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For the enrichment and enumeration of Vibrio fluvialis, a broth medium was designed by modifying alkaline peptone (AP) medium. This new V. fluvialis enrichment medium (FEM) was shown to be more effective than AP medium in field samplings where a total of 177 samples (estuarine waters and sediment, sewage, and crabs) were processed over a 14 month period. FEM was particularly superior to AP for water and sewage with low salinities (<6‰). V. vulnificus was shown to have a species-specific antigen by analyzing sonicated whole-cell antigens with two-dimensional immunoelectrophoresis. The antigen (designated as VVA) was purified by various protein chemistry techniques, and specific antiserum to VVA was prepared. Using anti-VVA serum, a simple and rapid microimmunodiffusion method was designed which allowed the specific identification of V. vulnificus in as early as 10 hrs after preparing a bacterial cell lysate from a single colony. A

suckling mouse assay was utilized to examine enteropathogenicity, i.e., potential to cause diarrhea, of environmental and clinical isolates of O-1 and non O-1 V. cholerae, V. mimicus, and V. fluvialis. Cultures from both environmental and clinical origins induced intestinal fluid accumulation (FA) in 3-day-old mice at 4 hr postinoculation followed by diarrheal feces and high mortality. The virulence-associated factor(s) that caused FA were different from cholera toxin (CT) in terms of kinetics of FA and serological behavior, but were correlated with extracellular cytotoxic factors active against Y-1 mouse adrenal tissue cultures. Furthermore, all clinical isolates of non O-1 V. cholerae, V. mimicus, and V. fluvialis, when grown in brain heart infusion broth supplemented with 0.5% sodium chloride, were found to produce a new extracellular heat-labile enterotoxin which induced significant FA in 3-day-old mice and was distinct from previously reported enterotoxins including CT. Most of the environmental isolates, however, did not produce the newly discovered enterotoxin.

Human Health Significance of Vibrios from the Aquatic Environment

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CONTRIBUTION OF AUTHORS

In Chapter II, Nell C. Roberts and Henry B. Bradford, Jr. conducted the field samplings to test the efficacy of the new enrichment medium for *Vibrio fluvialis*.

In Chapter IV, D. M. Rollins and S. W. Joseph performed Y-1 adrenal cell assay to compare cytotoxic and/or cytotonic factors with the virulence-associated factors that were found in the suckling mouse assay.

HUMAN HEALTH SIGNIFICANCE OF VIBRIOS FROM THE AQUATIC ENVIRONMENT

CHAPTER I

Introduction

Literature Review on Human-Pathogenic Vibrios in the Aquatic Environment

The literature will be reviewed on the 8 species of human-pathogenic *Vibrio*. Special emphasis will be placed on their pathogenicity and ecological distribution in the natural environment.

V. cholerae is conventionally divided into 2 major groups based on the O-antigen detected by an agglutination test. Even though over 60 serovars have been detected in *V. cholerae* (95), it is the serovar 1 (hereinafter called O-1 *V. cholerae*) that induces the most common cases of severe cholera inducing "rice-water stools." The other serovars are collectively designated as non O-1 *V. cholerae* or non-agglutinable (NAG) vibrios because they do not agglutinate with anti-O1 serum. The non O-1 strains have also been designated as non-cholera vibrio (NCV) because historically only O-1 *V. cholerae* has been considered as an etiological agent of Asiatic cholera. O-1 *V. cholerae* is further subdivided into 2 biotypes based on its hemolytic property: classical (nonhemolytic) and El Tor (hemolytic) biotypes. The former includes early

isolates from severe cholera cases and the latter recent isolates from relatively mild disease (33).

O-1 *V. cholerae* has been the subject of extensive studies since 1883 when Robert Koch first isolated this organism. Readers are referred to well-written books (4,85) and review articles (33,34) for information on the bacteriology, immunology, pathology, epidemiology, control, and treatment of O-1 *V. cholerae*.

In the last two decades, there has been a great advancement in understanding mechanisms of pathogenesis of O-1 *V. cholerae*. Since several timely reviews (24,35,57,69) document the progress of these studies, only a brief survey will be presented below. Until cholera toxin (CT) was discovered, there had been much controversy over the pathogenic mechanism(s) of O-1 *V. cholerae*. As reviewed by Lankford (70), many virulence factors such as mucinase, neuraminidase, endotoxin, and fimbriae had been suggested to explain pathogenesis. Above all, mucinase, which causes epithelial desquamation in the small intestine (13), attracted intense attention by many workers. The inherent weakness of mucinase as the primary mechanism of pathogenesis was pointed out by the fact that non O-1 *V. cholerae* isolates from the aquatic environment also produce this enzyme (81,97) and, more significantly, intestine biopsies from cholera patients revealed little, if any, degeneration (42).

In the early 1950s, symptoms similar to cholera diarrhea were experimentally reproduced with viable bacterial cells in the rabbit

ileal loop model (27) and infant rabbit model (30). Injection into the surgically segmented ileum (loop) caused distention of the loop due to outpouring of fluid while intraintestinal injection in infant rabbits caused fatal diarrhea. In 1965, Craig (23) found a heat-labile factor called permeability factor (PF) in filtrates of cholera stools and spent culture medium of O-1 V. cholerae. The factor caused induration and increased permeability in the skin of guinea pigs and rabbits. The skin reaction was neutralized by convalescent sera from cholera patients. Definitive evidence that an extracellular product related to cholera diarrheal disease was presented by Finkelstein et al. (37) who demonstrated cholera-like diarrhea in the infant rabbit model with culture supernates of O-1 V. cholerae. Subsequent purification of the protein enterotoxin, i.e., CT, from the culture supernate (36) eliminated other previously proposed hypotheses.

Up-to-date information (69) suggests that CT, with a molecular weight (MW) of 84,000, consists of 2 major components: subunit A (MW: 28,000-30,000) and subunit B (MW: ca. 56,000). Subunit A is composed of 2 smaller units A1 and A2 and are surrounded by subunit B which consists of 5 equal smaller units (MW of each: 10,300-14,000). The molecule lacking subunit A is named choleragenoid in contrast to choleragen which is a holotoxin containing both subunits A and B. Subunit B plays a role in attaching CT onto the intestinal membrane by combining with the receptor, GM1 ganglioside (glycoprotein), on the intestinal cell. Subunit A2 helps subunit

A1 to transverse the membrane to an intracellular site where it activates the enzyme adenylate cyclase. Activation of adenylate cyclase by subunit A2 is initiated with ADP-ribosylation of an unidentified regulator protein by NAD. The ADP-ribosylation is linked to the inhibition of GTP hydrolysis on adenylate cyclase, and thus the enzyme is activated. (GTP, when bound to the regulatory site of adenylate cyclase, activates the enzyme but, when hydrolysed to GDP, it is dissociated from the site and the enzyme maintains a basal level.) Activated adenylate cyclase causes derangement of ion transport of intestinal cells and thus stimulates hyper-secretion of chloride, bicarbonate, and water resulting in fluid accumulation (FA) within the intestinal lumen. That is why CT can be conveniently assayed with tissue cultures which respond to activated adenylate cyclase, e.g., induction of steroidogenesis in Y-1 adrenal cell culture (29), lysis of pigeon erythrocytes (43), and elongation of Chinese hamster ovary (CHO) cell cultures (47). CT and heat-labile enterotoxin (LT) of Escherichia coli are similar in their structure, biological activity and immunological reactivity (19,20). A gene probe encoding E. coli LT can be used to detect CT genes in isolates of O-1 V. cholerae (64) because of the DNA sequence homology of the LT and CT. Recently, CT genes were cloned from the chromosomal DNA of O-1 V. cholerae biotype El Tor (62).

Since the mechanism of fluid secretion from intestinal cells induced by CT is now almost understood and a new concept that

bacterial cells have to be in close contact with the host cells for effective delivery of a toxin to the target cells has been developed (7,16), a line of studies on the pathogenesis of O-1 V. cholerae has been focused on mechanisms which establish bacterial infection. These include the influence of gastric acidity on the effective oral dose in human volunteers (15), the role of chemotaxis in localizing bacterial cells on mucosal surfaces (3), the role of bacterial motility in pathogenesis (45,108), the association of bacterial cells with mucosal surfaces (58,92), the role of somatic antigens in adhesion (17), bacterial adhesion and the receptors on intestinal cells (48,59), virulence factors (proteases, mucinase, neuraminidase, etc.) associated with survival and multiplication of bacterial cells in the intestine (5,91), inhibition of O-1 V. cholerae multiplication by normal intestinal flora (96), and kinetics of multiplication and mechanical clearance of bacterial cells in the intestine (46,82).

The ecology of O-1 V. cholerae had been exemplified by a conventional notion that the human is the only reservoir and the occurrence of the organism in the extraintestinal environment (water, sewage, food) is a result of spread by human carriers (32). In the United States, therefore, people have been cautious about introducing the organism by travelers coming from endemic areas in Southeast Asia (100). However, there is a growing hypothesis that O-1 V. cholerae has become or always was an autochthonous aquatic organism. O-1 V. cholerae has been isolated

from aquatic environments where there was no report of cholera in England (6), in Maryland and Louisiana (22), and in Florida (53). Interestingly, some of the environmental isolates were nontoxigenic in bioassays and did not possess CT genes (64). Investigations on clinical and environmental (shellfish, water, sewage, etc.) isolates associated with cholera outbreaks in the U.S. Gulf Coast since 1973 revealed that cultures were identical in terms of their hemolytic properties, phage sensitivity (9), and CT genes (61). Furthermore, there are reports that indicate O-1 *V. cholerae* has the ability to persist in the natural aquatic environment. The organism survived in live shellfish under simulated natural conditions (40), and was absorbed onto and multiplied on chitin (80).

Non O-1 *V. cholerae* is isolated from humans, water, animals, fish, foods, and other environments in many parts of the world (88,95). In the ecological studies conducted in the United States (21), Germany (78), and Hungary (102), the ubiquitous distribution of non O-1 *V. cholerae* in the aquatic environment suggested this organism is an autochtonous estuarine bacterium. The incidence of O-1 *V. cholerae* was lower in the cool season and was limited to water with certain salinity ranges (21,78).

Non O-1 *V. cholerae* has also been isolated from the stools of diarrheal patients in outbreaks or sporadic cases which occurred in many countries (11). Generally non O-1 *V. cholerae* disease is not as severe as that of O-1 *V. cholerae* (2,54). In many cases the

vehicle of transmission was suspected or verified to be seafood (11,54). Despite epidemiological findings, the pathogenic mechanism(s) of non O-1 V. cholerae has not been clearly illustrated. Some isolates from clinical cases (111) and from the environment (25) produce PF that is serologically similar or identical with CT, and in fact some strains of non O-1 V. cholerae possess CT gene sequences detectable by the gene probe encoding E. coli LT (64). Most clinical isolates, however, do not produce CT (101).

Many workers have demonstrated the presence of virulence factors in clinical isolates of non O-1 V. cholerae with bioassay systems often used to detect enterotoxins. These include FA factor(s) in rabbit ileal loops (8), PF (83), hemorrhagic factor (83), and factors that cause changes in cellular morphology of CHO cells and Y-1 adrenal cells (101). However, nonclinical isolates (from healthly humans, domestic animals, water, sediment, and shellfish) also produce these factors (18,63,98,101). Therefore, at present there is no definitive assay to measure potential enteropathogenicity of non O-1 V. cholerae isolates (11).

V. mimicus, a recently described species, is phenotypically very similar to V. cholerae except it does not ferment sucrose (26). The organism, consisting of a wide variety of O-serotypes (99), was isolated from shellfish, water, human diarrhea, and wound infections in the United States, Mexico, New Zealand, Guam, Canada, and the Orient (26). Diarrheal cases are linked to consumption of

shellfish (26). Five out of 24 human stool isolates were found to produce enterotoxins similar to those of E. coli LT or ST and were detectable by the Y-1 adrenal cell assay (29), the enzyme-linked immunosorbent assay (109), or by the infant mouse assay (28).

V. parahaemolyticus was first isolated from improperly preserved sardines that caused food poisoning in 1950 in Japan (38). In the same country where seafoods are widely consumed, V. parahaemolyticus was responsible for 47-75% of bacterial cases of food poisoning during the years 1963-1972 (84). In the last decade, gastroenteritis associated with V. parahaemolyticus has been reported in North and Central America, Africa, Europe, and Asia (11). Major symptoms of gastroenteritis include watery diarrhea or dysenteric syndrome with mucoid or sanguinous stools (11). The organism is also isolated from some extraintestinal infections (11).

Pathogenicity of V. parahaemolyticus was initially found to be associated with the Kanagawa phenomenon (hemolysis on a special blood agar "Wagatsuma agar") (89). Later this Kanagawa phenomenon was shown to be due to a heat-stable hemolysin with a molecular weight of 42,000 (52). However, the significance of the Kanagawa phenomenon in the epidemiology of diarrheal disease has been doubted by some workers (11). As for bioassays, neither live bacterial cells nor culture filtrates induce FA in the rabbit ileal loop model (14,56). Possible virulence factors which have been experimentally demonstrated include the heat-labile factor that

caused positive CHO-cell reactions (51), the ability to penetrate the intestinal epithelium of infant rabbits (14), and adherence to human epithelial cells (44). However, no conclusive evidence can explain the pathogenic mechanisms of V. parahaemolyticus (11).

V. parahaemolyticus has been isolated from the estuarine and marine environments (water, sediment, fish, mollusks, plankton, etc.) throughout the world (39,11). The cell numbers of V. parahaemolyticus in the estuarine environment undergo a unique seasonal change. The organism over-winters in the sediment and, as the temperature rises, it is released from the sediment and becomes attached to zooplankton and proliferates in the water in warm seasons (60). Therefore, V. parahaemolyticus-associated food poisoning due to consumption of contaminated seafoods occurs mostly in summer (68).

V. alginolyticus, a biotype of V. parahaemolyticus (94), is widely distributed in the marine environment (11). Although it was isolated from occasional wound infections and an ear infection, its role in pathogenesis is not clear (11).

A species named V. vulnificus was established by Farmer (31) in 1980. It includes a group of Vibrio previously reported as lactose-positive or lactose-fermenting halophilic vibrios (50,103) including the isolates originally misidentified as V. parahaemolyticus (87,106,110). The organism was isolated from wound infections and septicemia in humans in the United States (10), Japan (75), and Belgium (76). Most patients with wound

infections recover but there is a 46% fatality rate with septicemia in the United States (10). Many of the infected had underlying diseases such as diabetes, alcoholism, chronic heart failure, and some hepatic diseases (10). Epidemiological studies implicate contaminated seawater and crabs in wound infections and consumption of raw oysters in septicemia cases (10).

Recently, V. vulnificus was isolated from a drowning victim and from seawater in Texas (65) and from crab feces in Oregon and Louisiana (105), suggesting that the reservoir of V. vulnificus is the marine environment. Also, vibrios isolated from diseased eels cultured in estuarine ponds in Japan (79) were found to be a new biotype of V. vulnificus (104).

Studies on the pathogenic mechanisms of V. vulnificus are currently in progress. Pathologically, severe edema and tissue necrosis were observed in biopsies of a person with septicemia (75) and in experimental mice (86) infected with V. vulnificus. Experimental injection of viable organisms into mice caused septicemia and high mortality, in which the presence of iron played an important role (107). An extreme hemoconcentration was observed in association with massive loss of intravascular fluid due to vascular permeability changes (12). Resistance of the organism to phagocytosis due to its antiphagocytic surface antigen was also suggested to play an important role in pathogenesis (66). Extracellular virulence factors that exhibit cytolytic activity against mammalian erythrocytes, cytotoxic activity for CHO cells, increased

vascular permeability in guinea pig skin, and lethal activity for mice are reported (67).

A new Vibrio species was given the name V. fluvialis in 1981 (72). These organisms were first designated as "Group F" and have been isolated from estuarine waters, shellfish, and diarrhea cases in Britain and elsewhere (41). The organism has also been isolated from many diarrheal patients in Bangladesh (55,101), from human diarrhea cases as well as aquatic environments (sewage, seawater, river water, canal water, crab, and prawn) in India (90), and from polluted coastal waters of the United States (93). Accordingly, the bacterium is considered an inhabitant of various aquatic environments and is transmissible to humans in ways which are yet to be understood.

Currently, the significance of V. fluvialis as an agent of diarrheal illness is uncertain (11). However, factors associated with non O-1 V. cholerae diarrheal infections are also produced by both clinical and environmental isolates of V. fluvialis. These include FA factor(s) in the rabbit ileal loop (1,90,93,101), CHO-cell elongation factor (73), and factors that caused positive Y-1 adrenal cell reactions (93).

V. metschnikovii, an unusual Vibrio because of its negative reaction in the Kovács oxidase test, is widely distributed in aquatic environments (rivers, estuaries, sewage, shellfish) and is sometimes isolated from human and animal intestines (71). However, there is no evidence to suggest that the organism causes enteritis

in humans or animals (71). Blake et al. (11) described this bacterium, named Enteric Group 16 by them, for a culture isolated from the blood of a woman with gallbladder disease.

In 1981, V. damsela was initially described as the causative agent of skin ulcers in the damsel fish (74). The bacterium was also isolated from marine water (74). Subsequently, it was reported that this organism was implicated in 6 human wound infections. In 5 of these cases the wounds were exposed to salt or brackish water at the time of injury. Infections are self-limited and this bacterium does not appear to have as severe pathogenic potential as V. vulnificus (77).

V. hollisae was designated as a new species in 1982 (49). It was isolated from 9 diarrheal stools in which no other enteric pathogen was identified (77). Epidemiologically, 6 out of 9 cases were traced to eating raw seafoods in the 5 days before the disease symptom started.

Statement of Research

Many microbial species isolated from clinical specimens can also be found in the natural environment. Isolates from the nonclinical environment are thus considered potentially pathogenic. However, the health significance of these so-called potential pathogens present in the environment has rarely been confirmed. Also, confusion exists over the taxonomic status of medically significant pathogens and their free-living counterparts. In addition, the mechanisms by which many pathogens cause clinical disease are at best, poorly understood.

The genus Vibrio is a good example of the problems alluded to above. Vibrios are one of the most dominant bacterial populations in the estuarine environment. In Bergey's Manual of Determinative Bacteriology (94), only 5 species are listed for the genus Vibrio, of which V. cholerae and V. parahaemolyticus (including biotypes parahaemolyticus and alginolyticus) are described as human pathogens that can be found in freshwater or marine environments. Since then there has been a considerable amount of activity in describing new species of the genus. Among them V. vulnificus (31), V. fluvialis (72), V. mimicus (26), V. metschnikovii (71), and V. damsela (74) can be listed as potentially human-pathogenic vibrios which exist in the environment. V. hollisae (49) was isolated from cases of human diarrhea. This species may also be found in estuaries since most human infections were associated with raw seafood consumption.

In order to determine the significance of these pathogens in the natural environment, the following steps must be taken: recovery of the organism from environmental samples, identification of the isolates, and assessment of pathogenicity in comparison with that of clinical isolates. For these Vibrio species which were recently described, methods of detection and enumeration are not established. Mechanisms of pathogenicity are partially understood only for O-1 V. cholerae which produces the well-characterized CT. Although several workers have reported virulence factors in the other potentially pathogenic vibrios, none of the factors can account for pathogenesis in a satisfactory fashion.

In an attempt to resolve some of the problems raised, I have undertaken several studies:

1) Development of a new enrichment medium to isolate V. fluvialis from the aquatic environment.

So far only 2 kinds of enrichment media have been used to isolate V. fluvialis. In our laboratory, however, neither of them were satisfactory. Therefore I have tried to develop a new enrichment medium that gives better recovery of V. fluvialis from the environment.

2) A search for a serological method as an aid for the simple and rapid identification of V. vulnificus.

Identification of environmental V. vulnificus cultures by routine biochemical tests is difficult and often one cannot be certain of identification until genetic analysis by DNA-DNA

hybridization experiments are performed. In addition, the course of human disease caused by *V. vulnificus* is very rapid and it is often fatal. For this reason, it is necessary to identify isolates from clinical specimens as quickly as possible. Accordingly, I have used a new approach to establish a simple and rapid identification of *V. vulnificus*.

3) Establishment of an animal model system to establish and study pathogenic mechanisms and to screen isolates for virulence associated mechanisms.

A group of vibrios that are often isolated from both human diarrhea and environmental samples are non O-1 *V. cholerae*, *V. fluvialis*, and *V. mimicus*. Currently, the mechanism by which these organisms induce diarrhea is not known, and therefore there is no way to answer the question as to whether the environmental strains are really a threat to human health. Unlike the previous studies conducted on these organisms, I have tried to reproduce diarrheal symptoms in the suckling mouse model that was originally developed to test enterotoxigenicity of other bacterial species. Whole bacterial cultures and culture supernates of clinical and environmental strains were examined for their capacity to produce diarrhea and the mechanism of diarrhea production was investigated.

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CHAPTER II

A Broth Medium for the Enrichment of *Vibrio fluvialis* from the Environment

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Abstract

A medium was designed for the enrichment and enumeration of Vibrio fluvialis from environmental samples. The medium contains 1 percent peptone plus 4% sodium chloride, and 5 µg/ml of novobiocin, pH 8.5. This V. fluvialis enrichment medium (FEM) was tested, in comparison with alkaline peptone (AP), in field samplings. A total of 177 samples (estuarine waters and sediment, sewage, and crabs) collected over a 14 month period were examined with FEM and with AP broth. Results showed that FEM was more effective than AP in detecting V. fluvialis, particularly from water and sewage samples with low salinities (<6°/oo). The greatest incidence of V. fluvialis occurred when both enrichment media were used simultaneously.

Introduction

In 1977 Furniss et al. (2) described organisms isolated from water and human diarrhea and designated them as Group F. These bacteria exhibited characteristics in common with both Vibrio and Aeromonas. The organisms were further characterized (4,5) and given the name V. fluvialis (6). This species was isolated from a number of patients with diarrhea in Bangladesh (3,13), from both human diarrhea cases and aquatic environments in India (9), and from polluted coastal waters of the United States (10). It has been reported that strains isolated from clinical specimens are anaerogenic and the strains isolated from the environment are either aerogenic or anaerogenic (2,5,10). Recent studies have shown that this organism produces various virulence factors and enterotoxins (1,7,9,10,11). Since V. fluvialis is capable of causing human disease it is desirable to have a procedure to detect this pathogen in natural environments, especially when there is a risk of transmission to humans. To the best of our knowledge, alkaline peptone (AP) broth and modified Rimler-Shotts (MRS) were used as enrichment media in previous studies (10,11). When the authors tried to isolate V. fluvialis from various aquatic samples using the above enrichment media, the former appeared superior to the latter. However, AP broth was not always satisfactory since other contaminating vibrios and terrestrial organisms, typically Aeromonas, competed with V. fluvialis. Diverse types of colonies appearing on the thiosulfate-citrate-bile salts (TCBS) agar presumptive medium also made it difficult to detect V. fluvialis.

Therefore an attempt was made to make AP enrichment broth more selective for the isolation and enumeration of this vibrio from environmental samples. In designing the improved enrichment medium, the usefulness of sodium chloride, 2,4-diamino-6,7-diisopropylpteridine (0/129), and novobiocin was assessed as additives to the basal AP medium. V. fluvialis, like other vibrios, can grow in the presence of high concentrations of sodium chloride and, like Aeromonas, is not as sensitive to 0/129 and novobiocin as other Vibrio species (2,5). A modified alkaline peptone broth was developed in the laboratory and field-tested. The overall results indicated the new V. fluvialis enrichment medium (FEM) was more effective in detecting V. fluvialis than AP broth, particularly with low-salinity water samples and raw sewage.

Materials and Methods

Bacterial strains. Reference strains used in this study are listed in Table 1. Aerogenic strains of V. fluvialis produced gas in MR-VP broth (Difco) and anaerogenic strains did not. Cultures were maintained in tryptic soy broth without dextrose (Difco) supplemented with 0.5% sodium chloride (TSB´) plus 5% glycerol and stored at -80°C. Frozen cultures were reconstituted on tryptic soy agar (Difco) supplemented with 0.5% sodium chloride (TSA´) and incubated at 25°C for 24 hr.

Effect of novobiocin, 0/129 and sodium chloride on growth. A transfer was made from TSA´ into 10 ml of TSB´ and incubated statically at 25°C for 12 hr. Then 0.1 ml of the broth culture was inoculated into 40 ml of TSB´ and incubated at 200 rpm at 25°C until the absorbance at 600 nm reached 1.0-1.2. The broth culture was diluted in phosphate buffered saline (PBS; 0.0033 M NaH_2PO_4, 0.0067 M Na_2HPO_4, 0.13 M NaCl, pH 7.2) to about 5×10^2 colony-forming units (CFU)/ml. One-tenth milliliter of the bacterial suspension was spread in duplicate over the surface of TSA´ and the test agar media, i.e., AP agar plates (peptone 1%, sodium chloride 1% unless otherwise specified, pH 8.5) containing different concentrations of novobiocin (sodium salt, Sigma), 0/129 (Calbiochem), or sodium chloride. The inoculated plates were incubated at 35°C and the number of colonies counted after 24 hr incubation. Percent relative growth was determined by comparing the CFU on the test

plate with that on the control TSA´ plate. For V. alginolyticus ATCC 17749, the control medium was AP agar with 5% sodium chloride.

Growth in FEM. FEM basal medium contained per liter: Bacto peptone 10 g, NaCl 40 g, adjusted to pH 8.5. The medium was autoclaved for 15 min at 121°C. Novobiocin solution was prepared by dissolving 0.005 g in 10 ml of sterile distilled water and added to the cooled basal medium. Test organisms were grown as described in the previous section. The broth culture in early stationary phase was diluted in PBS to 10^{-6} and 20 µl was inoculated into 5 ml of FEM in a colorimeter tube fitted with a plastic cap. The inoculated tube was incubated statically at 35°C. Growth was monitored by measuring turbidity (absorbance at 600 nm) with a Spectronic 20 (Bausch and Lomb). Absorbances below 0.03 were considered insignificant, i.e., within the variance of negative controls. Growth was also measured by determining total viable-cell numbers in FEM at incubation times of 0 hr (inoculum) and 14 hr when most V. fluvialis strains reached log phase. The number of viable cells was determined by the plate count method on TSA´.

Field tests. The efficacy of FEM, as compared with AP, in recovering V. fluvialis from environmental samples was examined by the most probable number (MPN) technique. Environmental samples were inoculated into 9 tubes of enrichment broth (3 tubes for each of three 10-fold dilutions) and incubated at 35°C for 18 hrs. Then each broth culture was streaked onto TCBS agar (Oxoid) and

incubated at 35°C. Typical yellow colonies on TCBS agar were tested in a screening medium and representative isolates were submitted to a battery of biochemical tests to identify V. fluvialis (N. C. Roberts and R. J. Seidler, Vibrios in the environment, in press). The MPN was calculated based on the number of tubes containing V. fluvialis. Sampling was performed at 20 stations in the state of Louisiana at the frequency of 2-23 times at each station in the period of November 1980-December 1981 (N. C. Roberts, R. J. Siebeling, J. Kaper, and H. B. Bradford, Microbial Ecology, in press). Samples collected included natural water (river, pond, swamp, and estuary), sewage, sediment, and crabs. Temperature and salinity of water samples ranged from 7°C to 32°C and from <1°/oo to 32 °/oo.

Results and Discussion

AP broth, originally designed for the isolation of V. cholerae (8), is used at pH 8-9 for the isolation of Vibrio species because vibrios, unlike other organisms, generally can grow at high pHs. The effect of pH on the growth of four representative strains of V. fluvialis was examined in order to verify that AP can also be an effective basal medium for the enrichment of V. fluvialis. All strains grew to approximately the same maximal levels in the pH range of 6-9. Therefore a pH of 8.5 was chosen for further development of the selective enrichment medium.

Using the AP broth as a basal medium, the effects of novobiocin, 0/129, and sodium chloride on the growth of various vibrios and aeromonads were examined (Table 2). Strains of V. fluvialis and Aeromonas spp. were resistant to novobiocin while V. parahaemolyticus CDC 8658 was moderately sensitive (no growth at 20 µg/ml) and the other vibrios were extremely sensitive. Sensitivity to 0/129 varied among strains of Vibrio spp. including V. fluvialis although all of the test strains were sensitive at the 20 µg/ml level. On the other hand, strains of Aeromonas spp. were not affected at this concentration. The growth of Aeromonas spp. was completely inhibited by 5% sodium chloride while the growth of V. fluvialis strains were variable at the same concentration. From the data obtained it was assumed that a combination of novobiocin and sodium chloride at appropriate concentrations could be used in AP medium to inhibit the growth of undesirable species while

allowing *V. fluvialis* to grow. A novobiocin concentration of 5 µg/ml was considered suitable because it totally inhibited the growth of 3 strains of *Vibrio* spp. and was moderately inhibitory to *V. parahaemolyticus* CDC A8658. Since the growth of *Aeromonas* spp. was influenced little by novobiocin or 3% NaCl, it appeared that 4% NaCl would be the concentration of choice.

In the next step, therefore, the growth of the reference strains in MAP containing 4% NaCl and 5 µg/ml novobiocin was compared. Small numbers of bacterial cells were inoculated into FEM (Table 3) and incubated statically at 35°C to simulate an actual enrichment procedure. When growth was monitored turbidimetrically, 9 out of 10 *V. fluvialis* strains, regardless of origin or aerogenicity, exhibited signs of growth in 9-15 hrs. Representative growth curves are shown in Fig. 1. *V. parahaemolyticus* CDC A8658 increased to significant turbidity levels only after 24 hr. The 9 strains of *V. fluvialis*, which showed increased turbidity in 9-15 hrs, contained total viable cells in the order of 10^7-10^8 CFU at 14 hr while *V. parahaemolyticus* CDC A8658 was still in the range of 10^5 CFU at the same incubation time (Table 3). Viable cells were not detected for *V. fluvialis* 5125 and other strains of *Vibrio* and *Aeromonas*.

The above results strongly suggest that enrichment in FEM under these conditions (static incubation, 35°C, >14 hr) should improve the detection of *V. fluvialis* by inhibiting the growth of other *Vibrio* and *Aeromonas* species.

FEM containing 4% sodium chloride and 5 µg/ml novobiocin was examined in field tests in Louisiana where V. fluvialis is often isolated from aquatic environments. A total of 177 samples were processed in a 14 month period with both FEM and AP. V. fluvialis was isolated from 71 of these samples (Table 4). Among them, 29.6% were isolated by both enrichment media, 45.1% through FEM only, and 25.4% through AP only. The superior results obtained with FEM are mainly attributable to the almost exclusive detection by FEM of V. fluvialis from sewage samples. It is suspected that this higher isolation frequency by FEM is due to the inhibition of growth of terrestrial species like Aeromonas by the high concentration of sodium chloride in FEM. Therefore the isolation of V. fluvialis from water was analyzed with regard to the salinity of the sample (Fig. 2). At low salinities ($<6^{o}$/oo) FEM was more effective than AP as illustrated by both a higher incidence of positive samples and a greater concentration recovered. At high salinities ($>6^{o}$/oo), AP may be slightly more effective than FEM as the samples generally exhibited higher MPN values with AP than FEM. This could be due to the overgrowth of novobiocin-resistant halophilic organisms. There was no apparent correlation between the MPN of V. fluvialis and other environmental factors measured such as water temperature, turbidity, dissolved oxygen, pH, and fecal coliforms (data not shown). To summarize, the results showed that FEM was an effective enrichment medium for detecting V. fluvialis from various environmental samples, particularly in samples with low salinities, including sewage.

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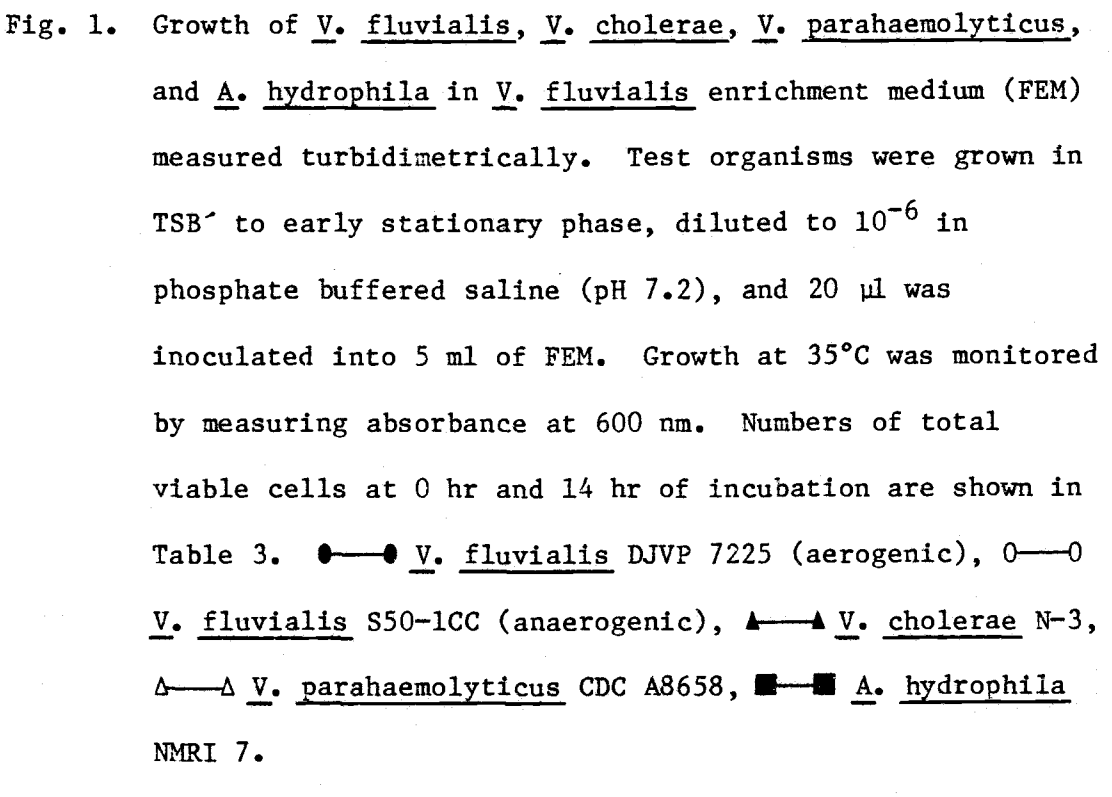

Fig. 1. Growth of V. fluvialis, V. cholerae, V. parahaemolyticus, and A. hydrophila in V. fluvialis enrichment medium (FEM) measured turbidimetrically. Test organisms were grown in TSB´ to early stationary phase, diluted to 10^{-6} in phosphate buffered saline (pH 7.2), and 20 µl was inoculated into 5 ml of FEM. Growth at 35°C was monitored by measuring absorbance at 600 nm. Numbers of total viable cells at 0 hr and 14 hr of incubation are shown in Table 3. ●—● V. fluvialis DJVP 7225 (aerogenic), 0—0 V. fluvialis S50-1CC (anaerogenic), ▲—▲ V. cholerae N-3, Δ—Δ V. parahaemolyticus CDC A8658, ■—■ A. hydrophila NMRI 7.

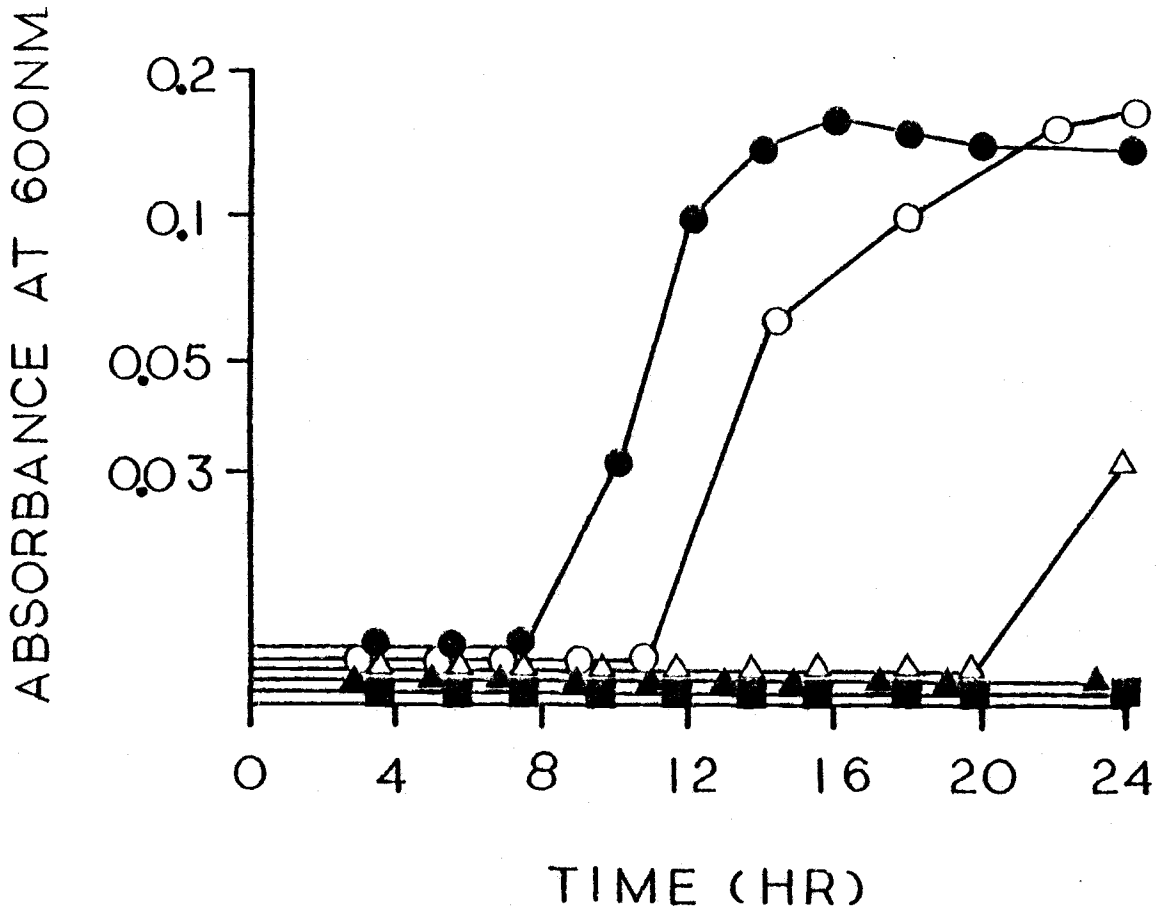

Fig. 1.

Fig. 2. Isolation of *V. fluvialis* from natural water samples with varying salinity using *V. fluvialis* enrichment medium and alkaline peptone enrichment medium. Height of the columns represent the number of positive samples. Numbers within each column indicate the MPN range of counts. A total of 63 samples were tested, of which 26 contained detectable counts of *V. fluvialis*.

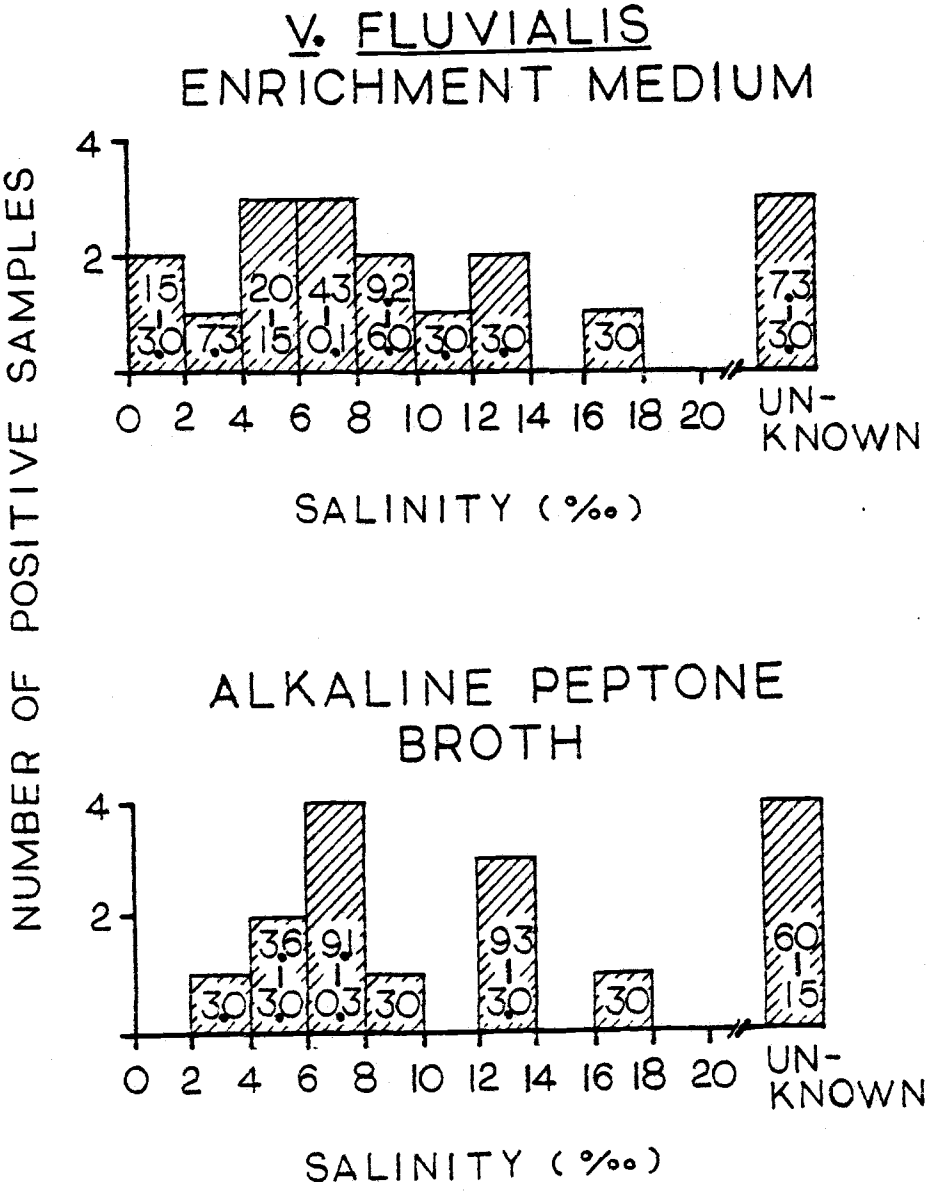
V. FLUVIALIS
ENRICHMENT MEDIUM
NUMBER OF POSITIVE SAMPLES
4
2
15/3.0
7.3/1.5
20/1.5
43/0.1
9.2/6.0
3.0/3.0
3.0
7.3/3.0
0 2 4 6 8 10 12 14 16 18 20 UN-KNOWN
SALINITY (‰)
ALKALINE PEPTONE
BROTH
4
2
3.0/3.0
3.6/3.0
9.1/0.3
3.0
9.3/3.0
3.0
6.0/1.5
0 2 4 6 8 10 12 14 16 18 20 UN-KNOWN
SALINITY (‰)

Fig. 2.

Table 1. Bacterial strains studied.

Organism	Strain no.	Source
Vibrio fluvialis, aerogenic[a]	DJVP 7225	Gastroenteritis-Indonesia
	2386	River water-England
	LSU 9-26a	Crab feces-Louisiana
	LSU 12-1G	Crab feces-Louisiana
	LSU 12-17V	Crab feces-Louisiana
V. fluvialis, anaerogenic[b]	5125	Human feces-Bangladesh
	DJVP 7440	Gastroenteritis-Indonesia
	S50-1CC	Sediment-New York
	H-5	Estuarine water-Maryland
	LSU 10-30a	Crab feces-Louisiana
V. cholerae, non O-1	N-3	Crab-Louisiana
V. parahaemolyticus	CDC A8658	Gastroenteritis-Maryland
V. alginolyticus	ATCC 17749	Seawater-Japan
V. anguillarum	LS-174	Salmon-Oregon
Aeromonas sobria	NMRI 6	Diver wound-Maryland
A. hydrophila	NMRI 7	Diver wound-Maryland

[a] Gas producer in MR-VP broth (Difco).

[b] No gas production in MR-VP broth.

Table 2. Percent relative growth of various organisms on alkaline peptone agar with different concentrations of novobiocin, 0/129 or sodium chloride at 35°C.

Organism		Test medium												
		TSA[a]		Alkaline peptone (NaCl 1%) with								Alkaline peptone with NaCl %		
				Novobiocin µg/ml				0/129 µg/ml						
Species	Strain	(NaCl 1%)	Control	2	5	10	20	2	5	10	20	3	5	7
Vibrio fluvialis	DJVP 7225	100[b]	87	95	83	105	89	29	38	0	0	119	48	0
	S50-1CC	100	139	114	76	79	79	90	60	63	0	105	10	0
	5125	100	118	91	91	94	59	84	105	120	0	83	0	0
	2386	100	105	179	93	120	98	95	98	108	0	109	108	57
V. cholerae non O-1	N-3	100	105	0	0	0	0	98	0	0	0	83	0	0
V. parahaemolyticus	CDC A8658	100	105	115	89	60	0	105	124	91	0	85	67	0
V. alginolyticus	ATCC 17749	SW[c]	0	0	0	0	0	0	0	0	0	SW	100	69
V. anguillarum	LS-174	100	100	0	0	0	0	0	0	0	0	0	0	0
Aeromonas sobria	NMRI 6	100	113	84	82	67	79	72	94	99	80	97	0	0
A. hydrophila	NMRI 7	100	51	116	78	64	76	81	69	72	76	74	0	0

[a] Tryptic soy agar (Difco) supplemented with 0.5% NaCl.

[b] Percent relative growth was determined as (number of colonies on test medium) ÷ (number of colonies on TSA) x 100. Number of colconies on TSA was taken as 100% growth except for V. alginolyticus ATCC 17749 strain where the numbers of colonies on alkaline peptone agar with 5% NaCl was taken as 100%.

[c] No separate colonies were observed due to swarming.

Table 3. Growth of *V. fluvialis*, *Vibrio* spp., and *Aeromonas* spp. in *V. fluvialis* enrichment medium (FEM) as measured by the number of viable cells.

Test organism[a]		Total viable cell numbers at an incubation time of[b]	
		0 hr	14 hr
Vibrio fluvialis, aerogenic	DJVP 7225	88	1.9×10^8 CFU
	2386	56	2.7×10^8
	LSU 9-26a	34	5.0×10^7
	LSU 12-1G	78	6.9×10^8
	LSU 12-17V	50	8.0×10^8
V. fluvialis, anaerogenic	5125	72	<50
	DJVP 7440	2.0	4.9×10^7
	S50-1CC	58	1.4×10^8
	H-5	40	5.1×10^8
	LSU 10-30a	59	5.9×10^8
V. cholerae	N-3	74	<50
V. parahaemolyticus	CDC A8658	42	1.1×10^5
V. alginolyticus	ATCC 17749	44	<50
V. anguillarum	LS-174	52	<50
Aeromonas sobria	NMRI 6	190	<50
A. hydrophila	NMRI 7	38	<50

[a] Test organisms were grown in TSB′ to early stationary phase (absorbance at 600 nm : 1.0-1.2) with shaking (200 rpm) at 25°C. Cultures were diluted to 10^{-6} in phosphate buffered saline (pH 7.2) and 20 µl was inoculated into 5 ml of FEM and incubated statically at 35°C. Turbidity data are shown in Fig. 1.

[b] Number of viable cells was determined by the plate count method on TSA′.

Table 4. Isolation of *Vibrio fluvialis* from environmental samples using *V. fluvialis* enrichment medium (FEM) and alkaline peptone (AP) enrichment medium.

	Water		Sediment	Crab	Total
	Natural water	Sewage			
Number of samples studied	63	45	60	9	177
Number of positive samples	26	14	27	4	71
Isolation with FEM / AP					
+ / +	8 (30.8%)	0 (0%)	12 (44.4%)	1 (25%)	21 (29.6%)
+ / -	10 (38.5%)	13 (92.9%)	7 (25.6%)	2 (50%)	32 (45.1%)
- / +	8 (30.8%)	1 (7.1%)	8 (29.6%)	1 (25%)	18 (25.4%)

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CHAPTER III

A Species-Specific Antigen of *Vibrio vulnificus*

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Running Head: Seroidentification of *Vibrio vulnificus*

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Abstract

An antigen common to all Vibrio vulnificus strains was detected in sonicated whole-cell preparations by Ouchterlony immunodiffusion. Its specificity was confirmed by two-dimensional immunoelectrophoresis. The antigen, designated VVA, was purified by ammonium sulfate precipitation, gel filtration, ion exchange column chromatography, and preparative gel electrophoresis. The molecular weight of VVA was 64,000 when estimated by gel filtration and 40,000 when measured by denaturing polyacrylamide gel electrophoresis. Specific antiserum prepared against purified VVA (anti-VVA serum) did not agglutinate whole cells of V. vulnificus. Therefore, VVA was considered an internal antigen. Using anti-VVA serum, a microimmunodiffusion method was designed to detect antigen VVA in bacterial cell lysates prepared from a single colony. This simple method allowed the specific identification of V. vulnificus as early as 10 hrs after antigen preparation.

Introduction

Vibrio vulnificus is a recently recognized human-pathogenic *Vibrio* which causes wound infections and primary septicemia (5,6). In early reports, isolates from patients were mistakenly identified as *V. parahaemolyticus* (18,24,26). Originally, this species was designated as the lactose-fermenting or lactose-positive *Vibrio* due to the unique ability of the isolates to ferment lactose. Often these isolates were labelled halophilic vibrios owing to their tolerance to NaCl (13,20). The "halophilic lactose-fermenting" *Vibrio* was shown to be distinct from other *Vibrio* species by DNA hybridization (8). Reichelt et al. (17), named the organism *Beneckea vulnifica*. It was subsequently transferred to the genus *Vibrio* and established as *V. vulnificus* (11,12). Recently, organisms that were very similar to clinical isolates of *V. vulnificus* in biochemical reactions were isolated from seawater (14), and *Vibrio* isolates from the estuarine environment were identified as *V. vulnificus* by DNA hybridization (22). *Vibrio* isolated from diseased eels cultured in estuarine ponds in Japan were also found to be taxonomically very similar to *V. vulnificus* and were included as a biotype within this species (21).

Now that *V. vulnificus* is considered a human pathogen existing in the estuarine environment, recent studies are directed toward defining its pathogenic mechanisms and role in the environment. As reflected in the stormy history of its taxonomy and its diverse

ecological habitats, identification of V. vulnificus based on biochemical tests is empirical, confusing, and not always correct. For example, it is now known that vibrios other than V. vulnificus ferment lactose (4,5, D. L. Tison, J. Greenwood, M. Nishibuchi, and R. J. Seidler. In R. R. Colwell (ed.), Vibrios in the environment, in press). Molecular taxonomy based on DNA hybridization, however, is time-consuming and not easy to perform on a routine basis. Identification must proceed as quickly as possible in the diagnosis of V. vulnificus since the course of the infection is very rapid (5).

Recently Bang et al. (3) used the moderately conserved enzyme, superoxide dismutase, to show that serological relationships among Vibrio species are generally in agreement with other taxonomic criteria including DNA homology. The present authors attempted to establish a seroidentification scheme for a specific, quick and convenient identification of V. vulnificus. Since strains of V. vulnificus have rather diversified surface antigens when examined by the rapid slide agglutination test (21), sonicated whole-cell antigens were investigated by precipitation tests in this study. A species-specific antigen was shown to exist by two-dimensional immunoelectrophoresis and antiserum was prepared against the purified antigen. An immunodiffusion technique was designed using specific antiserum for the identification of presumptive V. vulnificus. Growth from a single colony is the antigen source and identification of V. vulnificus specific-precipitation line is seen in as little as 10 hrs.

Materials and Methods

Bacterial strains. Bacterial strains used in this study, their experimental designation, and origins are listed in Table 5. Most of the strains were previously used in DNA hybridization studies (21,22, Tison et al., in press). All the organisms were stored in tryptic soy broth without dextrose (Difco) supplemented with 0.5% NaCl (TSB´) plus 5% glycerol at -80°C.

Antigens. Sonicated bacterial cell antigens used for Ouchterlony immunodiffusion analysis, exclusive of microimmunodiffusion test, and two-dimensional immunoelectrophoresis were prepared as follows. Organisms were grown on TSB´ plus 1.5% agar (TSA´) at 25°C for 24 hr. Bacterial growth was inoculated onto TSA´ prepared in a 32 oz. prescription bottle and incubated at 25°C for 20-24 hr. The bacterial cells were harvested with formol-PBS (0.01 M phosphate buffer, pH 7.2, NaCl 0.74%, formaline 0.3%) and washed with formol-PBS three times. The washed cells were suspended in formol-PBS (0.2 g wet cell weight/ml) and sonicated with a Branson Sonifer Cell Disruptor 200 equipped with a half-inch horn (50 watts, 30 seconds x 4 cycles) and stored at 4°C until used. The sonicated whole-cell antigens prepared in this manner are hereinafter named using experimental strain designation, e.g., antigen V1: sonicated whole-cell antigens prepared from bacterial strain V1 (= *V. vulnificus* E9315).

Antisera. Whole bacterial cells were prepared as follows. Washed bacterial cells were prepared as described for antigen preparation above. The cells were suspended in formol-PBS to an optical density of 1.4-1.5 at 600 nm. Two milliliters of the cell suspension were mixed with an equal volume of Freund's complete adjuvant (Difco) using a homogenizer (Virtis 23, Virtis Co., N.Y.) equipped with a small blade at 10,000 rpm and inoculated into a rabbit. A booster shot was given with Freund's incomplete adjuvant (Difco) 32 days after the initial injection. Serum was prepared from the blood obtained 2, 3, and 4 weeks after the booster shot. Pooled serum was filter-sterilized through a membrane with a pore size of 0.2 μ (Gelman) and stored at 4°C with added 0.01% of the preservative thimerosal (Sigma). These antisera prepared against whole bacterial cells were given experimental strain notations, e.g., anti-V1 serum which is antiserum prepared against whole cells of bacterial strain V1 (= V. vulnificus E9315).

Antiserum was raised against the purified species-specific antigen (VVA) from V. vulnificus V-4. A rabbit was immunized with macerated polyacrylamide gel containing VVA (estimated total protein: 0.27 mg) with Freund's complete adjuvant. Two weeks after the initial injection, eluted VVA from polyacrylamide gel (total protein: 0.04 mg) in 0.1 M Tris-HCl buffer (pH 6.8) was injected with Freund's incomplete adjuvant as a booster. Two weeks after the booster shot, the animal was bled and the serum prepared as described above.

Ouchterlony immunodiffusion analysis. Ouchterlony double-diffusion plates were prepared by solidifying buffered agarose, consisting of 0.8% agarose (Type II, Sigma) and 0.01% thimerosal in Veronal buffer (pH 8.6, ionic strength 0.05), in a plastic Petri dish (100 x 15 mm). Antisera and antigens placed in the wells were allowed to react in a moist chamber at room temperature for up to 3 days. The results were photographed against dark-field illumination.

Microimmunodiffusion test. The Ouchterlony immunodiffusion test to detect VVA in lysed bacterial cells was done on a microscope slide. Unless otherwise specified, 0.8% agarose gel was prepared with Veronal buffer on a microscope slide and wells (3 mm in diam.) were cut with a Gel Punch (Gelman). The test organism was grown on TSA' at 25°C for 24-36 hrs. Growth from a single large colony was suspended in 20 µl of distilled water. About 5 µl of this antigen was used to fill one of the surrounding wells and about 5 µl of anti-VVA serum was put into the center well. The test agar slide was incubated at room temperature in a moist chamber, and the results were read periodically for 24 hr.

Two-dimensional immunoelectrophoresis. About 4.6 ml of melted 0.8% agarose prepared in Veronal buffer was solidified over a glass plate (50 x 50 x 2 mm). Antigen(s) put in the well(s) made at the bottom-right position were electrophoresed at 8 V/cm, unless otherwise stated, for 50 min at room temperature for the first dimension. The upper portion of the gel (2/3) was then removed.

Antiserum was incorporated into 3.5 ml of melted 0.8% agarose gel in Veronal buffer (50°C), unless otherwise indicated, at the concentration of 1.7% (v/v) and the gel was casted to cover the top portion of the glass plate. Electrophoresis for the second dimension was conducted at 0.5 V/cm for 20 hr at room temperature. The gel plate was incubated at 4°C overnight before the results were photographed against dark-field illumination.

Rocket immunoelectrophoresis. To 4.6 ml of melted 0.8% agarose gel prepared in Veronal buffer (50°C), 0.025 ml of anti-V4 serum was mixed and poured onto a glass plate (50 x 50 x 2 mm) and allowed to solidify. Up to 10 wells (3 mm in diam.) were cut on each plate and 6 µl of sample was placed in each well and electrophoresed at 0.5 V/cm for 20 hr at room temperature. The gel was incubated overnight at 4°C and the heights of the precipitin peaks were measured.

Purification of VVA. The procedure used to purify VVA from strain V4 is illustrated in Fig. 3. All chromatography was performed at 4°C. The fractions from gel columns and from electrophoresis were assayed for VVA by rocket immunoelectrophoresis using agarose gels containing appropriately diluted antiserum (anti-V4 serum). This allowed only the reaction of VVA to be seen. When sonicated whole-cell antigen (V4) was assayed by rocket immunoelectrophoresis, there was a linear relationship between the relative concentration and the height of the precipitation lines in the range of 3.0-17.5

mm. Therefore, when necessary, samples were diluted so that the precipitin heights fell in this range. One millimeter in height was defined as one antigen unit. The shape of the rocket precipitin band became dome-shaped rather than pointed during the purification procedure. This may reflect the elimination of contaminating proteins (23). Therefore, the antigen concentration, as estimated by the height of the rocket, will not exactly quantitate the concentration of the more purified antigen.

For the purification of VVA, a bacterial cell suspension (42 g wet cell weight in 210 ml of formol-PBS) was disrupted by ultrasonication. The supernate was obtained from the sonicated cells by centrifugation (14,000 x g, 100 min) at 4°C, and was brought to 50% saturation with ammonium sulfate. The precipitate was removed by centrifugation (13,000 x g, 60 min) at 4°C. The supernate was raised to 70% saturation with ammonium sulfate and centrifuged (14,000 x g, 60 min). The pellet was dissolved in 20 ml of formol-PBS and dialyzed against the same buffer at 4°C. Any precipitate which appeared in the dialysis bag was removed by centrifugation (13,000 x g, 20 min) and the supernate was concentrated by ultrafiltration on YM-30 membrane (Amicon) to 5 ml.

The crude antigen preparation was loaded onto a Sephadex G-100 (Pharmacia) column (2.5 x 60 cm) prepared with formol-PBS buffer. Eluted fractions that had high concentrations of VVA were pooled, dialyzed against 0.02 M Tris-HCl buffer (pH 7.2, NaCl 0.05 M), and concentrated to 7 ml by ultrafiltration. The sample preparation

was applied to a 1.5 x 33 cm diethylaminoethyl cellulose (DE52, Whatman) column equilibrated with 0.02 M Tris-HCl buffer (pH 7.2, NaCl 0.05 M). VVA was eluted with a 0.05-0.25 M NaCl linear gradient. Chloride concentration of the fractions was determined by the argentometric method (16). One 5 ml fraction that had the highest concentration of VVA was dialyzed against 0.1 M Tris-HCl buffer (pH 6.8) and was further purified by preparative gel electrophoresis.

Preparative gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE), with a final acrylamide concentration of 7.5%, was carried out under nondenaturing conditions basically as described below in the PAGE section, using a vertical water-jacketed slab gel (18.5 x 7 x 0.4 cm) electrophoresis unit. The 5 ml sample (0.3 mg protein/ml) with 0.002% bromphenol blue (Fisher) and 10% glycerol (Sigma) was overlayed throughout the top of the stacking gel. Current was applied at 150 V for 2 hr with the gel continuously cooled by circulating cold water (4°C). Immediately after electrophoresis, the gel was transferred to a cold room (5°C) where all subsequent work was done. Two horizontal gel strips were cut using Rf values (= distance migrated by proteins ÷ distance migrated by dye) as a guide (0.57-0.62, 0.62-0.67). Each gel strip was macerated by homogenization followed by passage through a 15 gauge needle. The macerated gel was then mixed with 0.1 M Tris-HCl buffer (pH 6.8) and incubated overnight at 4°C. The supernate, obtained by centrifugation (5000 x g, 30 min), was concentrated

using polyethylene glycol 20,000 (J. T. Baker). The macerated gel sedimented by centrifugation was stored at -20°C until used for immunizing a rabbit.

Polyacrylamide-agarose gel two-dimensional immunoelectrophoresis. PAGE for the first dimension was carried out under nondenaturing conditions as described in the PAGE section below, using a vertical slab gel with a final acrylamide concentration of 7.5%, prepared between thin glass plates (10.2 x 15.3 x 0.08 cm). Two identical samples (10 µl/well) were loaded in two adjacent wells made close to the edge of the gel, and electrophoresed at 4°C until the tracking dye reached the end of the gel. The gel was sliced vertically and one strip was stained for proteins. The other gel strip was left on one of the glass plates, onto which about 3 ml of melted 0.8% agarose gel in Veronal buffer containing antiserum (anti-whole cell antigen of V. vulnificus V4) at the concentration of 3.15% (v/v) was casted to the thickness of 0.6-0.8 mm for the second dimension. The gel, connected to reservoir buffer (Veronal buffer) with wicks, was electrophoresed at 200 V for 4.5 hr at 4°C. The position of the protein bands in the first dimension was compared with the position of the precipitation-line peak of VVA in the second dimension by their Rf values.

Protein concentration. Protein concentrations were determined by the method of Bradford (7) as refined by Spector (19). The test reagent and bovine gamma globulin standard were from Bio-Rad

Laboratories (Richmond, California). Both were used according to the manufacturer's directions.

Molecular weight estimation. The molecular weight (MW) of native VVA was estimated by gel filtration. A Sephadex G-100 column (0.8 x 60 cm) was calibrated with bovine albumin (essentially fatty acid free, Sigma), ovalbumin (Grade V, Sigma), α-chymotrypsinogen-A (Type II, Sigma), and ribonuclease-A (Type III-A, Sigma) prepared in formol-PBS buffer as MW standards. The eluted molecular weight standards were detected by measuring the absorbance at 280 nm of the column fractions. The position of VVA in the fractions was determined by rocket immunoelectrophoresis. A calibration curve was obtained by plotting log MW vs. partition coefficient of the protein standards (9).

The MW of the antigen under denaturing conditions was determined by sodium dodecyl sulfate (SDS)-PAGE using a gel with a final acrylamide concentration of 10%. A mixture of MW markers (Dalton Mark VI) was purchased from Sigma Chemical Co. (Saint Louis, Missouri).

PAGE. Nondenaturing PAGE was performed with the high pH discontinuous buffer system of Davis (10). SDS-PAGE was carried out with essentially the same buffer system except that the gels and reservoir buffer contained 0.1% SDS (sodium lauryl sulfate, Fisher). Prior to SDS-PAGE, the antigen samples were boiled for 3 min in the buffer containing SDS (final concentration 1.8%) and 2-

mercaptoethanol (final concentration 4.2%). Protein bands in the gel were made visible with the silver stain of Allen (1).

Results and Discussion

Demonstration of VVA. The initial survey for V. vulnificus common antigens was conducted by Ouchterlony immunodiffusion analysis using sonicated whole-cell antigens and antisera prepared against whole cells. Example reactions with anti-V4 serum are illustrated in Fig. 4. The most distinct precipitation line (examples shown by arrows) appeared common to all strains of V. vulnificus (V1-V9 and V'1-V'3 in Figs. 4 A, B, and C), but not to the other organisms with the possible exception of SP1 (Fig. 4 C, D, and E). A very similar precipitation pattern was also observed when anti-V2 and anti-V'3 sera were used in the place of anti-V4 serum (Table 6).

Two-dimensional immunoelectrophoresis analysis, which gives much higher resolution than Ouchterlony immunodiffusion, was performed in order to determine whether this most distinct precipitation line is specific to V. vulnificus. One strain of V. vulnificus (V2) and its homologous antiserum (anti-V2 serum) were used to optimize the experimental conditions for this antigenic analysis. Preliminary experiments using different concentrations of antiserum and antigens indeed revealed one dominant precipitation line shown by the arrow in Fig. 5A. The concentration of antiserum in the gel was subsequently reduced to the extent that only the most distinct precipitation line could be seen (Fig. 5B).

To determine whether other strains of V. vulnificus have the same dominant precipitation line as seen in Fig. 5B, test antigens

were placed into a second well and analyzed in tandem with the V2 antigen and antiserum by immunoelectrophoresis. Some examples of these results are shown in Fig. 6. In Fig. 6A, when antigens V2 and V4 were tested, two fused precipitation lines formed revealing their identity. On the other hand, when antigen SP1 was examined in the same manner, there was no fused precipitation line (Fig. 6B).

The latter results suggest that the reaction observed in the Ouchterlony immunodiffusion (Fig. 4E) was caused by a different antigen superimposed over that of the species-specific antigen.

All antigen preparations listed in Table 6 were tested by the tandem two-dimensional immunoelectrophoresis with anti-V2 serum. The results demonstrate that all the V. vulnificus strains, including biotype 2 strains, displayed the same fused line of identity but none of the other test organisms showed any precipitation line. Furthermore, when anti-V2 serum was replaced by antisera prepared against other strains of V. vulnificus (anti-V1, V3, V4, V5, and V6 sera) for reciprocal tests, identical reaction patterns were observed (Fig. 6C).

From the results obtained, therefore, it was concluded that there exists a species-specific antigen in V. vulnificus that forms a distinct line in precipitation tests. The antigen is designated as antigen VVA. Using the presence or absence of the precipitation line in Ouchterlony immunodiffusion plate as the indicator, the VVA in crude sonicated cell preparations was briefly characterized.

VVA was not dialyzable, and it lost antigenicity by heating at 100°C but not at 70°C, and was precipitated by 70%, but not by 50%, saturated ammonium sulfate. Also, VVA was not found in concentrated (x20 by lyophilization) spent culture medium (TSB´). Based on these observations, VVA was considered to be a cell-associated protein.

Purification of VVA. When the concentrated ammonium-sulfate precipitate was separated by Sephadex G-100 column chromatography, the peak of VVA corresponded with the second peak of eluted proteins (Fig. 7). Fractions 29-37 were pooled, concentrated by ultrafiltration, and applied to a column containing diethylaminoethyl cellulose (DE52). In DE52 column chromatography, two very closely eluted protein peaks were observed and the peak of VVA activity was found in the first peak (Fig. 8). When fraction No. 11, which had the highest concentration of antigen, was subjected to PAGE under non-denaturing conditions, 3 closely migrating major protein bands were made visible by silver stain (Fig. 9B). Prior to further purification, 2-dimensional electrophoresis was performed (PAGE, in the first dimension, agarose gel immunoelectrophoresis in the second dimension), in order to determine which of the three protein bands corresponded to VVA. As a result of three independent trials, the peak of the precipitation line (Fig. 9A) was found to coincide most closely with the positions of the second and third major protein bands (Fig. 9B). Rf values for the precipitation line and the second and

third protein bands were 0.62 ± 0.03, 0.60 + 0.01 and 0.64 ± 0.01, respectively.

An attempt was then made to isolate the second and third protein bands by preparative gel electrophoresis, using a nondenaturing gel with a final acrylamide concentration of 7.5%. Slices A and B were made from the gel using Rf values as a guide (A: second protein band, B: third protein band) and the proteins were eluted by diffusion into 0.1 M Tris-HCl bufer (pH 6.8) (recovery of protein: 12.7%). When eluted materials were examined by PAGE under nondenaturing conditions the preparation from slice A showed a single protein band but the preparation from slice B was still contaminated with a very small amount of the other protein band. Nevertheless, when the protein concentration was adjusted to the same level (0.003 mg/ml), the concentration of the VVA in these two preparations was not very different (5.8 and 7.9 units for preparation A and B, respectively). In addition, when these preparations were electrophoresed under denaturing conditions (SDS-PAGE), they showed an exactly identical protein band although preparation B showed a very minor contaminating protein band (Fig. 10). It was concluded that the proteins corresponding to the second and third band (Fig. 9B) are both VVA and may be isomers. These two closely migrated protein bands were not separated very well in PAGE under nondenaturing conditions (Fig. 9B). This may be why they formed a single peak of precipitation rather than two continuous peaks in the second dimension of polyacrylamide-agarose

gel two-dimensional immunoelectrophoresis (Fig. 9A). Since preparation B still had contaminating protein, preparation A was designated purified VVA and used for specific antiserum production.

MW of antigen VVA. The estimated MW of purified VVA was 40,000 based on SDS-PAGE electrophoresis (Fig. 10). The MW of the partially purified native antigen was estimated at 64,000 by gel filtration on a calibrated Sephadex G-100 column (Fig. 11). While estimation of MW by SDS-PAGE is quite reliable (25), MW estimated by gel filtration cannot always be interpreted with confidence because this technique is useful for only globular proteins (2). Other problems limit this technique in estimating MW as well (15). Thus it is not unusual to see discrepancies in the results obtained using the two procedures. It is difficult, however, to speculate whether antigen VVA is a monomer or dimer from the results obtained because of the magnitude of the MW discrepancy obtained from the two techniques.

Specificity of VVA. After purification of the antigen by preparative gel electrophoresis, gel slice A and the material eluted from gel slice A (purified VVA) were used to immunize a rabbit for the production of anti-VVA serum. In Ouchterlony immunodiffusion plates, anti-VVA serum formed a single, visible precipitation line with purified VVA in 1 day or less. The reaction showed complete identity with the precipitation line formed between anti-VVA serum and sonicated whole-cell antigen V4

(Fig. 12). When other sonicated whole-cell antigens listed in Table 6 were reacted with anti-VVA serum, antigens of all strains of V. vulnificus exhibited the same precipitation line as the line formed by VVA and antigen V4. The other antigens did not exhibit the same precipitation line. However, after 3 days, antigens C, AL, AR, SP1, and SP3 formed a very faint precipitation line of partial identity with VVA, indicating the presence of cross-reacting antigens that have some antigenic determinant(s) in common with VVA. It is, therefore, believed that VVA is a moderately conserved antigen of taxonomic importance at the species level similar to superoxide dismutase (3).

When anti-VVA serum was used for rapid slide agglutination tests with formalin-fixed and subsequently washed whole-cell antigens of strain V4, no agglutination was observed. This indicates that VVA is not present on the cell surface but is an internal antigen made available for precipitation tests by destruction of cells.

Microimmunodiffusion test. A microimmunodiffusion test was designed which allows convenient and rapid identification of V. vulnificus by detecting VVA in lysed whole-cell suspensions. Vibrio cells usually autolyse and release internal substances in distilled water because they require salts to maintain cellular integrity. To take advantage of this characteristic, antigen was prepared by suspending bacterial cells in distilled water. When the reference strains listed in Table 1 were tested, all the V.

vulnificus strains, including biogroup 2, developed a distinct, detectable precipitation line in 10 hr. The line became more clear as incubation was continued until 24 hr, when the experiment was terminated. None of the other test organisms exhibited any precipitation line. The species specificity of precipitation reactions resulting from microimmunodiffusion of lysed cell suspensions is illustrated in Fig. 13. The same set of micro-immunodiffusion experiments were conducted with agarose gels prepared in phosphate buffered saline (0.01 M phosphate buffer, pH 7.2, NaCl 0.74%), instead of Veronal buffer, and identical results were obtained. Bacterial cell suspensions can also be treated with formalin for safety. There was no effect of formalin on the antigenicity of the preparation when, after completely suspending bacterial cells in distilled water, one tenth volume of 3% formalin was added to the antigen solution.

It was concluded that this simple double diffusion method allows the specific identification of V. vulnificus. The screening of many isolates is possible by this technique as only a single colony growing on agar medium is required to prepare antigen and the results can be judged in as little as 10 hr after antigen preparation.

Acknowledgements

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Fig. 3. Procedure for the purification of V. vulnificus species-specific antigen.

SONICATED BACTERIAL CELL SUSPENSION

↓

SUPERNATE

↓

PRECIPITATION WITH 50-70% SATURATED $(NH_4)_2SO_4$

↓*

SEPHADEX G-100 COLUMN CHROMATOGRAPHY

↓*

DE52 COLUMN CHROMATOGRAPHY

↓*

PREPARATIVE GEL ELECTROPHORESIS

*CONCENTRATION BY ULTRAFILTRATION ON YM-30 MEMBRANE

Fig. 3.

Fig. 4. Ouchterlony immunodiffusion analysis of antigens of various *Vibrio* and *Aeromonas* species. Center wells contain rabbit antiserum prepared against whole cells of *V. vulnificus* V4. Outer wells contain sonicated whole-cell antigens of *Vibrio* and *Aeromonas* species. Arrows indicate precipitation lines formed by a presumed species-specific antigen of *V. vulnificus*. See Table 6 for strain designations.

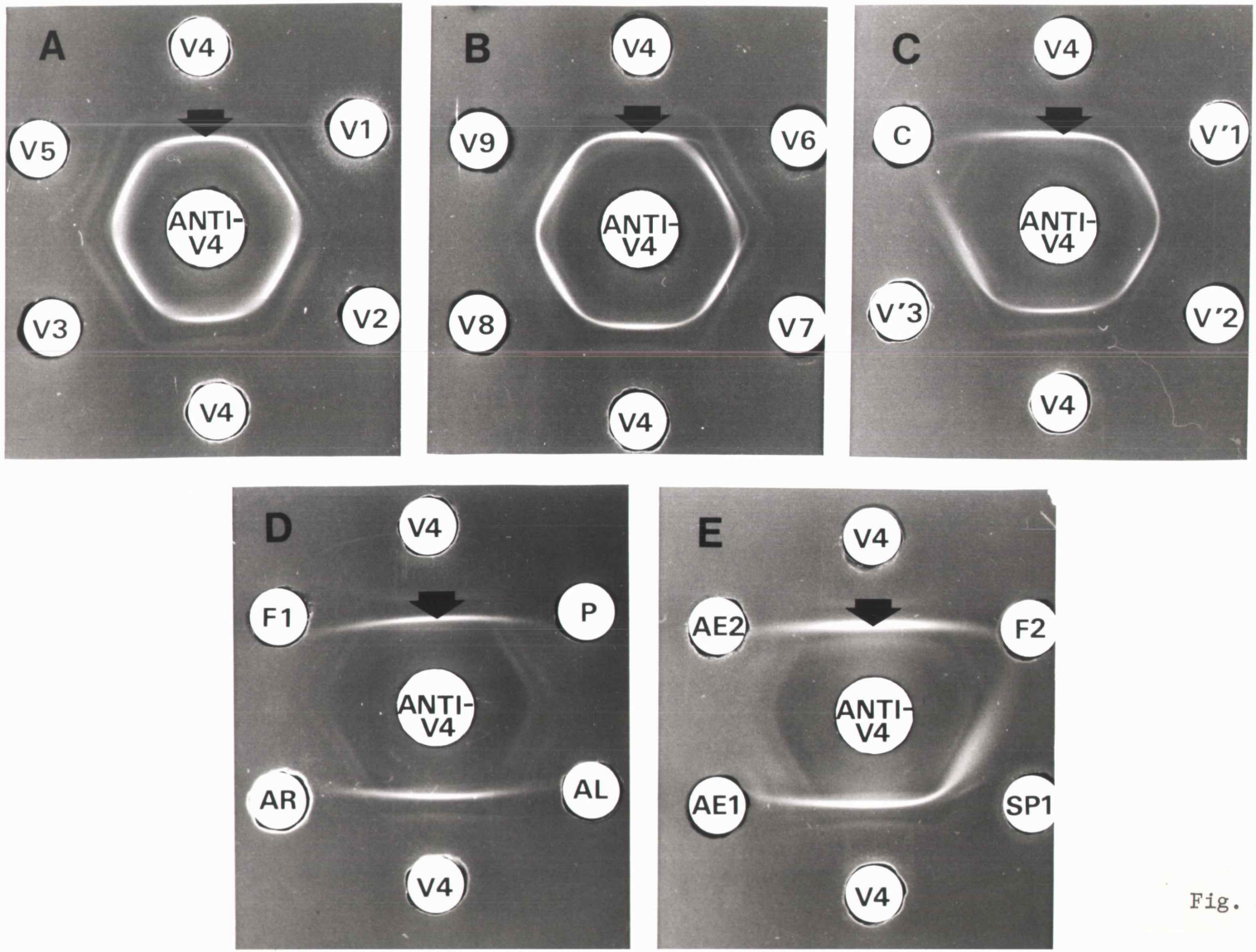
A
V4
V5
V1
ANTI-
V4
V3
V2
V4
B
V4
V9
V6
ANTI-
V4
V8
V7
V4
C
V4
C
V'1
ANTI-
V4
V'3
V'2
V4
D
V4
F1
P
ANTI-
V4
AR
AL
V4
E
V4
AE2
F2
ANTI-
V4
AE1
SP1
V4

Fig. 4.

Fig. 5. Antigenic analysis of *V. vulnificus* V2 by two-dimensional immunoelectrophoresis. Sonicated V2 antigen was placed in the well and electrophoresed in 0.8% agarose. Antiserum against whole cells of *V. vulnificus* V2 (anti-V2 serum) was incorporated into the top portion of agarose at the concentration (v/v) indicated. A, first-dimension electrophoresis for 70 min at 8 V/cm, antiserum concentration, 6% (v/v). The arrow indicates the species-specific antigen of *V. vulnificus*. B, first-dimension electrophoresis for 50 min, antiserum concentration: 1.7% (v/v).

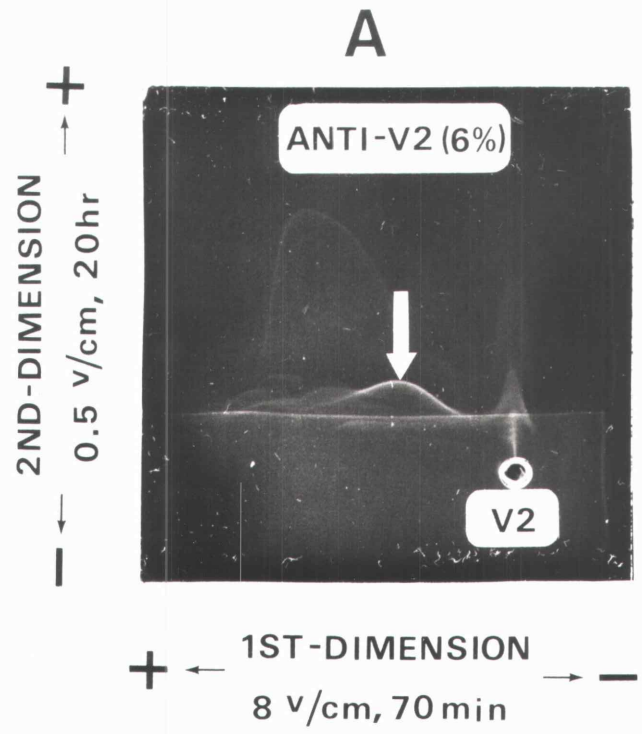
A
ANTI-V2 (6%)
V2
2ND-DIMENSION
0.5 v/cm, 20hr
+
−
+ ← 1ST-DIMENSION → −
8 v/cm, 70 min
B
ANTI-V2 (1.7%)
V2
2ND-DIMENSION
0.5 v/cm, 20hr
+
−
+ ← 1ST-DIMENSION → −
8 v/cm, 50 min

Fig. 5.

Fig. 6. Reaction of species-specific antigen of V. vulnificus by tandem two-dimensional immunoelectrophoresis. Sonicated whole-cell antigens were electrophoresed in 0.8% agarose gel at 8 V/cm for 50 min in the first dimension. Electrophoresis in the second dimension was performed in 0.8% agarose containing 1.7% antiserum (v/v) at 0.5 V/cm for 20 hr. A, reaction of antigens V2 and V4 with anti-V2 serum; B, reaction of antigens V2 and SP1 with anti-V2 serum; C, reaction of antigens V2 and V4 with anti-V4 serum.

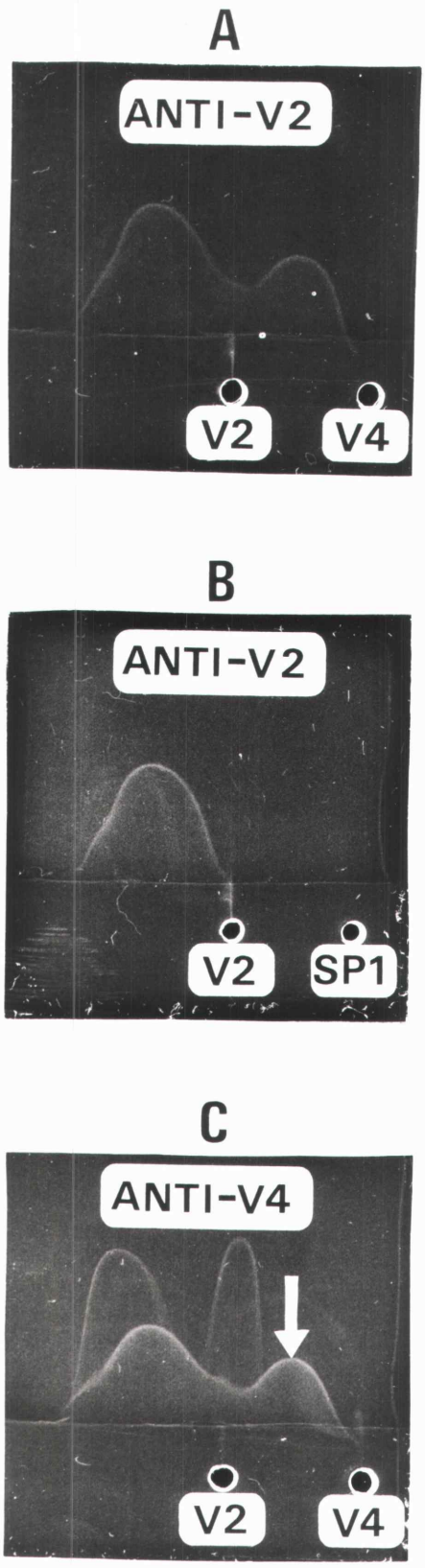

Fig. 6.

Fig. 7. Purification of the species-specific antigen of V. vulnificus V4 by Sephadex G-100 column chromatography. The concentrated ammonium sulfate precipitate of sonicated cells was applied to a Sephadex G-100 gel prepared in formal-PBS buffer. Protein concentration (▲). Concentration of the species-specific V. vulnificus antigen (VVA) (●). One antigen unit: antigen concentration in 6 µl sample that gives a height of 1 mm in rocket immunoelectrophoresis.

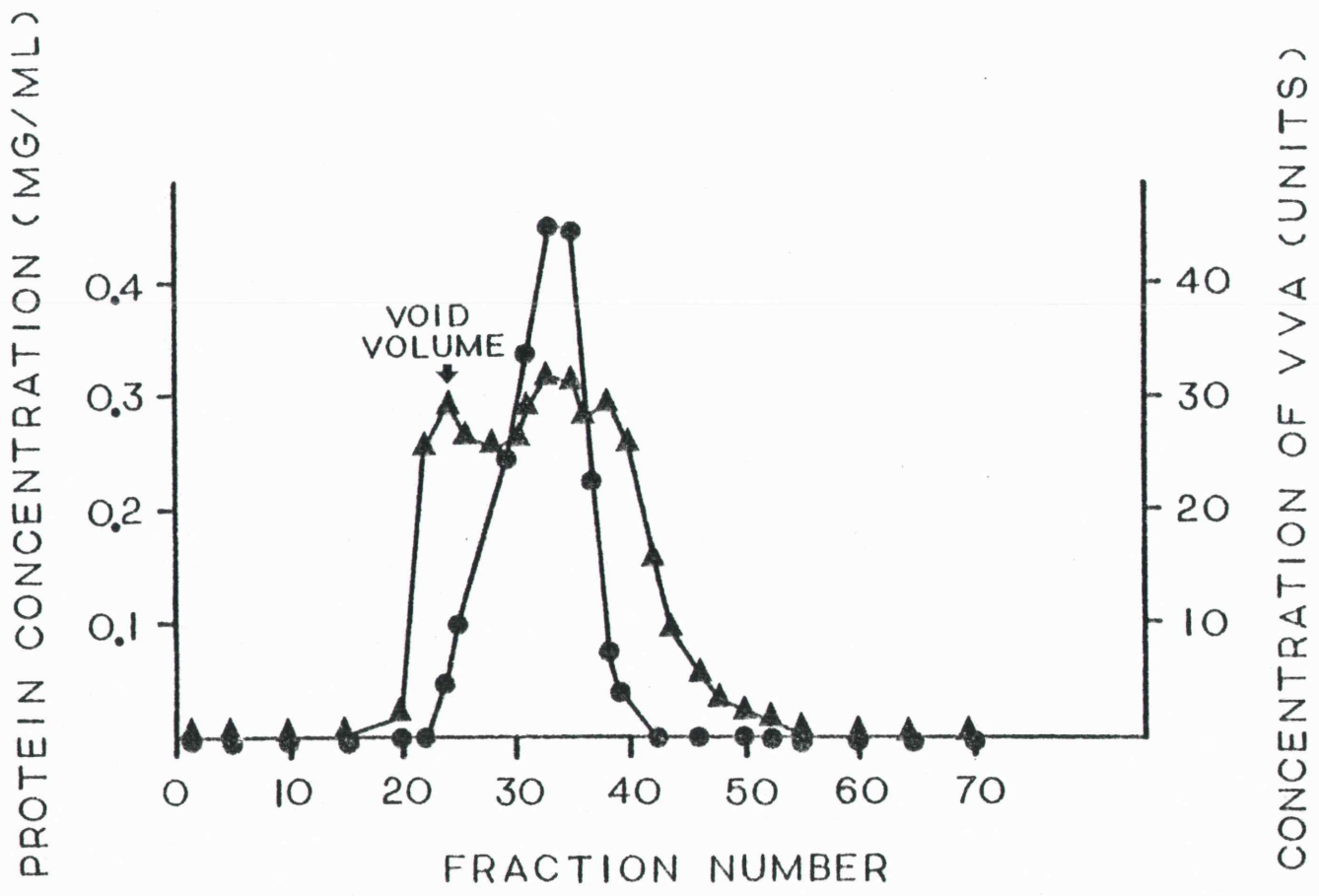
PROTEIN CONCENTRATION (MG/ML)
0.4
0.3
0.2
0.1
VOID
VOLUME
0
10
20
30
40
50
60
70
FRACTION NUMBER
40
30
20
10
CONCENTRATION OF VVA (UNITS)

Fig. 7.

Fig. 8. Purification of the species-specific antigen of V. vulnificus V4 by DE52 column chromatography. Fractions 29-37 from the Sephadex G-100 column were pooled, concentrated, dialyzed and applied to the DE52 column equilibrated with 0.02 M Tris-HCl buffer (pH 7.2, NaCl 0.05 M). The antigen was eluted with a 0.05-0.25 M NaCl linear gradient. Protein concentration (▲). Concentration of the species-specific V. vulnificus antigen (VVA) (●). Chloride concentration (■).

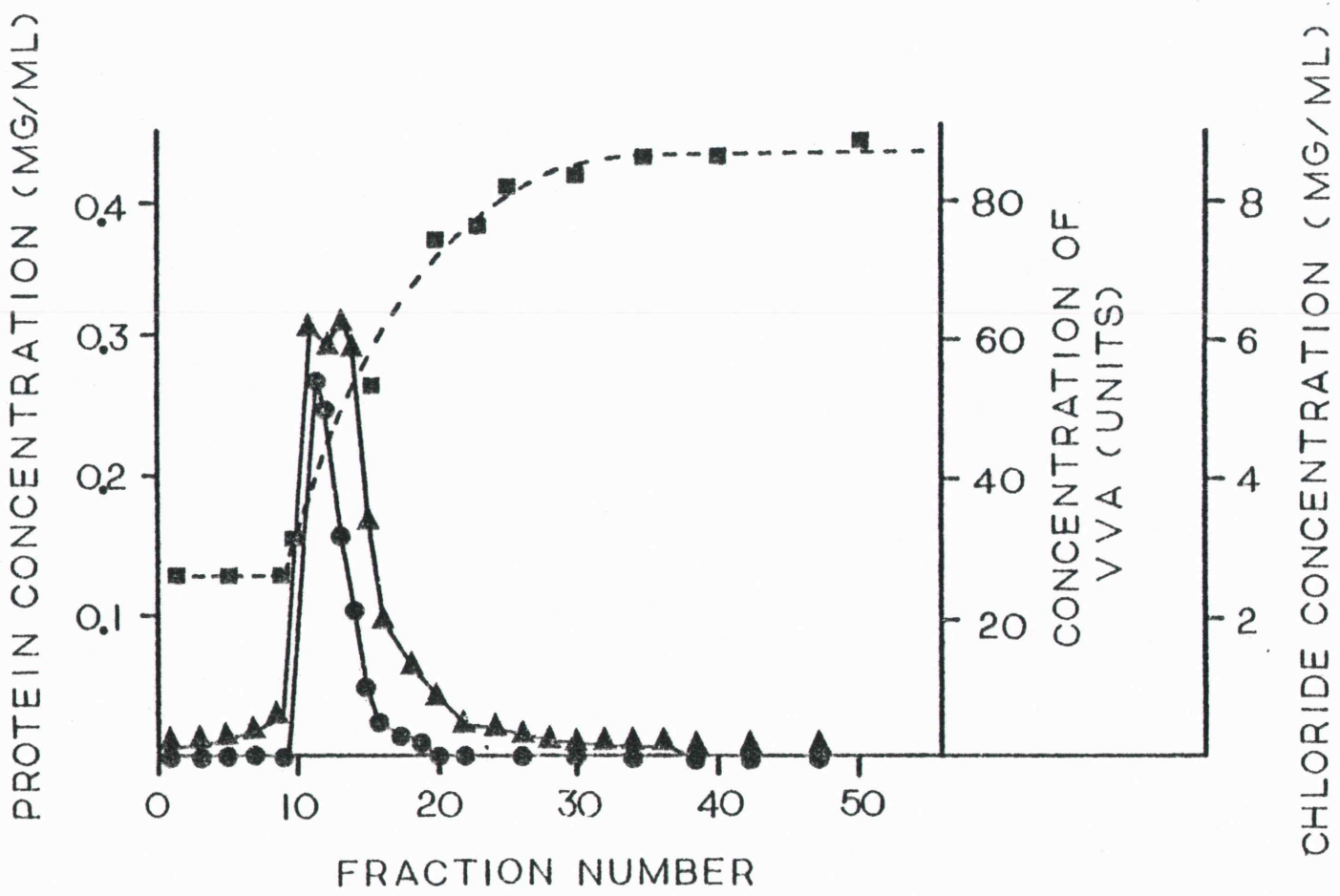
PROTEIN CONCENTRATION (MG/ML)
0.4
0.3
0.2
0.1
0
10
20
30
40
50
FRACTION NUMBER
80
60
40
20
CONCENTRATION OF VVA (UNITS)
8
6
4
2
CHLORIDE CONCENTRATION (MG/ML)

Fig. 8.

Fig. 9. Detection of the partially purified species-specific V. vulnificus antigen (VVA) by polyacrylamide-agarose gel two-dimensional immunoelectrophoresis. Two identical samples prepared from fraction no. 11 of the DE52 column were electrophoresed in polyacrylamide gel under nondenaturing conditions. The gel was sliced longitudinally and one strip was subjected to immunoelectrophoresis into agarose to identify the position of VVA (Fig. A). The other strip was subjected to silver stain for proteins (Fig. B). Zero position on the ruler is the top of the resolving gel. Rf value (distance migrated by protein or antigen ÷ distance migrated by dye) of the peak precipitation line of VVA (arrow in Fig. A) was 0.59, and those of 3 major protein bands (arrows in Fig. B) were 0.54, 0.58, and 0.64.

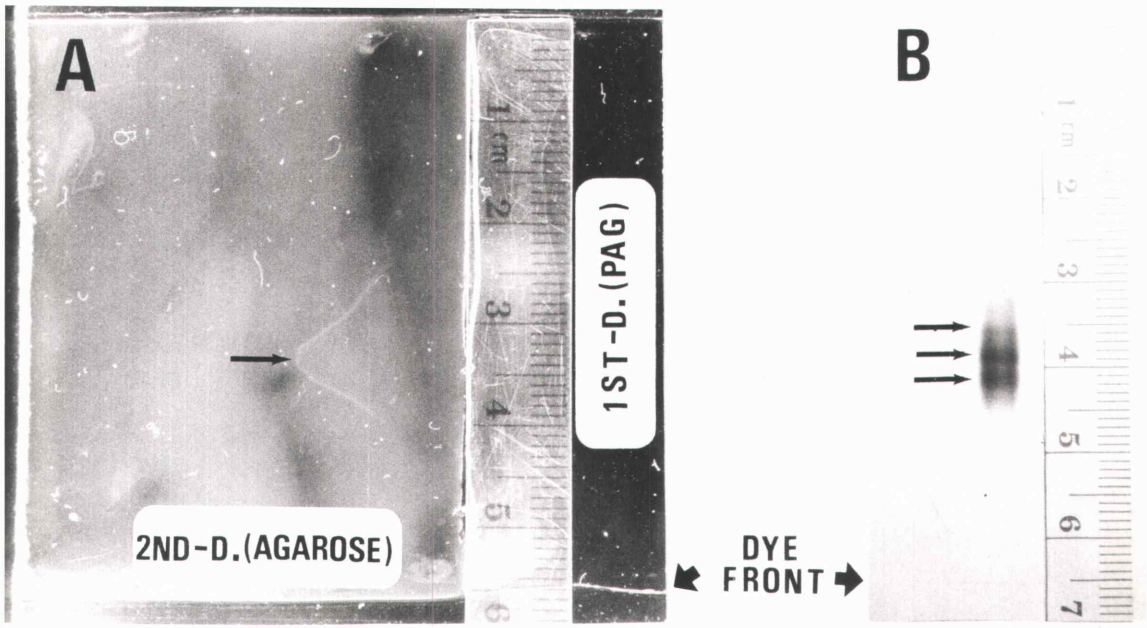

Fig. 9.

Fig. 10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified species-specific antigen prepared from V. vulnificus V4. Electrophoresis, using a gel with a final acrylamide concentration of 7.5% and the high pH discontinuous buffer system of Davis (10), was carried out under denaturing conditions. The proteins were made visible by the silver stain of Allen (1). Sample A and B are the materials eluted from the sliced gels corresponding in position, respectively, to the second and third protein bands of Fig. 9B. Sample C is the mixture of molecular weight markers.

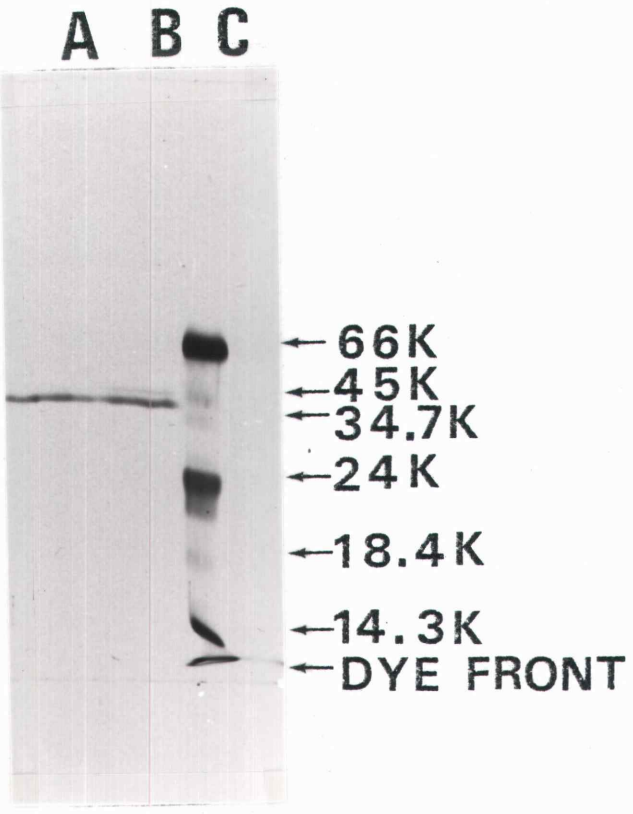

Fig. 10.

Fig. 11. Molecular weight estimation of the species-specific *V. vulnificus* antigen by gel filtration on Sephadex G-100. Kav (partition coefficient) = (Ve-Vo)/(Vt-Vo), where Ve is elution volume of protein, Vo is void volume, and Vt is total bed volume (9).

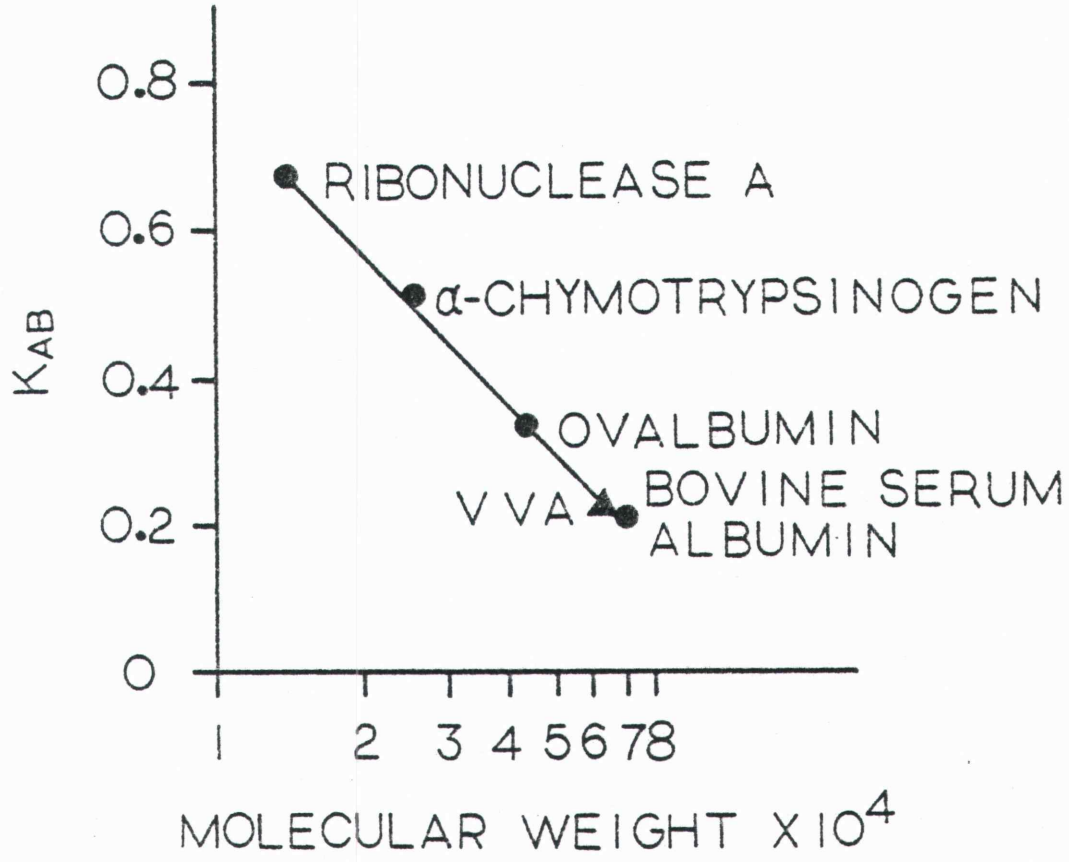
0.8
0.6
0.4
0.2
0
K_{AB}
RIBONUCLEASE A
α-CHYMOTRYPSINOGEN
OVALBUMIN
BOVINE SERUM ALBUMIN
V V A
1
2
3
4
5
6
7
8
MOLECULAR WEIGHT X 10^4

Fig. 11.

Fig. 12. Ouchterlony immunodiffusion analysis of purified species-specific V. vulnificus antigen. Anti-VVA : antiserum prepared against purified V. vulnificus antigen (VVA); VVA, purified antigen; V4, sonicated whole cells of V. vulnificus strain V4.

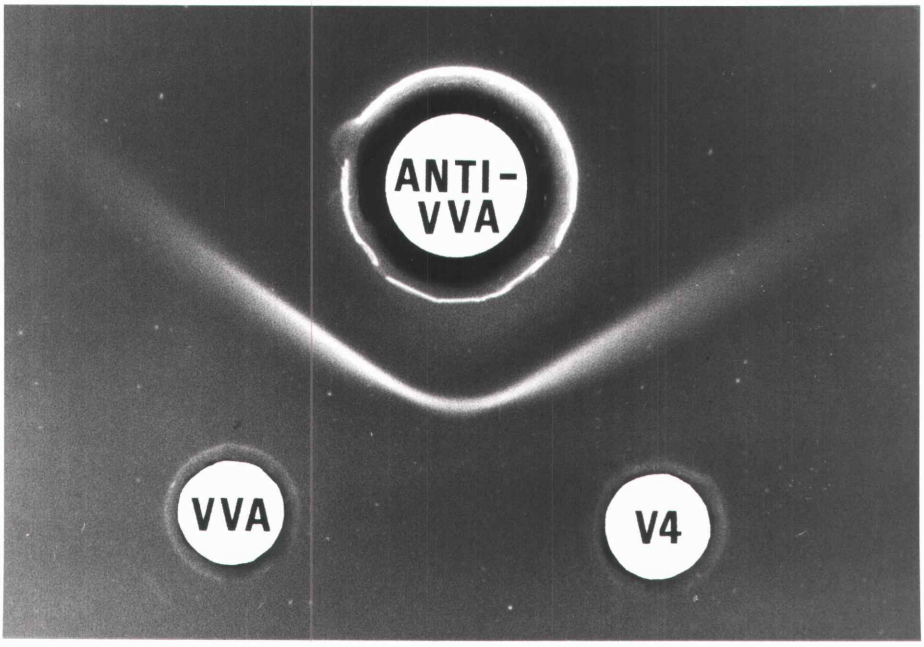

Fig. 12.

Fig. 13. Microimmunodiffusion test for detecting the species-specific V. vulnificus antigen (VVA). About 5 µl of antiserum against VVA prepared from strain V4 was placed in the center well. Generous bacterial growth from a single large colony of the test organism (See Table 5) was suspended in 20 µl of distilled water and about 5 µl of this antigen preparation was used to fill the surrounding well. After 24 hr of incubation at room temperature, the results were photographed.

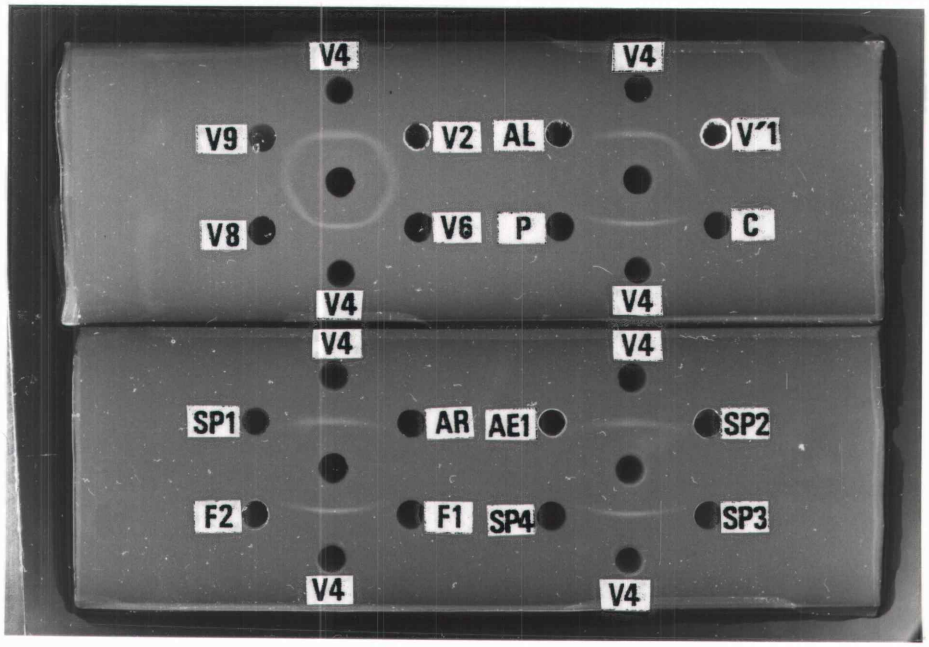
V4
V4
V9
V2
AL
V'1
V8
V6
P
C
V4
V4
V4
V4
SP1
AR
AE1
SP2
F2
F1
SP4
SP3
V4
V4

Fig. 13.

Table 5. Bacterial strains studied.

Experimental Designation	Species	Strain No.[a]	Source
V1	*Vibrio vulnificus*	E9315	Clinical, CDC
V2		CDC B51	Blood, Hawaii
V3		A8867	Finger wounds, Louisiana
V4		80-02-125	Blood, Texas
V5		79-08-9	Sea water, Texas
V6		A38	Crab, Oregon
V7		ATCC 27562	Blood, Florida
V8		LSU 11-3M-30	Crab, Louisiana
V9		79-11-114	Sea water, Texas
V'1	*V. vulnificus* biogroup 2[b]	ET-7617	Eel, Japan
V'2		ES-7601	Eel, Japan
V'3		KV-1	Eel, Japan
C	*V. cholerae* non O-1	N-3	Crab, Louisiana
P	*V. parahaemolyticus*	CDC A8659	Gastroenteritis, Maryland
AL	*V. alginolyticus*	ATCC 17749	Sea water, Japan
AR	*V. anguillarum*	LS-174	Salmon, Oregon
F1	*V. fluvialis*	2386	River water, United Kingdom
F2		DJVP 7225	Gastroenteritis, Indonesia

Table 5. Continued.

Experimental Designation	Species	Strain No.[a]	Source
SP1	*Vibrio* sp.	HOG-3	Oyster, Louisiana
SP2		OY-0-002	Oyster, Oregon
SP3		CL-0-001	Clam, Oregon
SP4		F43	Water, Louisiana
AE1	*Aeromonas* sp.	NMRI 1	River water, Maryland
AE2	*Aeromonas* sp.	NMRI 3	River water, Maryland

[a]Most of the strains were previously used for DNA hybridization studies (21,22, D. L. Tison, J. Greenwood, M. Nishibuchi, and R. J. Seidler. *In* R. R. Colwell (ed.), Vibrios in the environment, in press).

[b]Tison et al. (21).

Table 6. Occurrence of a V. vulnificus species-specific antigen as determined by Ouchterlony immunodiffusion analysis.[a]

Antigen[b]	Rabbit antiserum prepared against V. vulnificus[c]		
	V2	V4	V'3
V1	+	+	+
V2	+	+	+
V3	+	+	+
V4	+	+	+
V5	+	+	+
V6	+	+	+
V7	+	+	+
V8	+	+	+
V9	+	+	+
V'1	+	+	+
V'2	+	+	+
V'3	+	+	+
C	-	-	-
P	-	-	-
AL	-	-	-
AR	-	-	-
F1	-	-	-
F2	-	-	-
SP1	+[d]	+[d]	+[d]
SP2	-	-	-
SP3	-	-	-
SP4	-	-	-
AE1	-	-	-
AE2	-	-	-

[a]Presence (+) of a species-specific antigen was presumed by the distinct precipitation line shown by the arrows in Fig. 4.

[b]Sonicated whole-cell antigens. See Table 5 for designation.

[c]Antisera were prepared against whole-cell antigens.

[d]This was later shown to be a different antigen by two-dimensional immunoelectrophoresis.

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CHAPTER IV

Vibrio Virulence-Associated Factors Cause Rapid Fluid Accumulation in Suckling Mice

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Running Head: New virulence factors of *V. cholerae* and *V. fluvialis*

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503-754-4441

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Abstract

Non O-1 and O-1 *V. cholerae* and *V. 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 O-serovar, source of isolation, or ability to produce cholera toxin (CT), all strains of *V. cholerae* stimulated increased FA, which was measurable in the mice at 4 hr postinoculation. The virulence-associated factor(s) causing these symptoms was found to be distinct from CT by the kinetics of FA and serological difference from CT based on *in vivo* neutralization tests. In most instances FA was followed by high rates of mortality. Y-1 assays also showed that many *V. cholerae* produced extracellular heat-labile cytotoxic factor(s) and many CT-negative strains also caused a cytotonic-like morphological response. The majority of *V. fluvialis* strains produced smaller amounts of cytotoxic factor(s) but no cytotonic reactions. The factor which stimulates rapid FA in suckling mice could be one of several virulence-associated factors contributing to diarrheal disease by non-toxigenic vibrios but this is not verified at present.

Introduction

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 (2,8,16,21,25,26,27). 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. While 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 *V. fluvialis* from clinical and environmental origins. Virulence factor(s), associated with intestinal fluid accumulation, diarrhea and mortality, but distinct from CT were found. Cultures were also tested for reactions induced in Y-1 mouse adrenal tumor cells. *Vibrio 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 the suckling mouse but induced weaker responses in the Y-1 assay.

Materials and Methods

Bacterial cultures. Bacterial strains used in this study included 1 clinical and 5 environmental isolates of O-1 V. cholerae, 5 clinical and 7 environmental isolates of non O-1 V. cholerae, and 4 clinical and 5 environmental isolates of V. fluvialis. Escherichia coli and Citrobacter freundii were included as positive and negative controls, respectively (Table 9). All organisms were maintained in tryptic soy broth without dextrose (Difco) supplemented with 0.5% sodium chloride (TSB') plus 5% glycerol at -80°C. Serological properties and genetic characteristics regarding toxigenicity of V. cholerae and V. fluvialis strains listed in Table 9 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 "non-toxigenic" 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 using 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 fluid accumulation ratio (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 by the addition of 0.0053 value.

FA ratio. Frozen cultures were reconstituted on tryptic soy agar (Difco) with 0.5% added sodium chloride, and incubated at 25°C for 24 hr. A transfer was made into 10 ml of TSB´ and incubated in stationary culture at 25°C for 12 hr. 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) at 37°C for 18 hr. The final cell concentration reached 1.0-2.7 x 10^{10} colony-forming units (CFU) per milliliter. Culture supernates, were prepared by centrifugation of the broth culture with a clinical centrifuge (Beckman Microfuge B) and filtration through a 0.2 μm membrane filter (Acrodisk, Gelman). Animals were inoculated intragastrically and FA ratios determined by the method of Baselski et al. (1) with modifications as follows. Three-day-old suckling mice were submitted to experiments within 2 hr after they were removed from their mothers. One tenth milliliter of cell cultures or culture supernate with Evans blue dye added (0.01% w/v) was administered into the stomach of each test animal via polyethylene intramedic gastric tubing (PE60, Clay Adams). A group of 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. Fluid accumulation (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 hr and 12 hr post-administration into test animals. Purified cholera toxin diluted in TSB´ (Sigma, 100 µg/ml) and E. coli H10407, capable of producing both ST and LT, were used as positive controls. 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 hr as described above.

Neutralization test for cholera toxin. 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. One ml 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 blueing reaction in guinea pig skin (5). One-tenth milliliter of 1:50 diluted (20 µg protein/ml) cholera toxin (Sigma cat. no. C-3012, Lot No. 69C-0398), in PBS gave a blueing reaction 15 mm in diameter. This reaction was completely neutralized by mixing equal volumes of the diluted cholera toxin solution with anti-choleragenoid diluted to

10^{-3} in PBS. Anti-choleragenoid diluted to 10^{-4} did not prevent the blueing reaction.

The in vivo effect of anti-choleragenoid on FA in suckling mice was determined as follows. Three-day-old non-sibling mice were pooled and randomly assigned to 24 test groups. The test organisms were grown as described for FA ratio determinations. Cholera toxin was diluted 10-fold in TSB´ (100 µg protein/ml) and employed as a positive control while 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 (v/v), and 0.1 ml of the mixture was administered to each of the test animals within 30 min. Bacterial cultures, cholera toxin, and TSB´ were also treated with 0.01 M phosphate buffered saline, pH 7.2 (PBS) in place of anti-choleragenoid and were given to mice. Five animals in each test group were sacrificed at 4 hr and 12 hr, and the FA ratio was determined for each animal. The FA ratios obtained with and without added anticholeragenoid were statistically compared by t-test using a pooled sample estimator of population variance (18).

Mortality and diarrhea. The ability of cultures to induce death was measured on groups of 5 three-day-old mice. Each was orally administered 0.1 ml of bacterial culture and incubated on a white filter paper at 25°C for 18 hr. Mortality was recorded as (-), (+), and (++) if none or one, two or three, and four or five animals, respectively, died. Likewise for diarrhea scores, none 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 (-), (+), or (++).

LD_{50} and SF_{50}. The test organism grown in TSB´ as described above was diluted 10-fold in PBS. Three to five groups of 5 three-day-old mice was each administered intragastrically 0.1 ml of each dilution. 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 was quantitated by grading as 0%, 50%, and 100% response, respectively, in calculating the SF_{50}.

Y-1 assays. Preparations of cell cultures, culture supernate and heated supernate (56°C, 10 min) of the test organisms were examined for cytotoxic and cytotonic respones in Y-1 mouse adrenal tumor cells as previously described (17) except that the test organisms were grown in brain heart infusion (BHI, Difco) with shaking at 150 rpm at 37°C overnight.

Results

FA ratios. FA ratios induced by cholera toxin were higher at 12 hr than at 4 hr (Table 7). E. coli H10407 elicited FA ratios of 0.1104-0.1177 at 4 hr of incubation. The ratio was still high after 12 hr when whole cell culture was given to the animals, but effects of ST in the culture supernate became less evident at 12 hr. On the other hand drinking water isolates, i.e., 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 <0.08 as negative, 0.08-0.09 as positive, and ≥ 0.09 as strongly positive. Whole cultures of 5 strains of O-1 V. cholerae had positive FA ratios at 4 hr and negative or positive ratios at 12 hr, while culture supernates had negative FA ratios at 4 hr and 12 hr. The whole cell culture of non O-1 V. cholerae strains N-53, WA-0-001, and WA-0-028 and V. fluvialis strains DJVP6957 and H-5 exhibited higher FA ratios at 4 hr than at 12 hr. None of these culture supernates caused a positive response. High FA ratios at 4 hrs were also observed when the whole cell culture 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 7).

Kinetics of FA ratios. Figure 14 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 cholera toxin. The peak at 12 hr resulting from the administration of whole cell culture of the toxigenic strain SG-N-7277 was closely similar in time with the peak induced by cholera toxin alone. On the other hand the nontoxigenic strain SG-N-7077 caused rapid FA by 4 hrs and the ratios declined significantly thereafter. Toxigenic strain 569B induced little activity but ratios remained significantly above control levels in the period of 12-16 hrs. Figure 15 compares the time course of FA ratios induced by 2 strains of non O-1 V. cholerae, a toxigenic strain N-2002H and a nontoxigenic strain N-2030H, and by cholera toxin. Both strains induced peak FA ratios at 4 hr. Only the toxigenic strain N-2002H exhibited positive FA ratios after 8 hrs which corresponded in time to the activity caused by cholera toxin. The level of FA was analogous to that of strain 569B (Fig. 14). In order to confirm that the factor(s) produced at 4 hr is distinct from CT, in vivo neutralization tests were carried out with anticholeragenoid (Table 8). Stimulation of FA due to purified cholera toxin was completely eliminated at both 4 hr and 12 hr. Neutralization of cholera toxin produced in vivo was observed only with toxigenic strains of O-1 V. cholerae 569B and SG-N-7277 at 12 hr. FA ratios induced by the toxigenic non O-1 strain N-2002H, however, was not affected by anticholeragenoid treatment. It is evident that some factor that causes high FA ratios at 4 hrs is independent of cholera toxin. 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 are summarized in Table 9. All strains, except 1096-78, of O-1 and non O-1 V. cholerae tested and V. fluvialis, except strain S50-1CC, were lethal for 3 or more suckling mice. A positive control, E. coli H10407 caused death to all test mice, whereas 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 by those administered purified CT. Feces of a mucoid, pasty consistency was excreted from animals administered non-toxigenic O-1 and non O-1 V. cholerae and V. fluvialis. Mice administered toxigenic O-1 V. cholerae excreted mucoid, pasty feces first, followed by watery diarrhea. Animals administered sterile TSB', E. coli 701, and C. freundii 3321 did not excrete feces nor 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 supernates were diluted (1:2 to 1:16) to reduce the cytotoxic effect, exhibited a cytotonic effect on Y-1 cells (Table 9). 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 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 effected at minimal dilutions. Cytotonic and cytoxic 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 one log higher than SF_{50} values obtained for each test strain. Also, strains of non 0-1 *V. cholerae* generally had lower LD_{50} and SF_{50}values than the strains of *V. fluvialis* (Table 10).

Discussion

Until a few years ago, non O-1 *V. cholerae* generally was termed either non-cholera vibrio (NCV) or non-agglutinating vibrio (NAG). The terms NAG or NCV are no longer applicable since there are groups of *V. cholerae* other than O-1 which are genetically and physiologically indistinguishable with O-1 *V. cholerae*. In most instances, non O-1 *V. cholerae* do not produce CT, but 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* in the course of 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 O-1 *V. cholerae* as early as 4 hr postinoculation, and that all tested strains of *V. cholerae*, regardless of their source of isolation and toxigenicity, elicited intestinal fluid accumulation. 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 hrs although the amount of fluid accumulation 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 hr and later. Our results suggested the detection of another factor which was distinct from CT. The virulence associated factor reported in this study was also shown to be different from cholera toxin by the kinetics of fluid accumulation (12 hr) and by *in vivo* neutralization tests, e.g. FA by strain SG-N-7277 was not reduced at 4 hrs by anticholeragenoid but was significantly reduced at 12 hrs from an FA value of 0.0922 to 0.0808.

Spira et al. (26) found 2 strains of non O-1 *V. cholerae* isolated from Chesapeake Bay which produced a heat-stable toxin similar to the ST of *E. coli*. The non O-1 *V. cholerae* toxin caused peak fluid accumulation by 4 hrs in the suckling mouse assay and the rabbit ileal loop. The virulence associated factor responsible for the fluid accumulation at 4 hrs 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 supernate of the bacterial cultures.

None of the orally administered culture supernates of *V. cholerae* and *V. fluvialis* induced fluid accumulation, a finding which substantiates that of Baselski et al. (1). Apparently, detectable fluid accumulation caused by the concentrations of toxins found in culture broths requires the presence of bacterial cells in contrast to responses observed when purified CT is 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 CT 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. Also, regardless of CT production, there was a consistent correlation between diarrhea production, mortality, and ability to induce 4 hr 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 not due to CT production. We wish to designate these morphological reactions with Y-1 cells as "cytotonic-like" to formally distinguish this response from that produced by CT. The cytotonic-like 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). The typical morphological response induced in Y-1 cells by CT and the reaction designated cytotonic-like are very difficult to distinguish by morphological criteria only. Inactivation of the cytotonic responses with anticholeragenoid or evidence of steroidigenesis might be useful in distinguishing whether CT induced the effect. Such additional tests were not considered as necessary in the present studies since

suckling mice and tox-gene analyses verified the unique differences between the present virulence associated factor and CT.

Since all the V. cholerae tested induced rapid 4 hr 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 while no FA was observed in suckling mice unless living Vibrio cells were administered.

Two 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 hr FA virulence associated factor needs to be discussed in regard to these observations. Both strains induced the 4 hr FA response in mice, were cytotoxic, but 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 the human intestine (15). Only 40% (8/20) of the volunteers excreted vibrios and those cases were only detected for 1 or 2 days following ingestion. Although the clinical relevance of the virulence associated factor remains to be verified, we do not feel 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 while 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 hrs which is then followed, in toxigenic strains, by a second round of CT-induced FA which peaks at 8-12 hrs. This factor may be the substance or one of several substances contributing to Vibrio diarrheal disease in humans infected by non-toxigenic V. cholerae but this is not verified at present. It is also not clear whether the cytotoxic-like factor induced in Y-1 cells is caused by this or other virulence factors. Studies are currently underway to purify this factor which will help resolve some of the remaining questions of its mode of action and affect on various bioassay systems.

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Fig. 14. Kinetics of FA ratios induced by O-1 Vibrio cholerae. Test organism was grown in TSB´ (see text) for 18 hr 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. □-□ Strain SG-N-7277 (toxigenic), O-O strain 569B (toxigenic), Δ-Δ Strain SG-N-7077 (nontoxigenic), ●-● Cholera toxin (100 µg/1 ml of TSB´), X-X Control (TSB´).

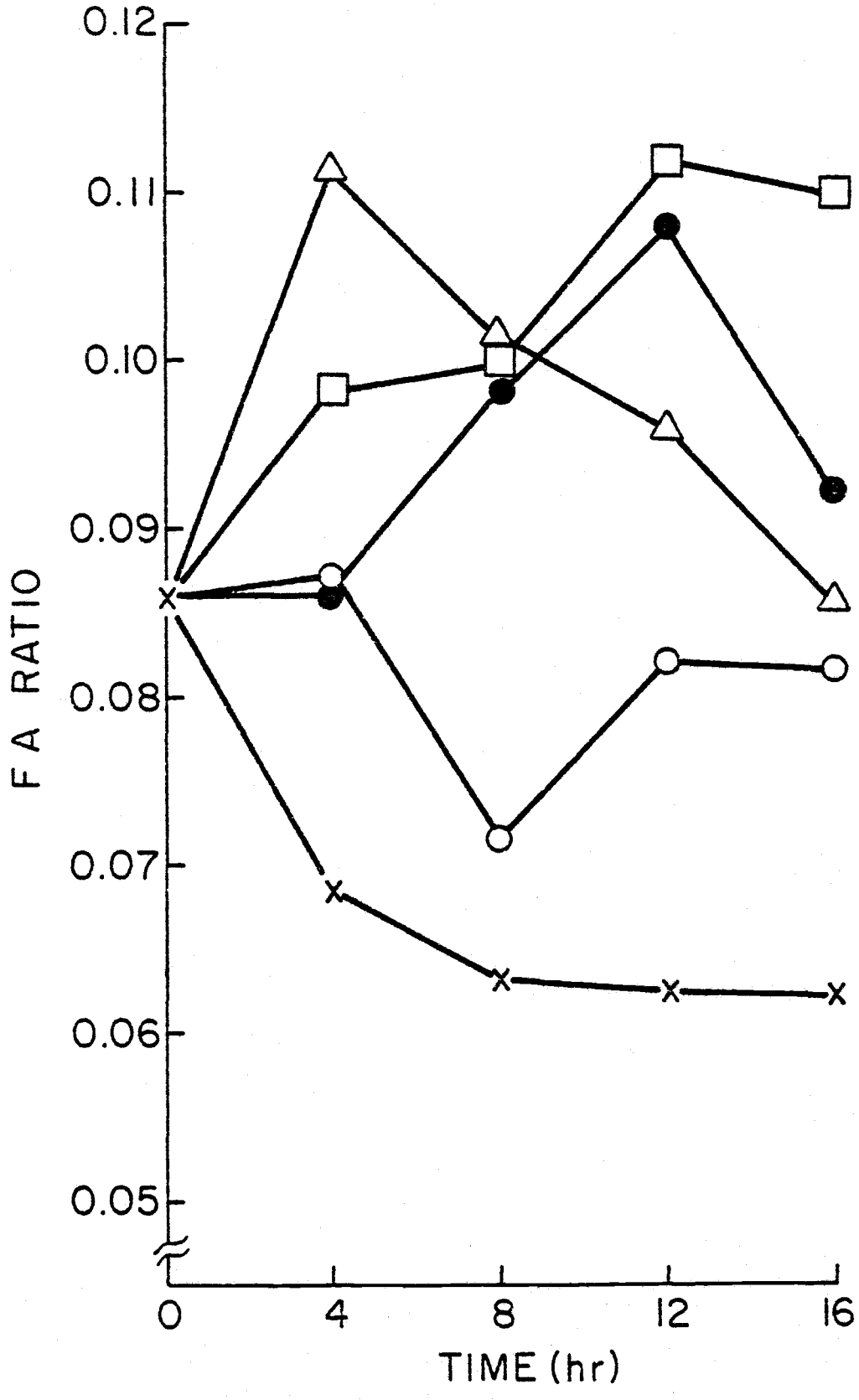

Fig. 14.

Fig. 15. Kinetics of FA ratios induced by non O-1 Vibrio cholerae. Test organism was grown in TSB´ (see text) for 18 hr 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. □-□ Strain N-2002H (toxigenic), Δ-Δ, Strain N-2030H (nontoxigenic), ●-● Cholera toxin (100 µg/1 ml of TSB´), X-X control (TSB´). Note: Strain N-2002H conforms to the description of the recently established species, V. mimicus.

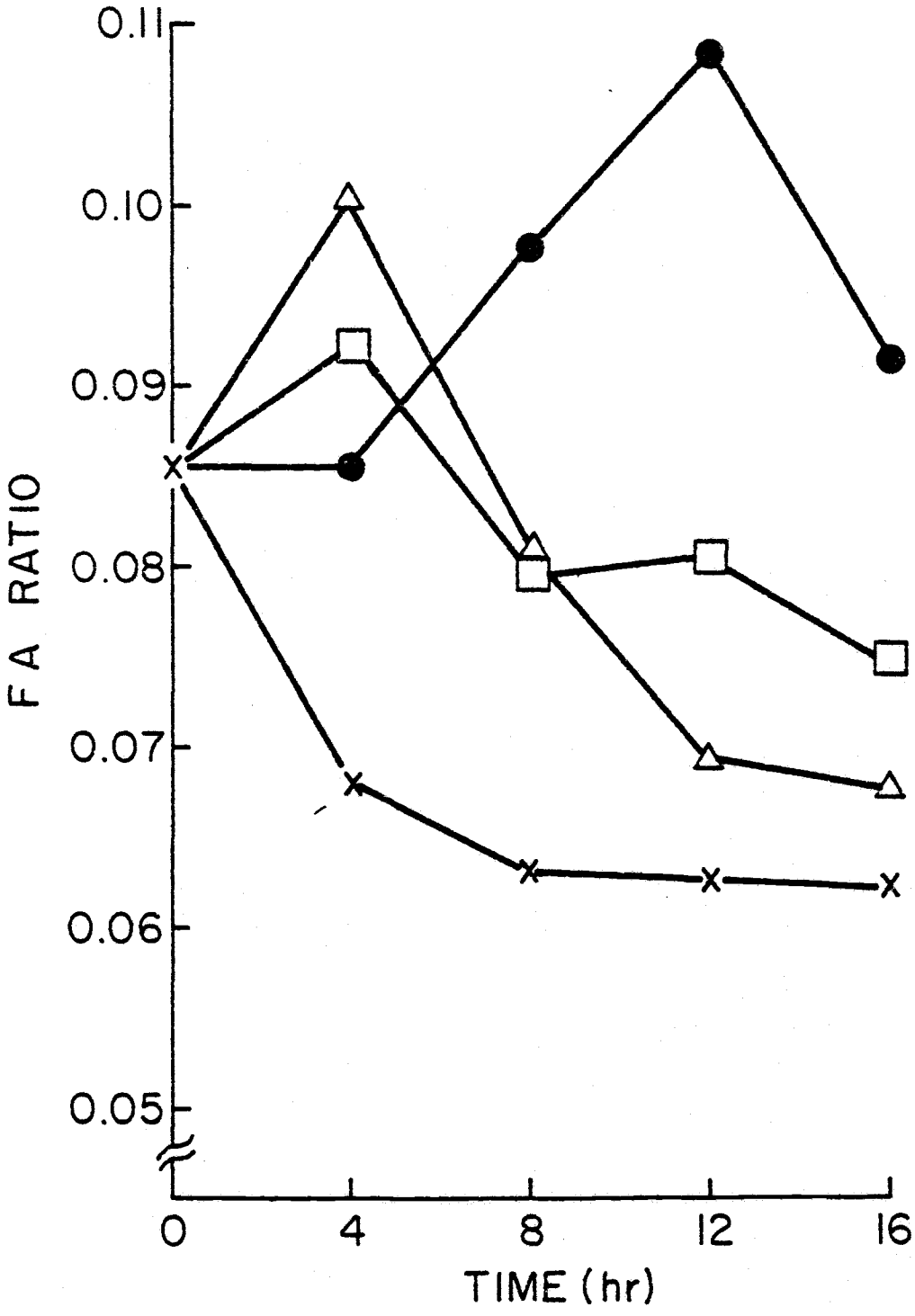

Fig. 15.

Table 7. Results of FA ratio determination in 3-day-old suckling mice.

Organism[a]			Whole culture (C) or culture supernatant (S)[c]	FA ratio at	
Species	Strain No.	Tox gene[b]		4 hr	12 hr
O-1 *Vibrio 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[f]	-	C	0.1007	0.0942
			S	0.0733	0.0627
	WA-O-001	-	C	0.0914	0.0858
			S	0.0761	0.0651
	WA-O-028	-	C	0.0993	0.0887
			S	0.0713	0.0703
	N-2002H[f]	+	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
Cholera Toxin				0.0920	0.1120
Escherichia coli	H 10407	NT	C	0.1177	0.1276
			S	0.1104	0.0861
	701	NT	C	0.0625	0.0668
			S	0.0633	0.0631
Citrobacter freundii	3321	NT	C	0.0694	0.0634
			S	0.0694	0.0620
TSB				0.0686±0.0061[e]	0.0640±0.0070[e]

Table 7. Continued.

[a]Test organism was grown in TSB´ (see text) for 18 hr at 37°C with agitation at 200 rpm.

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

[c]Each of the five 3-day-old mice received 0.1 ml of the test material intragastrically.

[d]NT, Not tested.

[e]Average of 3 determinations.

[f]These strains conform to the description of the recently established species, *V. mimicus*.

Table 8. Effects of anticholeragenoid on FA ratios induced by Vibrio cholerae in 3-day-old suckling mice.

Test organism[b]	FA ratio[a] at 4 hr: Culture + PBS[c]	4 hr: Culture + anti-choleragenoid[d]	4 hr: Effects[e]	12 hr: Culture + PBS	12 hr: Culture + anti-choleragenoid	12 hr: Effects
Cholera toxin[f]	0.0920±0.0122	0.0654±0.0055	+ (P<0.005)	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[g] (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]FA ratio of each of 5 animals was determined. Each animal received 0.1 ml of the test material.
[b]Test organism was grown in TSB´ (see test) for 18 hr at 37°C with agitation at 200 rpm.
[c]Culture : 0.01 M phosphate buffered saline, pH 7.2 (PBS) = 9:1 (v/v).
[d]Culture : anticholeragenoid = 9:1 (v/v).
[e]Compared by t-test using a pooled sample estimator of population variance.
[f]Diluted in TSB´ (100 µg protein/ml).
[g]See the footnote f of Table 7.

Table 9. Results of lethality and stained feces deposition caused by Vibrio cholerae and V. fluvialis in suckling mice and Y-1 mice adrenal cells.

Organism					Suckling mouse assays[a]		Y-1 assays[b]	
Species	Strain No.	Source	Tox gene[d]	Other characters	Diarrhea score	Mortality	Unheated supernate (1:16)	Heated supernate[c] (1:2)
O-1 Vibrio	5698	Clinical, India	+	Serotype Inaba	+	+	CN[e]	-[g]
cholerae	N20	Estuary water, Louisiana	+	"	++	++	CX[f]	(-)
	SG-N-7277	Estuary Water, Louisiana	+	"	++	++	CN,CX	-
	SG-N-7077	Crab, Louisiana	-	"	++	++	CN,CX	-
	1074-78	Sewage water, Brazil	-	Serotype Ogawa	++	+	NT	NT
	1096-78	Sewage water, Brazil	-	"	+	-	NT	NT
Non O-1	N-2002H[k]	Clinical, Louisiana	+		+	+	CN,CX	-
V. cholerae	N-2011H[k]	Clinical, Louisiana	+		+	++	CN,CX	-
	N-2030H	Clinical, Louisiana	-		+	++	CX	-
	N-2031H[k]	Clinical, Louisiana	-		++	++	CN,CX	-
	N-53[k]	Clinical, Louisiana	-	Sakazaki serovar 8	++	++	CN,CX	(-)
	N-3	Crab, Louisiana	-		+	++	CN,CX	-
	SG-N-7210	Crab, Louisiana	-		++	++	CN,CX	-

Table 9. Continued.

Organism					Suckling mouse assays[a]		Y-1 assays[b]	
Species	Strain No.	Source	Tox gene[d]	Other characters	Diarrhea score	Mortality	Unheated supernate (1:16)	Heated supernate[c] (1:2)
	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. fluvalis	DJVP6957	Clinical, Indonesia	-	Gas[h](-)	++	++	(-)	-
	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(+)	++	++	(-)	-
	LSU9-26a	Crab, Louisiana	-	Gas(+)	++	++	(-)	-

Table 9. Continued.

Organism					Suckling mouse assays[a]		Y-1 assays[b]	
Species	Strain No.	Source	Tox gene[d]	Other characters	Diarrhea score	Mortality	Unheated supernate (1:16)	Heated supernate[c] (1:2)
Escherichia coli	H10407	Clinical, Bangladesh	NT[j]	ST, LT[i]	++	++	CN	-
	701	Drinking Water, Oregon	NT		-	-	-	-
Citrobacter freundii	3321	Drinking Water, Oregon	NT		-	-	-	-

[a]Test organism was grown in TSB[k] (see text) at 37°C for 18 hr 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 hr. Signs are explained in the text.

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

[c]Culture supernate was heated at 56°C for 10 min.

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

[e]Cytotonic when culture supernate was diluted 1:16 based on morphologic appearance. Toxin was not confirmed as cholera toxin (CT) by anticholeragenoid neutralization or by trypan blue exclusion.

[f]Cytotoxic.

[g]Negative.

[h]Gas production from glucose. (-), negative; (+), positive.

[i]Capable of producing heat-stable and heat-labile enterotoxoins.

[j]NT, Not tested.

[k]See the footnote f of Table 7.

Table 10. LD_{50} and SF_{50} in suckling mice for selected strains of *Vibrio cholerae* and *V. fluvialis*.

Test organism[a]				
Species	Strain No.	Tox gene[c]	LD_{50}	SF_{50}[b]
O-1 *Vibrio 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[d]	+	4.68×10^8	4.68×10^6
	N-53[d]	-	1.11×10^7	1.11×10^6
	N-3	-	7.02×10^7	1.41×10^7
	WA-O-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 hrs with agitation at 200 rpm. The culture was diluted 10-fold in 0.01 M phosphate buffered saline with pH 7.2. Five 3-day-old mice were intragastrically administered 0.1 ml of each dilution. Inoculated animals were incubated at 25°C for 18 hr.

[b]Effective dose for stained feces. See the text for definition.

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

[d]See the footnote f of Table 7.

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CHAPTER V

Medium-Dependent Production of Extracellular Enterotoxins by Non O-1 *Vibrio cholerae*, *V. mimicus*, and *V. fluvialis*

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Abstract

Fluid accumulation at 4 hrs in the intestines of suckling mice distinguished clinical from environmental isolates of non O-1 *Vibrio cholerae*, *V. mimicus*, and *V. fluvialis*. Enterotoxin production was culture medium dependent. Filtrates of cultures grown in tryptic soy broth without dextrose but with added 0.5% NaCl did not exhibit marked enterotoxin activity in the assay. Filtrates of cultures of all clinical strains grown in brain heart infusion supplemented with 0.5% NaCl (BHI′) induced large amounts of fluid accumulation in the mouse intestine. Most environmental strains grown in BHI′ however, were unable to induce fluid accumulation. The enterotoxin present in culture filtrates lost activity at 56°C, and appears distinct from previously described virulence factors, including the well-described cholera toxin. The new enterotoxin could represent an important virulence mechanism common to all three species.

Introduction

Emerging agents of water- and food-borne disease of the genus Vibrio, other than O-1 Vibrio cholerae, include non O-1 V. cholerae (4,21), V. fluvialis (4,13), and V. mimicus (7) which is biochemically similar to V. cholerae. These organisms are usually found in marine/estuarine environments, and their mechanisms for establishing gastroenteritis are not definitely established. A few strains of non O-1 V. cholerae produce cholera or cholera-like toxin (6,16,22) although most clinical and environmental strains do not (15,20,21). Clinical isolates of non O-1 V. cholerae have induced diarrheal disease even though genes encoding cholera toxin were not demonstrable by DNA hybridization procedures (12). Other extracellular products produced by non O-1 V. cholerae have been reported including permeability and hemorrhagic factors (3,5,6,11,16,19,20) and factors toxic to Y-1 adrenal (11,20) and to CHO cells (20). Similarly, V. fluvialis also produces factors which induce fluid accumulation in rabbit ileal loops (1,17,18,20), factors which are toxic to Y-1 adrenal (18) and to CHO cells (14), as well as hemorrhagic factors (20).

Evidence for an enterotoxin should be based at least on documented in vivo intestinal fluid accumulation in an acceptable animal model. Fluid accumulation in the suckling mouse intestine was used originally to detect extracellular heat-stable enterotoxin (ST) of Escherichia coli (8,9). This assay was used subsequently to monitor enterotoxin production by clinical isolates of non O-1

V. cholerae (15) and *V. fluvialis* (10). However, there was no evidence of enterotoxin production in this bioassay. Therefore the search for virulence mechanisms which could account for human diarrhea caused by CT-negative strains of non O-1 *V. cholerae* and other *Vibrio* species is important.

Our research indicates that heat-labile extracellular enterotoxins, distinct from cholera toxin, are produced almost exclusively by clinical strains of non O-1 *V. cholerae*, *V. mimicus*, and *V. fluvialis* as demonstrated by the suckling mouse assay. Enterotoxin production was influenced by the culture medium used to grow the test organisms.

Materials and Methods

Bacterial cultures. The origins of strains used in this study are listed in Table 11. Cultures previously identified as non O-1 *Vibrio cholerae* were tested for fermentation of sucrose and Voges-Proskauer (VP) reaction, and sucrose-negative and VP-negative strains were identified as *V. mimicus* (7).

Growth of cultures. Stock cultures were stored at -80°C. Frozen cultures were revived on tryptic soy agar (Difco) plus 0.5% added NaCl with incubation at 25°C for 24 hr. In preparation for animal virulence assays, cultures were grown at 25°C for 12 hr in tryptic soy broth without dextrose (Difco) but with added 0.5% NaCl (TSB´) or brain heart infusion (Difco) with 0.5% NaCl added (BHI´). One tenth ml of the cultures was transferred into 40 ml of the respective medium (TSB´ or BHI´) in a 250 ml Erlenmeyer flask and incubated with shaking at 200 rpm at 37°C until late stationary phase (18 hr). Cultures were centrifuged using a clinical centrifuge (Microfuge, Beckman) and filtered through a 0.2 μm sterile membrane filter (Acrodisc, Gelman). Culture filtrate activity was usually tested immediately in the suckling mouse. However, the activity was stable for at least several months of storage at -20°C and -80°C.

Suckling mouse assays. Three-day-old CF-1 mice were obtained from Laboratory Animal Resources of Oregon State University. Mice were submitted to experiments within 2 hr after they were removed from

their mothers. A group of 4 or 5 animals was used for each test. One tenth ml of the culture filtrate containing Evans blue dye (0.01%) was administered intragastrically into the stomach of each test animal with polyethylene intramedic tubing (PE60, Clay Adams). Inoculated mice were held at 25°C for 4 hr and decapitated. The intestine and stomach were removed and the ratio of the weight of the pooled intestines and stomachs to the remaining body weight was measured to calculate the fluid accumulation ratio (FA ratio), a modification of Baselski, et al. (2).

Heat inactivation. Loosely capped tubes with culture filtrates (1 ml) were heated to 56 °C in a waterbath or boiled (100°C) for 30 min and FA ratios induced by heated and unheated culture filtrates were determined in suckling mice to test the effect of heating on enterotoxin activity.

Results

FA ratios in suckling mice determined after oral administration of culture filtrates are summarized in Table 11. The upper limit of the FA ratio confidence interval for the sterile TSB´ medium control was 0.0864 ($p = 0.01$) and for the sterile BHI´control was 0.0799 ($p = 0.01$). FA ratios due to culture filtrates of all the TSB´-grown organisms, except strain V-15 of *V. mimicus*, were less than the upper limit of the sterile TSB´ control. When the organisms were grown in BHI´, however, all clinical strains of non 0-1 *V. cholerae*, *V. mimicus*, and *V. fluvialis* induced very high FA ratios ranging from 0.1155 to 0.1716. The culture filtrates of environmental isolates of *V. cholerae* and *V. fluvialis*, except strain LSU 10-41C, did not induce FA ratios above the upper limit of the BHI´ control (0.0799). The culture filtrate of environmental strain V-15 of *V. mimicus*, known to produce a toxin similar to the heat-stable enterotoxin (ST) of *E. coli* (20), showed an extremely high FA ratio when grown in both TSB´ and BHI´. None of the culture filtrates of 0-1 *V. cholerae* strains, regardless of their genetic capability to produce cholera toxin, induced fluid in suckling mice in 4 hr.

The culture filtrate of one clinical strain of each species was heated at 56°C and 100°C for 30 min. The results presented in Table 12 indicate that the enterotoxin in the culture filtrate was inactivated at 56°C and at 100°C after 30 min. The enterotoxins produced from each of these organisms were similar with respect to

their thermal stability. On the other hand, the ST-like enterotoxin produced by V. mimicus V-15 and ST of E. coli H 10407 were stable at 56°C as well as 100°C.

Discussion

Several media were used for stimulating the production of fluid-accumulating activities in culture supernatants from clinical isolates of vibrios. These media included casamino acids plus yeast extract, syncase, TSB´ and BHI´. BHI´ was used in previous studies for detecting virulence factors produced by environmental non 0-1 *V. cholerae* (11). When assayed by the suckling mouse procedure in this study, all clinical isolates of non 0-1 *V. cholerae* and *V. fluvialis* produced detectable levels of enterotoxins when grown in BHI´ but not when grown in other media. The original suckling mouse assay of Dean et al. (8), was developed to detect the ST of *E. coli*. This assay was subsequently used by others in an attempt to detect enterotoxin production by clinical isolates of non 0-1 *V. cholerae* (15) and *V. fluvialis* (10), but without success. The original method of Dean et al. (8) employed tryptic soy broth to grow test organisms, and their standard index for a strongly positive FA ratio in suckling mice was 0.09 or higher. In agreement with the previous studies (10,15) which used the Dean technique (8), none of our strains of non 0-1 *V. cholerae* and *V. fluvialis*, exhibited FA ratios $\geq$ 0.09, after growth in TSB´. Therefore we suspect that failure to detect intestinal fluid accumulating activities in those studies were related to the choice of culture medium.

There is much confusion in the literature on whether strains of non 0-1 *V. cholerae* and *V. fluvialis* are enterotoxigenic

(1,10,14,15,19,20). This confusion is derived in part from the synthesis of other virulence factors (permeability factor, cell elongation factor, hemolysins, etc.), the use of different biological assay systems (tissue cultures, mice, rabbits), route of culture administration (injection, intragastric), and selection of growth media. In the present study the choice of growth medium and the 4 hr suckling mouse incubation period were crucial in revealing the potent fluid accumulating enterotoxin. Spira et al. (20) reported that some strains of V. cholerae isolated from Chesapeake Bay produced a toxin similar to ST of E. coli. We found that environmental strain V-15 of Spira et al. (20) is actually the sucrose negative V. mimicus, and a recent communication also reported ST production by V. mimicus strains (7). The heat-labile enterotoxin of non O-1 V. cholerae and V. fluvialis reported in this study is different from the ST-like toxin of V-15 since the latter is heat-stable and is produced extracellularly in TSB´.

Baselski et al. (2) have shown that orally administered cholera toxin caused significantly increased intestinal fluid accumulation in suckling mice at 8 hr postinoculation. Similar observations were made in the CF-1 mice used in these studies (unpublished observations). Since FA ratios in the current studies were measured at 4 hrs postinoculation, there was no detectable influence from cholera toxin. Furthermore, culture filtrates of BHI´-grown strains of known cholera toxin producers (569B and SG-N-7277) of O-1 V. cholerae did not elicit a significant increase in

FA ratios. FA ratios induced by strains N-2002H and N-2011H of V. mimicus, also known to produce cholera toxin (J. B. Kaper, personal communication), did not exceed the FA ratios caused by strain N-2031H of V. mimicus, a cholera-toxin nonproducer.

Our recent observations indicate that the enterotoxin of strain N-2030H of non O-1 V. cholerae is a protein (heat sensitivity, precipitated with ammonium sulfate, behavior on Sephadex, stained with Coomasie blue on polyacrylamide) with a molecular weight of about 40,000.

Based on the kinetics of action and heat sensitivity, similar enterotoxins were produced predominantly by clinical isolates in all three Vibrio species studied. The enterotoxin could therefore represent an important virulence mechanism common to all three species.

That occasional environmental organisms have the same or similar ability to produce enterotoxins as clinical isolates is not surprising. This was evident in strain LSU 10-41C of V. fluvialis and strain V-15 of V. mimicus. The production of enterotoxin by occasional environmental isolates may better establish the public health significance of Vibrio species which are abundant in marine/estuarine waters and in marine foods consumed by the human population.

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Table 11. FA ratios in suckling mice at 4 hr following oral administration of sterile culture filtrates of *Vibrio cholerae*, *V. mimicus*, and *V. fluvialis* grown in different media.

Test organism[a]				FA ratio[b] obtained with	
Species	Strain	Origin	Character-istics	TSB[c]	BHI[d]
Vibrio cholerae	569B	C[e] (India)	Tox[g+]	0.0708	0.0711
O-1	SG-N-7277	E[f] (Estuary water, Louisiana)	Tox$^+$	0.0700	0.0711
	SG-N-7077	E (Crab, Louisiana)	Tox$^-$	0.0714	0.0687
V. cholerae	N-2030H	C (Lousiana)	Tox$^-$	0.0673	0.1716
non O-1	N-3	E (Crab, Louisiana)	Tox$^-$	0.0838	0.0759
	WA-O-001	E (Estuary water, Oregon)	Tox$^-$	0.0761	0.0690
	WA-O-028	E (Estuary water, Oregon)	Tox$^-$	0.0713	0.0680
V. mimicus	N-2002H	C (Louisiana)	Tox$^+$	0.0689	0.1392
	N-2011H	C (Louisiana)	Tox$^+$	0.0761	0.1321
	N-2031H	C (Louisiana)	Tox$^-$	0.0893	0.1601
	N-53	C (Louisiana)	Tox$^-$	0.0733	0.1367
	V-15	E (Chesapeake Bay, Maryland)	Tox$^-$	0.1833	0.1820
V. fluvialis	DJVP6957	C (Indonesia)	Tox$^-$	0.0595	0.1365
	DJVP7225	C (Indonesia)	Tox$^-$	0.0742	0.1232
	H-5	E (Estuary water, Maryland)	Tox$^-$	0.0677	0.0731

Table 11. Continued.

Test organism[a]				FA ratio[b] obtained with	
Species	Strain	Origin	Character-istics	TSB[c]	BHI[d]
	LSU9-26a	E (Crab, Louisiana)	Tox$^-$	0.0711	0.0794
	LSU10-41c	E (Crab, Louisiana)	Tox$^-$	0.0880	0.1155
Control (sterile culture medium)				0.0686±0.0061	0.0742±0.0010

[a]Test organism was grown in the respective medium for 18 hr at 37°C with agitation at 200 rpm.

[b]Each of five 3-day-old mice received 0.1 ml of the culture filtrate intragastrically. FA ratio was determined at 4 hr postinoculation.

[c]Tryptic soy broth without dextrose (Difco) with added 0.5% sodium chloride.

[d]Brain heart infusion (Difco) added with 0.5% sodium chloride.

[e]Clinical isolate (diarrheal stool).

[f]Environmental isolate.

[g]Genes coding for cholera toxin, i.e., hybridizing to LT probe of Escherichia coli (personal communication, J. B. Kaper). +, the genes present; -, the genes absent.

Table 12. Effect of heat treatment on enterotoxin activity present in culture filtrates of *Vibrio* species and enterotoxigenic *Escherichia coli*.

Test Organism[a]	Strain	FA ratio induced by heat-treated culture filtrate		
		Untreated	56°C, 30 min	100°C, 30 min
Non O-1 *Vibrio cholerae*	N-2030H	0.1400	0.0719	0.0796
V. mimicus	N-53	0.1316	0.0707	0.0721
V. fluvialis	DJVP6957	0.1527	0.0701	0.0749
V. mimicus	V-15	0.1975	0.1651	0.1426
Escherichia coli	H10407 (clinical)[b]	0.1650	0.1869	0.1372
Control (sterile culture medium)		0.0712		

[a]Test organism was grown in brain heart infusion (Difco) with added 0.5% NaCl with shaking at 200 rpm at 37°C for 18 hr.

[b]Produces both heat-stable and heat-labile enterotoxins.

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APPENDICES

APPENDIX 1

Partial Purification of Enterotoxin Produced by Non O-1 *Vibrio cholerae*

The newly discovered enterotoxin of non O-1 *V. cholerae*, *V. mimicus*, and *V. fluvialis* was produced only in BHI′ medium, was heat-labile, and induced significantly high FA ratios in 3-day-old suckling mice at 4 hr postinoculation. It is distinct from the well-known cholera toxin (CT) (Chapter V).

In this study, an attempt was made to purify the enterotoxin produced by the clinical strain 2030H of non O-1 *V. cholerae*.

The enterotoxin produced in BHI′ medium was concentrated and partially purified using the flow scheme of Fig. 16. Table 13 summarizes the numerical results of the partial purification procedure.

Suckling mice were used to monitor the purification progress by detection of FA activities in the various fractions. A standard curve of FA ratios was obtained by plotting various concentrations of crude enterotoxin (definition given below) vs. FA ratios induced in mice (Fig. 17). The results indicated that the following linear relationship exists between FA ratios and crude enterotoxin in the FA ratio range of 0.0806-0.1312: (FA ratio) = 0.506 x (relative concentration of crude enterotoxin) + 0.0806 (correlation coefficient = 0.89). Accordingly, the following equation, derived from the above relationship, was used to monitor enterotoxin units

during the purification for FA ratios ranging from 0.09 to 0.13: (Enterotoxin unit) = 51.3 x (FA ratio) - 4.13, where 1 enterotoxin unit was defined as the amount of the enterotoxin that induces 0.10 FA ratio in a 3-day-old suckling mouse. Thus, when the experimentally observed FA ratio is 0.1, there is 1 unit of enterotoxin activity.

Quantitative protein measurements were performed by the coomassie blue method (3,4).

The culture was stored at -80°C and was streaked onto TSA´ as needed for experimentation. Several colonies from TSA´ were inoculated into each of eight tubes containing 10 ml of BHI´ and incubated statically at 25°C for 12 hrs. Five ml of the broth culture from each tube was inoculated into 500 ml of fresh BHI´ in a 2.8 liter culture flask (x8) and incubated at 37°C with shaking (200 rpm) for 18 hrs. The broth culture was centrifuged (10,000 x g, 30 min) at 4°C and the supernate was filtered through a 0.45 μm sterile membrane filter (Gelman). The filtrate was brought to 70% saturation with ammonium sulfate, stirred for several hrs on ice and the precipitate was collected by centrifugation (10,000 x g, 30 min) at 4°C. The pellet was dissolved in 100 ml of phosphate buffered saline (PBS: 0.01 M phosphate, 0.12 M NaCl, 0.01 M NaN_3, pH 7.8) at 4°C, concentrated to 25 ml by ultrafiltration on a YM-30 membrane (Amicon), and dialyzed against PBS at 4°C. The preparation was further concentrated by ultrafiltration to 13 ml at 4°C. Any precipitate that appeared

during concentration was collected by centrifugation and discarded. This concentrated sample was designated crude enterotoxin. About one-half (7.5 ml) of the crude enterotoxin preparation was applied to a Sephadex G-100 column (2.5 x 60 cm) prepared with PBS. Fractions containing enterotoxin had a major peak of activity (shown by the large arrow in Fig. 18A) corresponding to an approximate molecular weight (MW) of 40,000. However, fractions containing enterotoxin appeared over a broad range, indicating overloading of the sample. Fractions around the major peak of enterotoxin activity (no. 33-44) were pooled, concentrated by ultrafiltration to 6.0 ml, and rechromatographed on the same Sephadex G-100 column. The fractions then exhibited two peaks of enterotoxin activity. The peaks were designated I and II (Fig. 18B) and corresponded to apparent MW of 49,000 and 31,000, respectively, on the calibration curve for the column. These preparations were called partially purified enterotoxin I and II (corresponding to peak I and II, respectively). The partially purified enterotoxin II, however, had lower specific activity than that of the original culture filtrate (Table 13). Therefore further purification by isoelectric focusing was only carried out using partially purified enterotoxin I. The 5 ml sample from fraction no. 33 was dialyzed against 0.01 M Tris-HCl buffer (pH 6.8), concentrated to 1.3 ml with polyethylene glycol 20,000 (J. T. Baker) at 4°C and stored at -80°C. Isoelectric focusing was performed in a linear gradient of 0-60% glycerol containing

Isolytes (final concentration: 2.8%) buffer with pH range 3-10 (Isolab) in a LKB electrofocusing column (LKB Instruments). Current was applied to the water-jacketed column at a constant power (5 W) at 5°C for 24 hr. The voltage across the column increased from 400 to 1500 V during the run. Fractions (2 ml each) were stored at -20°C after pH measurement. To assay the fractions for enterotoxin activity, 0.2 ml from each of 5 consecutive fractions were pooled, dialyzed against PBS at 4°C and concentrated to 0.4-0.6 ml with polyethylene glycol. One of the pooled fractions induced significantly high FA ratios in suckling mice. Each of the 5 individual fractions in that pool which exhibited enterotoxin activity were dialyzed against PBS and assayed for enterotoxin activity in suckling mice. As a result, 3 consecutive fractions (38-40) (pI: 5.6-5.8) illustrated enterotoxin activity (FA ratios 0.117, 0.108, and 0.125 for fractions 38, 39, and 40, respectively). But enterotoxin activity was not observed in the same preparations after 1-week of storage at 4°C. Nevertheless these fractions were then dialyzed against 0.01 M Tris-HCl buffer (pH 6.8) and concentrated (x 10) by lyophilization. When these samples were boiled in the presence of 4.2% 2-mercaptoethanol and 1.8% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver stain (1), the 3 fractions appeared to have protein bands in common (shown by the arrows in Fig. 19, approximate MW 32,000 and 21,000) although some other minor bands were found to exist. Assuming that

these 2 proteins are subunits of the enterotoxin, judged by estimated molecular weight, partially purified enterotoxin I (MW ca. 49,000 by gel filtration) could be the holotoxin composed of both subunits, and partially purified enterotoxin II (MW ca. 31,000 by gel filtration), which showed relatively low enterotoxin activity, could be one of the subunits with MW ca. 32,000 (by SDS-PAGE). Currently, experiments are underway to verify these observations.

Fig. 16. Methods used for purification of an enterotoxin produced by non 0-1 *V. cholerae* N-2030H.

Method of Purification	Designation of Preparation
Growth in BHI´	
↓	
Culture filtrate	1
↓	
Ammonium sulfate precipitation (70% saturation)	
↓	
Dissolution in PBS, concentration, and dialysis against PBS	2
↓	
Ultrafiltration on YM-30 membrane (Amicon)	3 (=Crude enterotoxin)
↓	
Sephadex G-100 chromatography	
↓	
Concentration by ultrafiltration	
↓	
Sephadex G-100 chromatography	4 (=Partially purified enterotoxin)
↓	
Concentration by ultrafiltration	
↓	
Isoeletric focusing	

Fig. 16.

Fig. 17. Relationship between the FA ratio and concentration of crude enterotoxin. Crude enterotoxin was prepared by concentrating BHI'-culture filtrate of non O-1 Vibrio cholerae N-2030H with ammonium sulfate precipitation followed by ultrafiltration on YM-30 membrane as shown in Fig. 16 and Table 13. Appropriate dilutions of the crude enterotoxin preparation were made in phosphate buffered saline (pH 7.8). One-tenth milliliter of each diluted crude enterotoxin was given to each of two 3-day-old suckling mice. At 4 hr postinoculation, the pooled intestines and stomaches from 2 animals were used to determine the FA ratio.

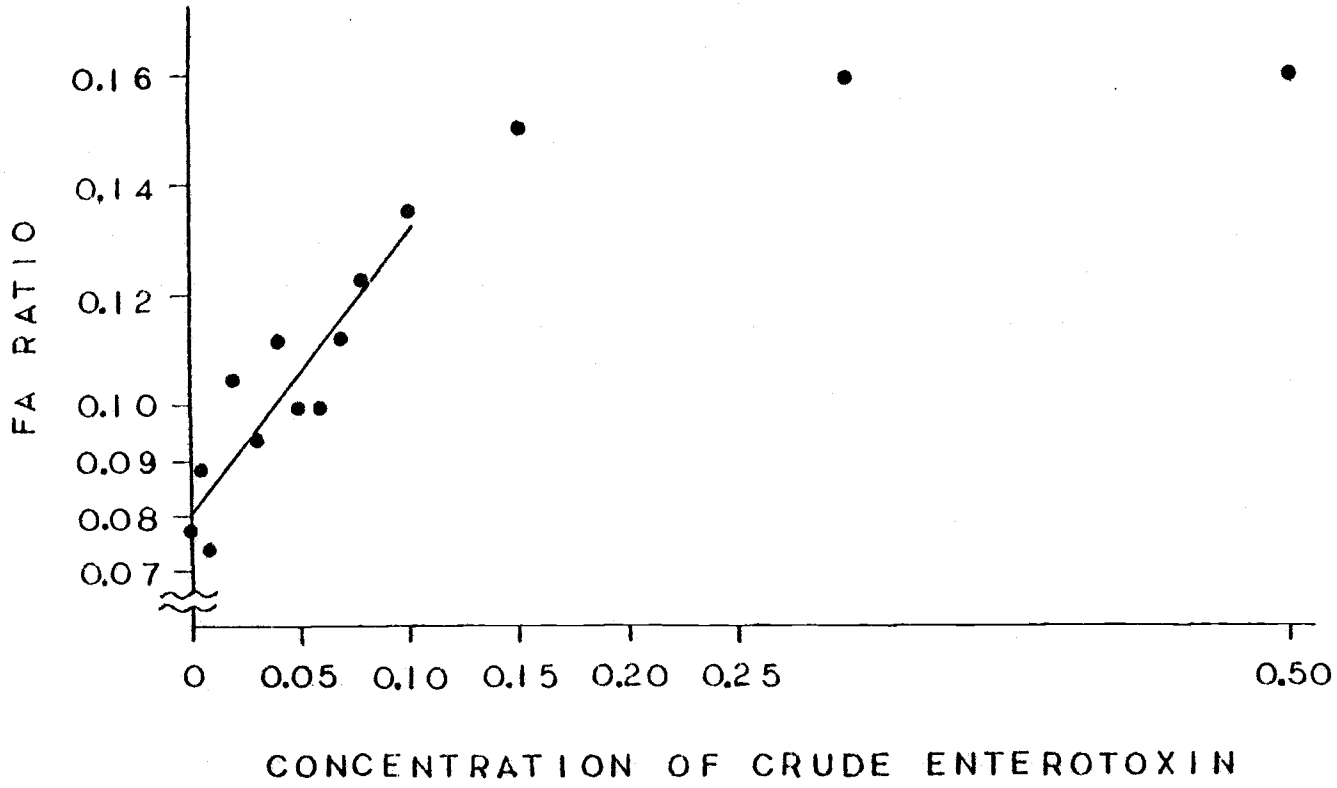
FA RATIO
0.16
0.14
0.12
0.10
0.09
0.08
0.07
0
0.05
0.10
0.15
0.20
0.25
0.50
CONCENTRATION OF CRUDE ENTEROTOXIN

Fig. 17.

Fig. 18. Recovery of crude enterotoxin from Sephadex G-100 column chromatography. Seven and a half milliliters of crude enterotoxin (See Fig. 16 and Table 13) were chromatographed on a Sephadex G-100 column (Fig. A). Fractions 33-44 exhibiting the major peak (large arrow in Fig. A) of enterotoxin activity were pooled, concentrated, and rechromatographed on the same Sephadex G-100 column (Fig. B), where 2 separate peaks I and II of enterotoxin activity were observed. ●——●, protein concentration determined by the coomassie blue method (2,4). ▲——▲ Enterotoxin activity determined from FA ratios in suckling mice (1 unit = amount of enterotoxin that induces 0.10 FA ratio in a 3-day-old suckling mouse). Vo: void volume.

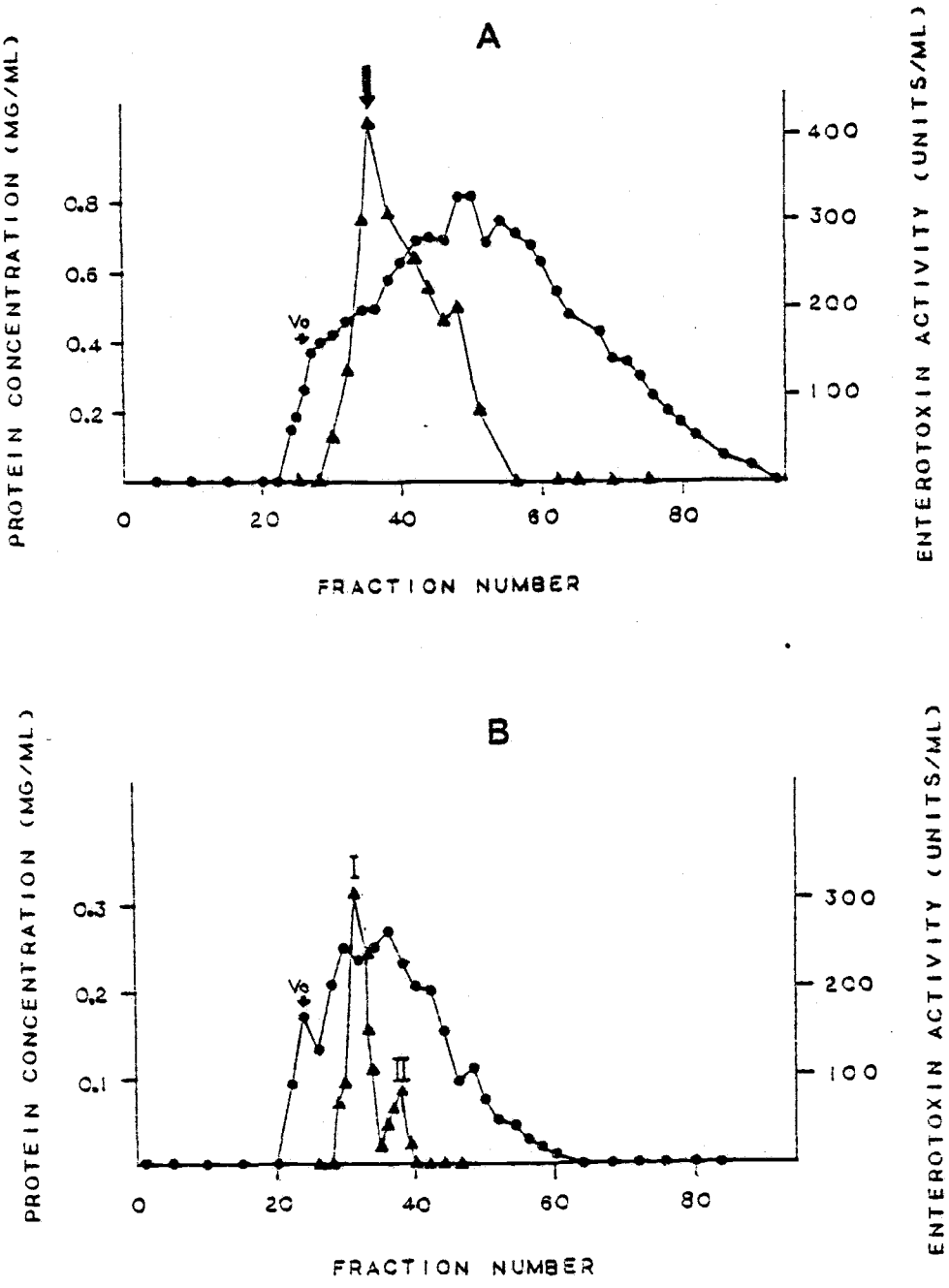
A
PROTEIN CONCENTRATION (MG/ML)
ENTEROTOXIN ACTIVITY (UNITS/ML)
FRACTION NUMBER
Vo
0.2
0.4
0.6
0.8
100
200
300
400
0
20
40
60
80
B
PROTEIN CONCENTRATION (MG/ML)
ENTEROTOXIN ACTIVITY (UNITS/ML)
FRACTION NUMBER
Vo
I
II
0.1
0.2
0.3
100
200
300
0
20
40
60
80

Fig. 18.

Fig. 19. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions from isoelectric focusing which exhibited enterotoxin activity. Partially purified enterotoxin I obtained after 2 successive Sephadex G-100 chromatographies (fraction no. 33 Fig. 18B) was subjected to isoelectric focusing at pH 3-10. Fractions (No. 38-41) were concentrated, boiled in the presence of 4.2% mercaptoethanol and 1.8% sodium dodecyl sulfate (SDS) and subjected to SDS-polyacrylamide gel electrophoresis, using a discontinuous gel with a final acrylamide concentration of 10% for the resolving gel, in the high pH buffer system of Davis (3). The protein bands in the gel were visualized by a silver stain (1). Fraction nos. 38-40 showed enterotoxin activity with no. 40 the highest. A neighboring fraction, no. 41, was included for comparative purposes. The two protein bands showed by the arrows (approximate molecular weights 34,000 and 21,000) appear to be common to fraction nos. 38-40. MS: molecular weight markers (Dalton Mark VI, Sigma).

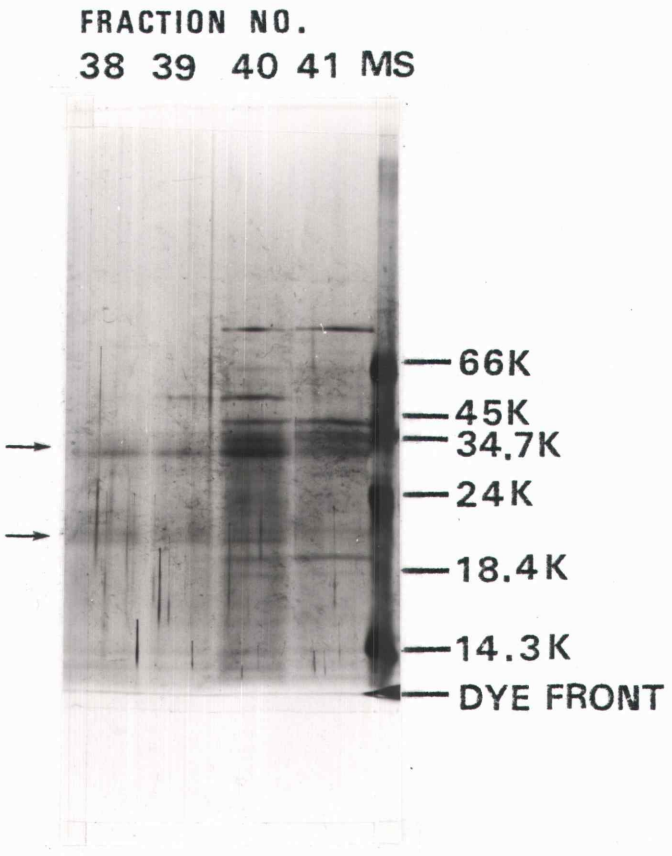

Fig. 19.

Table 13. Purification of enterotoxin produced by non O-1 *Vibrio cholerae* N-2030H.

Designation of Preparation[a]	Method of Purification	Volume (ml)	Enterotoxin Activity (units/ml)	Protein (mg/ml)	Specific Activity (units/ml)	Purification (X)	Total Activity (units)	Yield (%)
1	Culture filtrate	3590	24.8	0.35	71.7	-	89,100	100
2	Ammonium sulfate precipitation	48.5	1630	13.45	121.2	1.69	79,100	88.8
3	Ultra-filtration	13.0	5410	27.9	193.8	2.70	70,300	78.9
4	Sephadex G-100 chromato-graphy[b]							
	Peak I	2.5	549.8	1.6	343.6	4.79	1370	1.5
	Peak II	2.0	54.6	1.6	34.1	0.47	110	0.1

[a]See Fig. 16.

[b]About half (7.5 ml) of preparation 3 was used for purification by Sephadex G-100 chromatography.

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APPENDIX 2

Illustrated Procedures of the Suckling Mouse Assay

In chapters IV and V, the various virulence-associated factors and enterotoxins of O-1 and non O-1 *Vibrio cholerae*, *V. mimicus*, and *V. fluvialis*, were described. The measured responses in the suckling mice included fluid accumulation in intestines, diarrhea and mortality. The following 3 figures illustrate the key points of the procedures and provide photographic documentation of the responses observed in the various assays.

Fig. 20. Intragastric administration of test material into a suckling mouse. Three-day-old suckling mice were submitted to experiments within 2 hr after they were removed from their mothers. One tenth milliliter of cell culture or culture supernate with Evans blue dye (0.01% w/v) was administered into the upper region of the esophagus of each test animal via polyethlene intramedic gastric tubing. Contents of the culture entered the stomach as evidenced by a lack of fluid loss from the mouth and the appearance of blue dye in the stomach.

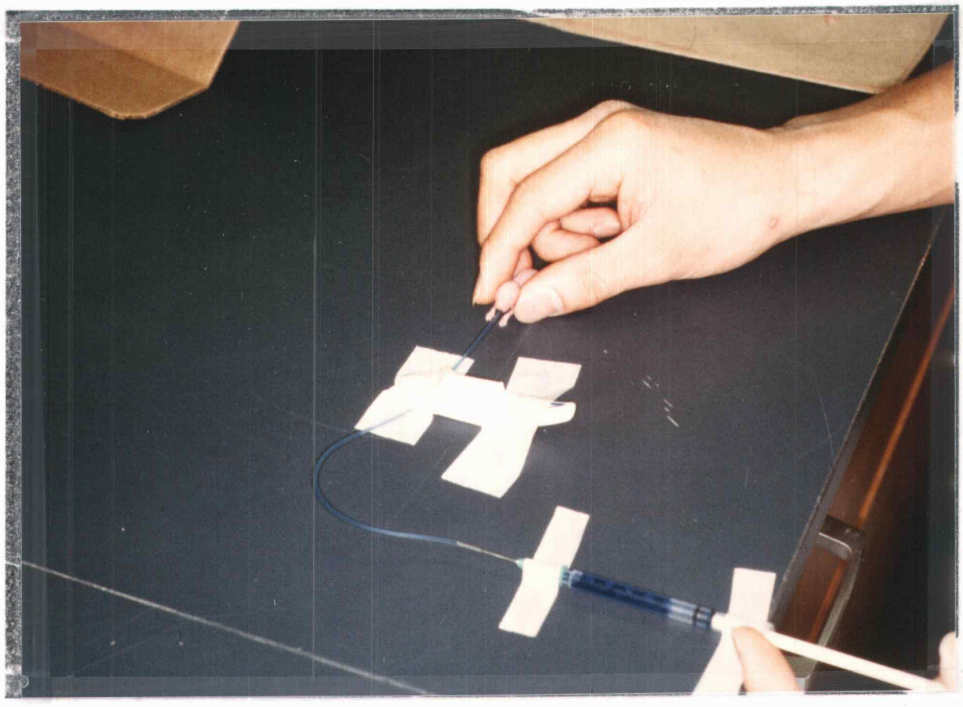


Fig. 20.

Fig. 21. Fluid accumulation in the intestine. A BHI´-culture filtrate of non O-1 Vibrio cholera N-2030H, which contains the new enterotoxin, induced intestinal fluid accumulation (FA) in 3-day-old mice at 4 hr postinoculation. For FA ratio determinations, at 4 hr postinoculation, a group of 5 inoculated animals were placed in a pre-weighed paper cup (3 oz. Bathroom Refill Cup, Dixie) and the total body weight of the animals were measured to the nearest 0.1 mg using an analytical balance (Mettler, Sergent-Welch). Then, the animals were sacrificed by decapitation and dissected. The entire intestines and stomachs were removed and placed into a pre-weighed plastic micro-centrifuge tube (1.5 ml capacity, VWR), and the weight was measured to the nearest 0.1 mg. FA ratio was measured as the ratio of the weight of the stomach plus the intestine to the remaining body weight. For example, a negative control group (5 animals) weighed 9.3000 g (total body weight) and pooled intestines plus stomachs weighed 0.6024 g. Accordingly, the FA ratio was 0.6024 ÷ (9.3000 - 0.6024) = 0.0693. The photographs show the extent of intestinal fluid accumulation relative to FA ratios. A: a negative control that received sterile culture medium (FA ratio: ca. 0.07); B, a mouse that received BHI´-culture filtrate of non O-1 V. cholerae N-2030H (FA ratio: ca. 0.12); C, a

Fig. 21. Continued.

mouse that received concentrated (x5) culture filtrate of non O-1 *V. cholerae* N-2030H by ammonium sulfate precipitation (FA ratio: ca. 0.16).

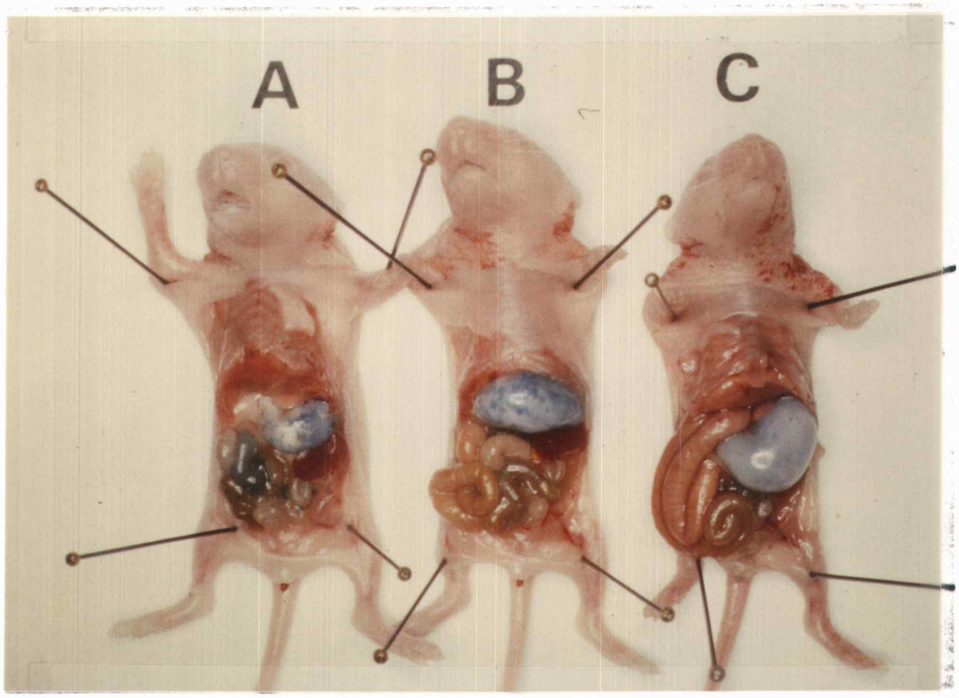
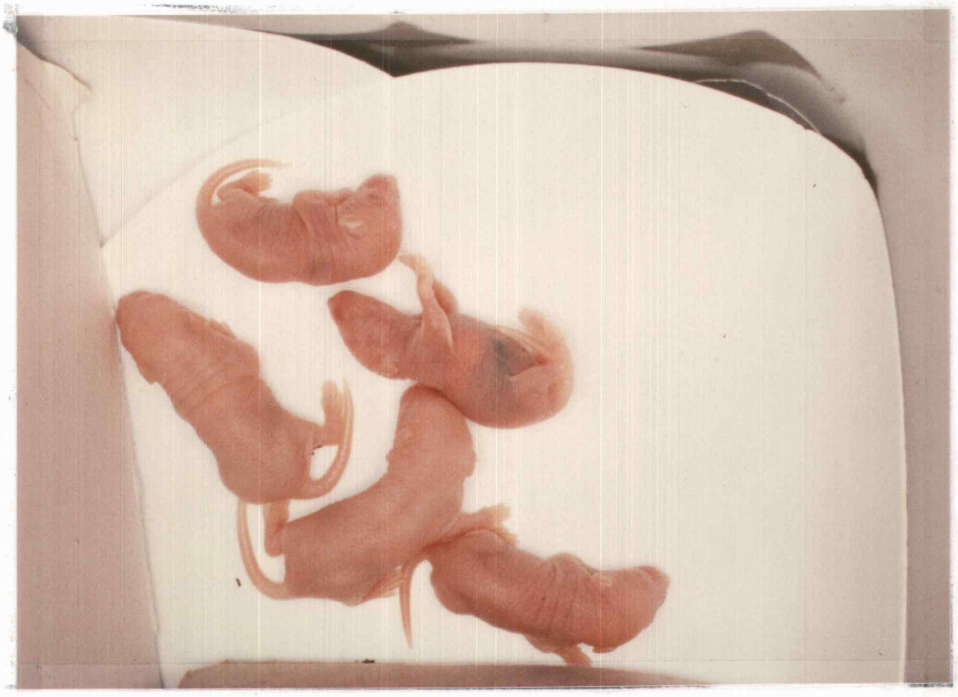


Fig. 21.

Fig. 22. Mortality and diarrhea in suckling mice. A group of 5 mice were incubated on a white filter paper at 25°C for 18 hr, following intragastric administration of test material. Diarrhea was observed as spots of feces stained with Evans blue dye. Mortality was recorded at 18 hr post-administration of the sample. A, Animals received sterile culture medium. No mortality or diarrhea was observed; B, Animals received 0.1 ml of whole cell culture of non O-1 *V. cholerae* N-53. Three out of 5 animals died and many spots of stained feces were observed.

A



B

Fig. 22.