INFECTION AND IMMUNITY, June 1983, p. 1083–1091
0019-9567/83/061083-09$02.00/0

Vol. 40, No. 3

Vibrio Factors Cause Rapid Fluid Accumulation in Suckling Mice†

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Received 26 August 1982/Accepted 1 March 1983

Non-O-1 and O-1 *Vibrio cholerae* and *Vibrio fluvialis* isolated from clinical and environmental sources were examined for virulence factor production in 3-day-old suckling mice and in Y-1 tissue culture. The responses of the suckling mice to intragastrically administered bacterial cultures were measured by intestinal fluid accumulation (FA), diarrhea, and mortality. Regardless of the O-serovar, source of isolation, or ability to produce cholera toxin, all strains of *V. cholerae* stimulated increased FA, which was measurable in the mice at 4 h post-inoculation. The factor(s) causing these symptoms was found to be distinct from cholera toxin by the kinetics of FA and serological difference from cholera toxin based on in vivo neutralization tests. In most instances, FA was followed by high rates of mortality. Y-1 mouse adrenal tumor cell assays also showed that many *V. cholerae* produced extracellular heat-labile cytotoxic factor(s), and many cholera toxin-negative strains also caused a cytotonic-like morphological response. The majority of *V. fluvialis* strains produced smaller amounts of cytotoxic factor(s) but no cytotoxic reactions. The factor which stimulates rapid FA in suckling mice could be one of several virulence-associated factors contributing to diarrheal disease by nontoxigenic vibrios, but this is not verified at present.

Diarrhea caused by serovar O-1 *Vibrio cholerae* has been extensively studied, and it is now known that an extracellular cholera toxin (CT) produced by this organism is often associated with severe human diarrheal disease (10). Although human gastroenteritis caused by non-O-1 *V. cholerae* has been well documented, the mechanisms of pathogenesis are still not well understood (3). Studies on human isolates (30) and environmental isolates (6) of non-O-1 *V. cholerae* revealed that some of these strains produce CT. Recently, some non-O-1 strains as well as O-1 strains of *V. cholerae* were shown to possess DNA polynucleotide sequences which hybridized with the genes encoding *Escherichia coli* heat-labile enterotoxin (LT) (13). In addition, some reports have indicated the existence of other uncharacterized virulence factors of non-O-1 *V. cholerae*, frequently in the absence of CT (2, 8, 16, 21, 25–27). Because of the wide distribution of non-O-1 *V. cholerae* in the environment and increasing human exposure, it is important to develop an understanding of its potential pathogenicity and mechanisms of virulence. One significant problem in the elucidation of virulence has been the appropriate development and implementation of a suitable bioassay system. A number of bioassays are available; the suckling mouse test was chosen in the present studies because of its value in simulating the actual course of gastroenteritis, as shown with studies of enterotoxigenic *E. coli* (7), *Yersinia enterocolitica* (22, 23), and O-1 *V. cholerae* (1, 4, 11, 28).

In this study, we examined responses in suckling mice induced after intragastric administration of whole cultures of O-1 and non-O-1 *V. cholerae* and *Vibrio fluvialis* of clinical and environmental origins. Virulence factor(s) associated with intestinal fluid accumulation (FA), diarrhea, and mortality, but distinct from CT, were found. Cultures were also tested for reactions induced in Y-1 mouse adrenal tumor cells. *V. fluvialis*, a newly described human pathogen of aquatic origin (14, 24), was found to elicit pathogenic responses similar to those of non-O-1 *V. cholerae* in suckling mice but induced weaker responses in the Y-1 assay.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains used in this study included one clinical and five environmental isolates of O-1 *V. cholerae*, five clinical and seven environmental isolates of non-O-1 *V. cholerae*, and

† Technical Paper no. 6644, Oregon Agricultural Experiment Station.

four clinical and five environmental isolates of *V. fluvialis*. *E. coli* and *Citrobacter freundii* were included as positive and negative controls, respectively (see Table 3). All organisms were maintained in tryptic soy broth without glucose (Difco Laboratories) supplemented with 0.5% sodium chloride (TSB') plus 5% glycerol at −80°C. Serological properties and genetic characteristics regarding the toxigenicity of the *V. cholerae* and *V. fluvialis* strains listed in Table 3 were investigated by R. Siebeling, Department of Microbiology, Louisiana State University, and J. B. Kaper, Center for Vaccine Development, University of Maryland School of Medicine, respectively. In this report, the terms toxigenic and nontoxigenic refer to the presence and absence of genes encoding for CT.

Animals. Three-day-old Swiss-Webster and CF1 strains of mice were obtained from Laboratory Animal Resources of Oregon State University. Most of the experiments were performed with the former strain of mice. There was no significant difference in the sensitivity of the mouse strain to test organisms as determined by mortality and diarrhea production. The FA ratio was slightly but consistently higher in the Swiss-Webster strain by approximately 0.0053. For the purpose of comparison, the FA ratios obtained with CF1 mice were adjusted to those of the Swiss-Webster mice by the addition of 0.0053.

FA ratio. Frozen cultures were reconstituted on tryptic soy agar (Difco) with 0.5% added sodium chloride and were incubated at 25°C for 24 h. A transfer was made into 10 ml of TSB' and incubated in stationary culture at 25°C for 12 h. Then, 0.1 ml of the broth culture was transferred into 40 ml of fresh TSB' in a 250-ml Erlenmeyer flask and incubated with agitation at 200 rpm on a reciprocal shaker (New Brunswick Scientific Co.) at 37°C for 18 h. The final cell concentration reached 1.0×10^{10} to 2.7×10^{10} CFU/ml. Culture filtrates were prepared by centrifugation of the broth culture with a clinical centrifuge (Microfuge B; Beckman Instruments, Inc.) and filtration through a 0.2-μm membrane filter (Acrodisk; Gelman Science, Inc.). Animals were inoculated intragastrically, and FA ratios were determined by the method of Baselski et al. (1) with modifications as follows. Three-day-old suckling mice were submitted to experiments within 2 h after they were removed from their mothers. Portions (0.1 ml) of cell culture or culture supernatant with Evans blue dye added (0.01%, wt/vol) were administered into the stomach of each test animal via polyethylene intramedic gastric tubing (PE60, Clay Adams). Five inoculated mice were held in the absence of their mothers at 25°C until examination. The intestines and stomachs were pooled from the animals of each group. FA was expressed as the ratio of the weight of stomachs plus intestines to the remaining body weight. Selected strains of O-1 and non-O-1 *V. cholerae* and *V. fluvialis* were used to compare the effect of whole culture and cell-free culture filtrates at 4 and 12 h post-administration into test animals. Purified CT diluted in TSB' (Sigma Chemical Co., 100 μg/ml) and *E. coli* H10407 capable of producing both heat-stable toxin (ST) and LT were used as positive controls (20). Sterile TSB' was employed as a negative control.

Kinetics of FA ratios. FA ratios induced by the cell cultures of selected toxigenic and nontoxigenic strains of O-1 and non-O-1 *V. cholerae* were determined at 4, 8, 12, and 16 h as described above.

Neutralization test for CT. Horse anti-choleragenoid serum prepared by Finkelstein (9) was obtained from J. I. Smith of the Clinical and Epidemiological Studies Branch, National Institute of Allergy and Infectious Diseases. A 1-ml portion of this anti-choleragenoid serum would neutralize between 53 and 140 μg of choleragen in the guinea pig skin test (J. I. Smith, personal communication). In our laboratory, the potency of the anti-choleragenoid was measured by inhibition of the bluing reaction in guinea pig skin (5). A 0.1-ml portion of CT (Sigma catalog no. C-3012, lot no. 69C-0398) diluted 1:50 (20 μg of protein per ml) in phosphate-buffered saline (PBS) gave a bluing reaction 15 mm in diameter. This reaction was completely neutralized by mixing equal volumes of the diluted CT solution with anti-choleragenoid diluted to 10^{-3} in PBS. Anti-choleragenoid diluted to 10^{-4} did not prevent the bluing reaction.

The in vivo effect of anti-choleragenoid on FA in suckling mice was determined as follows. Three-day-old nonsibling mice were pooled and randomly assigned to 24 test groups. The test organisms were grown as described for FA ratio determinations. CT was diluted 10-fold in TSB' (100 μg of protein per ml) and employed as a positive control; TSB' was used as a negative control. The concentrated anti-choleragenoid was added to the test material (living cells, CT, or TSB') at a ratio of 1:9 (vol/vol), and 0.1 ml of the mixture was administered to each of the test animals within 30 min. Bacterial cultures, CT, and TSB' were also treated with 0.01 M PBS, pH 7.2, in place of anti-choleragenoid and were given to mice. Five animals in each test group were sacrificed at 4 and 12 h, and the FA ratio was determined for each animal. The FA ratios obtained with and without added anti-choleragenoid were statistically compared by the Student *t* test with a pooled sample estimator of population variance (18).

Mortality and diarrhea. The ability of cultures to induce death was measured on groups of five 3-day-old mice. Each mouse was given 0.1 ml of bacterial culture orally and was incubated on a white filter paper at 25°C for 18 h. Mortality was recorded as −, +, or ++ if no or one, two or three, or four or five animals, respectively, died. Likewise for diarrhea scores, no or one, two or three, and four or more spots of feces stained with Evans blue dye observed on the filter paper were indicated, respectively, as −, +, and ++.

Lethal doses and stained feces doses. The test organism grown in TSB' as described above was diluted 10-fold in PBS. Three to five groups of five 3-day-old mice were given 0.1 ml of each dilution per mouse intragastrically. The 50% lethal dose (LD_{50}) was determined by the method of moving averages (19). The effective oral dose producing stained feces (SF_{50}) was defined as the number of cells that caused a 50% stained feces response. The −, +, and ++ responses for feces were quantitated by grading as 0, 50, and 100% responses, respectively, in calculating the SF_{50}.

Y-1 assays. Preparations of cell cultures, culture supernatants, and heated supernatants (56°C, 10 min) of the test organisms were examined for cytotoxic and cytotonic responses in Y-1 mouse adrenal tumor cells as previously described (17), except that the test

TABLE 1. FA ratios in 3-day-old suckling mice

Organism[a]		Tox gene[b]	Test material[c]	FA ratio	
Species	Strain no.			4 h	12 h
O-1 *V. cholerae*	569B	+	C	0.0870	0.0822
			S	0.0708	0.0632
	SG-N-7277	+	C	0.0845	0.0991
			S	0.0700	0.0635
	SG-N-7077	−	C	0.1156	0.0963
			S	0.0776	0.0650
	1074-78	−	C	0.0989	0.0936
			S	NT[d]	NT
	1196-78	−	C	0.0843	0.0703
			S	NT	NT
Non-O-1 *V. cholerae*	N-53	−	C	0.1007	0.0942
			S	0.0733	0.0627
	WA-0-001	−	C	0.0914	0.0858
			S	0.0761	0.0651
	WA-0-028	−	C	0.0993	0.0887
			S	0.0713	0.0703
	N-2002H	+	C	0.0921	0.0818
	N-2030H	−	C	0.1022	0.0695
	N-3	−	C	0.1186	NT
	1S7-1	−	C	0.0932	NT
	1W4-1	−	C	0.0998	NT
V. fluvialis	DJVP6957	−	C	0.0909	0.0729
			S	0.0595	0.0621
	H-5	−	C	0.0990	0.0766
			S	0.0677	0.0678
	5125	−	C	0.0834	NT
	LSU 10-41C	−	C	0.0857	NT
	2386	−	C	0.0897	NT
CT				0.0920	0.1120
E. coli	H10407	NT	C	0.1177	0.1276
			S	0.1104	0.0861
	701	NT	C	0.0625	0.0668
			S	0.0633	0.0631
C. freundii	3321	NT	C	0.0694	0.0634
			S	0.0694	0.0620
TSB'				0.0686 ± 0.0061[e]	0.0640 ± 0.0070[e]

[a] Test organism was grown in TSB' (see text) for 18 h at 37°C with agitation at 200 rpm.
[b] Presence (+) or absence (−) of the genes encoding for CT, i.e., hybridizing to LT probe of *E. coli* (J. B. Kaper, personal communication).
[c] Each of the five 3-day-old mice received 0.1 ml of whole culture (W) or culture supernatant (S) intragastrically.
[d] NT, Not tested.
[e] Average of three determinations.

organisms were grown in brain heart infusion (Difco) with shaking at 150 rpm at 37°C overnight.

RESULTS

FA ratios. FA ratios induced by CT were higher at 12 h than at 4 h (Table 1). *E. coli* H10407 elicited FA ratios of 0.1104 to 0.1177 at 4 h of incubation. The ratio was still high after 12 h, when whole cell culture was given to the animals, but effects of ST in the culture filtrates became less evident at 12 h. On the other hand, the drinking water isolates (*E. coli* 701 and *C. freundii* 3321) and culture medium (TSB') that served as negative controls showed FA ratios ranging from 0.0620 to 0.0755. Therefore, we considered FA ratios of <0.08 as negative, 0.08 to 0.09 as positive, and ≥0.09 as strongly positive. Whole cultures of five strains of O-1 *V.*

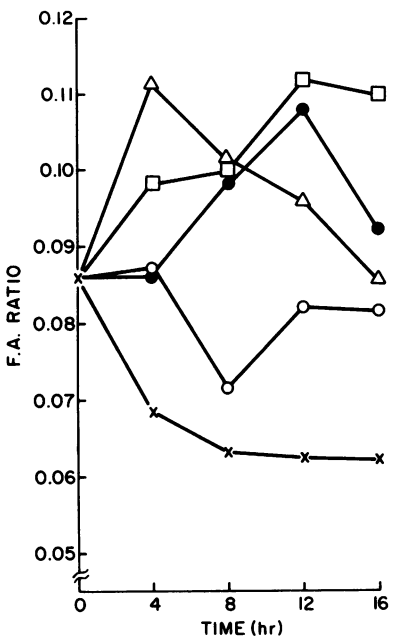

FIG. 1. Kinetics of FA ratios induced by O-1 *V. cholerae*. Test organism was grown in TSB' (see text) for 18 h at 37°C with agitation at 200 rpm. Each animal was given 0.1 ml of the test material intragastrically. Five animals were sacrificed for each FA ratio determination. Symbols: □, strain SG-N-7277 (toxigenic); ○, strain 569B (toxigenic); △, strain SG-N-7077 (nontoxigenic); ●, CT (100 μg/ml of TSB'); ×, control (TSB').

cholerae had positive FA ratios at 4 h and negative or positive ratios at 12 h, whereas culture supernatants had negative FA ratios at 4 and 12 h. The whole cell culture of non-O-1 *V. cholerae* N-53, WA-0-001, and WA-0-028 and *V. fluvialis* DJVP6957 and H-5 exhibited higher FA ratios at 4 h than at 12 h. None of these culture supernatants caused a positive response. High FA ratios at 4 h were also observed when the whole cell cultures of additional strains of each of these organisms were tested. The FA ratios induced by non-O-1 *V. cholerae* were generally higher than those caused by *V. fluvialis* (Table 1).

Kinetics of FA ratios. Figure 1 depicts the kinetics of FA ratios induced by two toxigenic strains (569B and SG-N-7277) and a nontoxigenic strain (SG-N-7077) of O-1 *V. cholerae* and by CT. The peak at 12 h resulting from the administration of whole cell culture of the toxigenic strain SG-N-7277 was closely similar in time to the peak induced by CT alone. On the other hand, the nontoxigenic strain SG-N-7077 caused rapid FA by 4 h, and the ratios declined significantly thereafter. Toxigenic strain 569B induced little activity, but ratios remained significantly above control levels in the period from 12 to 16 h. Figure 2 compares the time course of FA ratios induced by two strains of non-O-1 *V. cholerae* (toxigenic strain N-2002H and nontoxigenic strain N-2030H) and by CT. Both strains induced peak FA ratios at 4 h. Only the toxigenic strain N-2002H exhibited positive FA ratios after 8 h, which corresponded in time to the activity caused by CT. The level of FA was analogous to that of strain 569B (Fig. 1). To confirm that the factor(s) produced at 4 h is distinct from CT, in vivo neutralization tests were carried out with anti-choleragenoid (Table 2). Stimulation of FA owing to purified CT was completely eliminated at both 4 and 12 h. Neutralization of CT produced in vivo was observed only with toxigenic strains of O-1 *V. cholerae* 569B and SG-N-7277 at 12 h. FA ratios induced by the toxigenic non-O-1 strain N-2002H, however, were not affected by anti-choleragenoid treatment. It is evident that some factor that causes high FA ratios at 4 h is independent of CT. Toxigenic and nontoxigenic strains of non-O-1 as well as O-1 *V. cholerae* produced this factor.

Mortality and diarrhea. Results of suckling mouse mortality tests are summarized in Table 3. All *Vibrio* strains tested except *V. cholerae* 1096-78 and *V. fluvialis* S50-1CC were lethal for three or more suckling mice. A positive control,

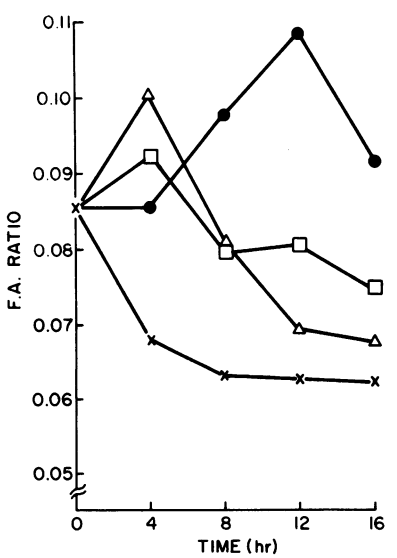

FIG. 2. Kinetics of FA ratios induced by non-O-1 *V. cholerae*. Test organism was grown in TSB' (see text) for 18 h at 37°C with agitation at 200 rpm. Each animal was given 0.1 ml of the test material intragastrically. Five animals were sacrificed for each FA ratio determination. Symbols: □, strain N-2002H (toxigenic); △, strain N-2030H (nontoxigenic); ●, CT (100 μg/ml of TSB'); ×, control (TSB').

TABLE 2. Effects of anti-choleragenoid on FA ratios induced by *V. cholerae* in 3-day-old suckling mice

Test material[a]	FA ratio[b]					
	4 h			12 h		
	Culture + PBS[c]	Culture + anti-choleragenoid[d]	Effects	Culture + PBS	Culture + anti-choleragenoid	Effects
CT[e]	0.0920 ± 0.0122	0.0654 ± 0.0055	+ ($P < 0.005$)[f]	0.1120 ± 0.0133	0.0676 ± 0.0051	+ ($P < 0.005$)
Control (TSB')	0.0678 ± 0.0041	0.0668 ± 0.0045	−	0.0625 ± 0.0024	0.0640 ± 0.0016	−
O-1 *V. cholerae* 569B (toxigenic)	0.0870 ± 0.0081	0.0820 ± 0.0165	−	0.0822 ± 0.0106	0.0713 ± 0.0088	+ ($0.050 < P < 0.100$)
O-1 *V. cholerae* SG-N-7277 (toxigenic)	0.0862 ± 0.0048	0.0912 ± 0.0105	−	0.0922 ± 0.0052	0.0808 ± 0.0094	+ ($0.010 < P < 0.025$)
Non-O-1 *V. cholerae* N-2002H (toxigenic)	0.0917 ± 0.0111	0.0958 ± 0.0051	−	0.0819 ± 0.0087	0.0800 ± 0.0051	−
Non-O-1 *V. cholerae* N-2030H (nontoxigenic)	0.0872 ± 0.0178	0.0897 ± 0.0129	−	0.0664 ± 0.0082	0.0677 ± 0.0091	−

[a] Test organism was grown in TSB' (see text) for 18 h at 37°C with agitation at 200 rpm.
[b] FA ratio of each of five animals was determined. Each animal received 0.1 ml of the test material.
[c] Culture:0.01 M PBS (pH 7.2) ratio, 9:1 (vol/vol).
[d] Culture:anti-choleragenoid ratio, 9:1 (vol/vol).
[e] Diluted in TSB' (100 μg of protein per ml).
[f] Compared by Student *t*-test with a pooled sample estimator of population variance.

TABLE 3. Lethality and stained feces deposition caused by *V. cholerae* and *V. fluvialis* in suckling mice and response of Y-1 mouse adrenal cells

Organism		Source	Tox gene[a]	Other characteristics	Suckling mouse assays[b]		Y-1 assays with supernatant[c]:	
Species	Strain no.				Diarrhea	Mortality	Unheated (1:16)	Heated (56°C, 10 min) (1:2)
O-1 *V. cholerae*	5698	Clinical, India	+	Serotype Inaba	+	+	CN[d]	–
	N-20	Estuary water, Louisiana	+	Serotype Inaba	++	++	CX[e]	(–)
	SG-N-7277	Estuary water, Louisiana	+	Serotype Inaba	++	++	CN,CX	–
	SG-N-7077	Crab, Louisiana	–	Serotype Inaba	++	++	CN,CX	–
	1074-78	Sewage water, Brazil	–	Serotype Ogawa	++	+	NT	NT
	1196-78	Sewage water, Brazil	–	Serotype Ogawa	+	–	NT	NT
Non-O-1 *V. cholerae*	N-2002H	Clinical, Louisiana	+		+	+	CN,CX	–
	N-2011H	Clinical, Louisiana	+		+	++	CN,CX	–
	N-2030H	Clinical, Louisiana	–		+	++	CX	–
	N-2031H	Clinical, Louisiana	–		++	++	CN,CX	–
	N-53	Clinical, Louisiana	–	Sakazaki serovar 8	++	++	CN,CX	(–)
	N-3	Crab, Louisiana	–		+	++	CN,CX	–
	SG-N-7210	Crab, Louisiana	–		++	++	CN,CX	–
	1S7-1	Estuary sediment, Oregon	–	Sakazaki serovar 6	++	++	CX	–
	1W4-1	Estuary water, Oregon	–	Sakazaki serovar 51	++	++	CN,CX	–
	WA-0-28	Estuary water, Oregon	–	Sakazaki serovar 8	++	++	CX	–
	2W2-1	Estuary water, Oregon	–	Sakazaki serovar 39	++	++	CX	–
	WA-0-001	Estuary water, Oregon	–	Sakazaki serovar 26	++	++	CX	–
V. fluvialis	DJV6957	Clinical, Indonesia	–	Gas[f] (–)	++	++	(–)	–
	5125	Clinical, Bangladesh	–	Gas (–)	+	++	(–)	–
	DJVP7147	Clinical, Indonesia	–	Gas (–)	++	++	–	–
	DJVP7225	Clinical, Indonesia	–	Gas (+)	+	+	(–)	–
	H-5	Estuary water, Maryland	–	Gas (–)	++	++	(–)	–
	LSU10-41C	Crab, Louisiana	–	Gas (–)	++	++	–	–
	S50-1CC	Estuary sediment, New York	–	Gas (–), nonmotile	–	–	(–)	–
	2386	Estuary water, England	–	Gas (+)	++	++	(–)	–
	LSU 9-26a	Crab, Louisiana	–	Gas (+)	++	++	(–)	–
E. coli	H10407	Clinical, Bangladesh	NT[g]	ST, LT[h]	++	++	CN	–
	701	Drinking water, Oregon	NT		–	–	–	–
C. freundii	3321	Drinking water, Oregon	NT		–	–	–	–

[a] Presence (+) or absence (–) of the genes encoding for CT, i.e., hybridizing to LT probe of *E. coli* (J. B. Kaper, personal communication).

[b] Test organism was grown in TSB′ (see text) at 37°C for 18 h with agitation at 200 rpm. Each of five 3-day-old mice received 0.1 ml of the bacterial culture intragastrically. Inoculated animals were incubated at 25°C for 18 h. Signs are explained in the text.

[c] Test was performed by the method of Maneval et al. (17) with minor modifications. +, >50% of cells exhibited positive changes; (–), <50% of cells exhibited positive changes; –, no positive cells.

[d] Cytotonic when culture supernatant was diluted 1:16 based on morphological appearance. Toxin was not confirmed as CT by anti-choleragenoid neutralization or by trypan blue exclusion.

[e] Cytotoxic.

[f] Gas production from glucose. (–), Negative; (+), positive.

[g] NT, Not tested.

[h] Capable of producing ST and LT.

E. coli H10407, caused death to all test mice; *E. coli* 701 and *C. freundii* 3321, employed as negative controls, did not cause mortality.

Watery diarrhea was discharged from the mice that received enterotoxigenic *E. coli* H10407 and from those that received purified CT. Feces of a mucoid, pasty consistency was excreted from animals that were given nontoxigenic O-1 or non-O-1 *V. cholerae* or *V. fluvialis*. Mice that were given toxigenic O-1 *V. cholerae* excreted mucoid, pasty feces first, followed by watery diarrhea. Animals that were given sterile TSB', *E. coli* 701, or *C. freundii* 3321 did not excrete feces or Evans blue dye.

Y-1 assay. *E. coli* H10407, which produces LT, was cytotonic for Y-1 adrenal cells. The majority of the toxigenic strains and some nontoxigenic strains of *V. cholerae*, when their culture supernatants were diluted (1:2 to 1:16) to reduce the cytotoxic effect, exhibited a cytotonic effect on Y-1 cells (Table 3). With one exception, *V. cholerae* N-20, the presence of the CT gene and cytotonic effect in Y-1 cells were in good agreement. With the exception of *V. cholerae* 569B, all of the strains tested, including *V. cholerae* N-20, SG-N-7077, and SG-N-7277, showed cytotoxicity to Y-1 cells. The effect of *V. fluvialis* on Y-1 cells was essentially negligible; <50% of cells were affected at minimal dilutions. Cytotonic and cytotoxic factors were inactivated by heat treatment at 56°C for 10 min.

LD_{50} and SF_{50}. The LD_{50} values ranged from 10^7 to 10^9 CFU and were about 1 log higher than the SF_{50} values obtained for each test strain. Also, strains of non-O-1 *V. cholerae* generally had lower LD_{50} and SF_{50} values than the strains of *V. fluvialis* (Table 4).

DISCUSSION

Until a few years ago, non-O-1 *V. cholerae* was generally termed either noncholera vibrio or nonagglutinating vibrio. These terms are no longer applicable since there are groups of *V. cholerae* other than O-1 which are genetically and physiologically indistinguishable from O-1 *V. cholerae*. In most instances, non-O-1 *V. cholerae* do not produce CT but do possess other mechanisms, possibly including toxins, which make them pathogenic for humans (3). Spira et al. (26) found CT-producing O-1 and non-O-1 *V. cholerae* during their clinical study in Bangladesh. Conversely, non-CT-producing O-1 *V. cholerae* have been isolated from human and environmental sources (26). Therefore, it is becoming more and more apparent that not all diarrheal disease caused by *V. cholerae* is attributable to the well-characterized CT.

A significant finding in our system was that high FA ratios resulted from the administration of whole cell cultures of non-O-1 as well as of O-1 *V. cholerae* as early as 4 h post-inoculation and that all tested strains of *V. cholerae*, regardless of their source of isolation and toxigenicity, elicited intestinal FA. Also, the whole cell culture of all tested strains of *V. fluvialis* isolated from both clinical and environmental origins produced positive FA ratios at 4 h, although the amount of FA was less than with *V. cholerae* strains. In the suckling mouse model of Baselski et al. (1), orally administered CT-producing O-1 *V. cholerae* cultures induced significantly high FA ratios at 8 h and later. Our results suggest the detection of another factor, distinct from CT. The factor reported in this study was shown to be different from CT by FA kinetics (12 h) and by in vivo neutralization tests; e.g., FA by strain SG-N-7277 was not reduced at 4 h by anticholeragenoid but was significantly reduced at 12 h from an FA ratio of 0.0922 to 0.0808.

Spira et al. (26) found two strains of non-O-1 *V. cholerae* isolated from Chesapeake Bay which produced an ST similar to that of *E. coli*. The non-O-1 *V. cholerae* toxin caused peak FA by 4 h in the suckling mouse assay and the rabbit ileal loop. The factor responsible for the FA at 4 h in our test system appears to differ from this ST-like toxin of *V. cholerae* and the ST of enterotoxigenic *E. coli* in that it was not present in the filtrate of the bacterial cultures.

None of the orally administered culture filtrates of *V. cholerae* and *V. fluvialis* induced FA, a finding which substantiates that of Baselski et al. (1). Apparently, detectable FA caused by the concentrations of toxins found in culture broths required the presence of bacterial cells, in contrast to the responses observed when purified CT was administered. Interestingly, the toxigenic strain N-2002H, a non-O-1 *V. cholerae*, was not noticeably affected by anti-CT treatment and caused approximately the same levels of FA before and after treatment. Recently, it was found that the toxin genes of this strain behaved somewhat differently from those of O-1 *V. cholerae* in gel electrophoresis (J. B. Kaper, personal communication).

Almost all strains of O-1 and non-O-1 *V. cholerae* and *V. fluvialis* induced diarrhea (stained feces) and were lethal to mice. The single *V. fluvialis* strain unable to induce diarrhea or mortality was nonmotile. Motility in *V. fluvialis* may also be significant in virulence, as previously reported for *V. cholerae* (12, 29). Also, regardless of CT production, there was a consistent correlation of diarrhea production, mortality, and ability to induce 4-h FA in mice. It is tempting to speculate that these responses are caused by the same factor(s).

The Y-1 cytotonic reactions produced by vibrios which lack CT genes is a most interesting enigma. The morphological response is clearly

TABLE 4. LD_{50} and SF_{50} in suckling mice for selected strains of *V. cholerae* and *V. fluvialis*.

Text organism[a]		Tox gene[b]	LD_{50} (CFU)	SF_{50} (CFU)[b]
Species	Strain no.			
O-1 *V. cholerae*	N-20	+	4.28×10^8	2.13×10^7
	SG-N-7077	−	5.50×10^8	3.48×10^7
Non-O-1 *V. cholerae*	N-2002H	+	4.68×10^8	4.68×10^6
	N-53	−	1.11×10^7	1.11×10^6
	N-3	−	7.02×10^7	1.41×10^7
	WA-0-028	−	3.94×10^8	4.96×10^7
V. fluvialis	DJVP6957	−	6.01×10^8	3.79×10^7
	H-5	−	1.61×10^8	4.05×10^7
	LSU 10-41C	−	1.88×10^9	1.49×10^8

[a] Test organism was grown in TSB′ (see text) at 37°C for 18 h with agitation at 200 rpm. The culture was diluted 10-fold in 0.01 M PBS, pH 7.2. Five 3-day-old mice were given 0.1 ml of each dilution intragastrically. Inoculated animals were incubated at 25°C for 18 h.

[b] Presence (+) or absence (−) of the genes encoding for cholera toxin, i.e., hybridizing to LT probe of *E. coli* (J. B. Kaper, personal communication).

[c] Effective dose for stained feces. See text for definition.

not due to CT production. We wish to designate these morphological reactions with Y-1 cells as cytotonic-like to distinguish this response formally from that produced by CT. The cytotonic-like morphological reactions are similar to the reactions observed with other cultures (S. W. Joseph, S. T. Donta, D. R. Maneval, J. B. Kaper, R. R. Colwell, and W. M. Spira, *in* R. R. Colwell, ed., *Vibrios in the environment*, in press).

Since all the *V. cholerae* tested induced rapid 4-h FA in mice and nearly all strains produced the cytotoxic response in Y-1 cells, it is conceivable that both responses are due to the same factor. However, this possibility seems unlikely since the unique morphological change in Y-1 cells was caused by culture supernatants, whereas no FA was observed in suckling mice unless living *Vibrio* cells were administered.

The nontoxigenic O-1 *V. cholerae* strains used in the present studies (1074-78 and 1196-78) were isolated from sewage in São Paulo, Brazil. There was no known cholera outbreak associated with their isolation. These same strains were also used in human volunteer ingestion experiments, and neither caused any of the symptoms associated with cholera (15). The clinical relevance of the 4-h FA virulence-associated factor needs to be discussed in regard to these observations. Both strains induced the 4-h FA response and were cytotoxic in mice but were not cytotonic in Y-1 assays (J. B. Kaper, personal communication). The lack of virulence in the human volunteer studies may likely be due to the inability of these strains to colonize human intestines (15). Only 40% (8 of 20) of the volunteers excreted vibrios, and those cases were only detected for 1 or 2 days after ingestion. Although the clinical relevance of the virulence-associated factor remains to be verified, we do not believe that its significance can be discounted by the outcome of the human volunteer studies. Strain 1196-78 also induced the smallest amount of FA of any *V. cholerae* strain tested in this study, whereas 1074-78 produced an FA ratio of 0.0989, about midrange for the cholera vibrios.

In summary, this study has revealed a new virulence-associated factor, produced by environmental and clinical strains of *V. cholerae* and *V. fluvialis*, which induces rapid FA in suckling mice. In mice, the factor produces peak FA in 4 h, followed, in toxigenic strains, by a second round of CT-induced FA which peaks at 8 to 12 h. This factor may be the substance or one of several substances contributing to *Vibrio* diarrheal disease in humans infected by nontoxigenic *V. cholerae*, but this is not verified at present. It is also not clear whether the cytotoxic factor induced in Y-1 cells is caused by this or other virulence factors. Studies are currently under way to purify this factor and help resolve some of the remaining questions of its mode of action and effect on various bioassay systems.

ACKNOWLEDGMENTS

The authors wish to express appreciation to Rita R. Colwell, Henry H. Bradford, Nell Roberts, Ronald J. Siebeling, and Thomas M. Evans for providing bacterial cultures. The authors also thank J. B. Kaper for the CT gene hybridization analyses.

This research was supported by the Oregon State University Sea Grant College Program, National Oceanic and Atmospheric Administration Office of Sea Grant, U.S. Department of Commerce, under grant number NA79AA-D-00106, project number R/FSD8.

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