughes *et al.*, 1978).

Some cholera toxin was detected in the accumulated fluid challenged by *V. cholerae* classical biotype but hemolysin was not detected. Therefore, the enterotoxicity of *V. cholerae* classical biotype may be responsible only for cholera toxin and another explanation should be considered for the hemolytic character of the one strain of *V. cholerae* classical biotype, for example, hemodigestion. However, the enterotoxicity of *V. cholerae* non-O1 may not be mainly responsible for cholera toxin but for other toxic substances including hemolysin, because hemolysin was detected among six of the 10 strains of *V. cholerae* non-O1 but cholera toxin was detected only from one strain. Furthermore, other factors cannot be excluded for the enterotoxicity of *V. cholerae* non-O1 because no correlation between FA ratio and the hemolysin titer was seen, although the abdomen of rabbits was reopened 13h after inoculation so that the FA ratio might not reflect exactly the production of hemolysin because purified hemolysin induces maximum fluid accumulation 8h after inoculation (Ichinose *et al.* 1987). Biotype El Tor *V. cholerae* O1 strains produce cholera toxin and/or hemolysin. Only hemolysin was detected in 6 of the 15 strains of *V. cholerae* O1, biotype El Tor. Therefore, the enterotoxicity of *V. cholerae* O1 biotype El Tor may be responsible for not only cholera toxin but also hemolysin. However, we cannot exclude the possibility that toxins under the level of detection limit might be produced. Proteolytic enzymes seem not to be directly related to enterotoxicity, although its application to the intestinal wall prior to challenge of cholera vibrios increase the FA ratio (data not shown). It would be more suitable to consider that protease inoculated may nick and activate cholera

enterotoxin in the intestinal loop as previously reported by Booth *et al.* (1984). Protease activities also correspond well to hemagglutinin activities and this fact supports the assumption that hemagglutinin has inherent protease activity as previously described by Finkelstein *et al.* (1983). The crude soluble HAs correlate very much with cell-associated HAs. Thus, by inference, soluble HA may be excreted into media from the surface of organisms, where cell-associated HA and proteolytic exzyme exist as a compound.

Acknowledgements

The author would like to thank Mr. M. Iwami and Miss M. Yokota in our department for their very kind help during the course of this study. The animal experiments in this study were performed at the Animal Research Center for Infectious Tropical Diseases, the Institute of Tropical Medicine, Nagasaki University.

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コレラ及び non-O1 コレラ菌による下痢症の腸管毒性因子に関する研究

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コレラ菌感染における液体貯留活性と，下痢原性因子であるコレラエンテロトキシン，溶血素及び蛋白分解酵素との関連を明らかにするため，クラシック型，エルトール型及び non-O1 コレラ菌，各々10，15，10株を用いて，プロテアーゼ及び血球凝集活性，コレラトキシン及び溶血素の産生性，成熟ウサギループテストにおける腸管内液体貯留率を測定しそれらの相関を検討した．その結果，クラシック型コレラ菌を投与したループ内貯留液からはコレラトキシンが検出されたが溶血素は検出されず，エルトール型コレラ菌を投与したループ内貯留液からはコレラトキシンだけでなく溶血素も検出された．non-O1 コレラ菌を投与したループ内貯留液からは10株中1株からコレラトキシンが，6株から溶血素が検出された．大腸菌 ST 様毒素はループ内貯留液から検出されず，腸炎ビブリオ耐熱性毒素様毒素は non-O1 コレラ菌の1株のみから検出された。またコレラトキシンと投与後13時間の FA 比との間の相関は有意であったが (Spearman's rank correlation $p<0.01$)，溶血素と FA 比，プロテアーゼ活性と FA 比との間には有意な相関はみられなかった．クラシック型コレラ菌の液体貯留活性はコレラトキシンに依存するがエルトール型コレラ菌はコレラトキシンだけでなく溶血素の関与も十分考えられた．また non-O1 コレラ菌は溶血素を含めたコレラトキシン以外の下痢因子の関与が示唆された．

熱帯医学　第29巻　第4号　211－219頁，1987年12月