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Diarrhea Caused by *Escherichia coli* That Produce Only Heat-Stable Enterotoxin

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To determine the role of *Escherichia coli* heat-stable enterotoxin (ST) as a virulence factor in human diarrhea, a strain that elaborates only ST (*E. coli* 214-4) was fed to free-living volunteers in doses of 10^6, 10^8, and 10^{10} organisms. Short-lived (1 day) mild illness consisting of abdominal cramps with vomiting or diarrhea occurred in three of five individuals fed 10^8. Typical travelers' diarrhea (loose stools, abdominal cramps, and low-grade fever for 2 to 3 days) was seen in four of five volunteers given 10^{10}; two had brief cholera-like purging of rice-water stools. Despite fever, there was no evidence of mucosal invasion. *E. coli* 214-4 became the predominant coliform in stools; coproculture isolates were uniformly negative for heat-labile enterotoxin (LT), whereas most produced ST. Ten of 13 individuals developed rises in antibody to somatic *E. coli* antigen, and none had rises in LT antitoxin. *E. coli* that elaborate only ST can cause diarrheal disease in adults.

Enterotoxigenic *Escherichia coli* have been implicated as etiological agents in travelers' diarrhea (8, 16), infantile diarrhea (10, 24), and colibacillosis in infant animals (28), and they have been shown to elaborate a cholera-like, heat-labile enterotoxin (LT) and/or a heat-stable enterotoxin (ST) (7, 11, 19). LT has attracted the attention of investigators because of its pharmacological, biological, and immunological similarity to cholera enterotoxin. In contrast to LT, ST is of low molecular weight, nonimmunogenic, and not neutralized by cholera or LT antitoxin (7, 11, 19). Most enterotoxigenic *E. coli* produce LT, either alone (ST^-/LT^+) or in conjunction with ST (ST^+/LT^+) (10, 16, 21, 24). Strains that produce only ST (ST^+/LT^-) are less common (10, 16, 21, 24). *E. coli* producing only ST were shown by Ryder et al. (22) to be responsible for epidemic diarrhea in an infant nursery. The role of these *E. coli* in endemic or adult travelers' diarrhea has not been clarified, although some reports suggest that ST^+/LT^- strains are pathogens (20, 26). These reports, however, have been questioned for the following reasons: (i) other enteropathogens often were concomitantly isolated (26); (ii) ST^+/LT^- *E. coli* were excreted sporadically (26); (iii) a control population was not included (20), though in other studies ST^+/LT^- strains have been found as commonly in healthy individuals as in those with diarrhea (10); (iv) some patients from whom ST^+/LT^- *E. coli* were isolated had fever, which certain workers suggest is atypical of enterotoxin-mediated diarrhea (6); and (v) in one study (20), *E. coli* were tested for ST in dog intestine rather than infant mice (10, 20, 26), making comparison and interpretation difficult (17).

This study was undertaken to determine whether *E. coli* that produce only ST are capable of causing enteric disease in adult volunteers, to describe the clinical syndrome, and to investigate the host immune response.

MATERIALS AND METHODS

Volunteers. Volunteers were free-living (mostly college students), healthy young adults (age 18 to 29 years). Challenge studies were carried out in the Isolation Unit of the Center for Vaccine Development. The protocol was reviewed by the University's Human Volunteer Research Committee and the Clinical Review Committee at the National Institute of Allergy and Infectious Diseases. Studies were explained to volunteers in detail, and a signed, witnessed consent was obtained. The informed nature of consent was documented prior to inoculation by having all volunteers satisfactorily pass a written examination (15) containing multiple choice and true-false questions on all aspects of the study including purpose, hazards, procedures, and pertinent bacteriology and immunology. The preinoculation health status of volunteers was ascertained from medical history, physical examination, chest radiograph, electrocardiogram, complete blood count, urinalysis, blood chemistries (including serum glucose, urea nitrogen, and electrolytes), and tests for

liver function, syphilis, and hepatitis B surface antigen.

Challenge strain. The challenge strain, *E. coli* 214-4 (26), a nontypable strain, was obtained from George Morris of the Center for Disease Control, Atlanta, Ga. This strain is sensitive to tetracycline, ampicillin, neomycin, and colymycin. It was isolated from a physician who, while in Mexico, acquired travelers' diarrhea characterized by watery diarrhea, abdominal cramps, nausea, and fever (26). The strain was shown to be noninvasive (negative Sereny test) (26); it did not produce LT, but it did elaborate ST (26). This particular strain was selected for use because no other bacterial or protozoal enteropathogens were isolated from the patient (26).

Clinical observation. Volunteers were examined and interviewed daily beginning 2 to 3 days prior to inoculation. Oral temperatures were taken every six h and repeated within 5 min if the reading was 100°F (ca. 37.8°C) or above. Stools or rectal swabs were obtained daily for culture; loose stools were collected in a cholera seat for measurement of volume and examination for fecal leukocytes (12). Diarrhea was defined as two or more loose stools in 24 h or at least one voluminous (>200 ml) liquid stool.

Inocula and challenge. Methods used were identical to those previously described (5). Briefly, 18-h Trypticase soy agar cultures of strain 214-4 were harvested with saline, and appropriate dilutions in saline were made. Inocula of 10^6, 10^8, and 10^{10} viable organisms in 45 ml of milk were fed to groups of volunteers who took no food or water for 2.5 h before or after challenge. Inoculum size was quantitated by replicate pour-plate technique.

Typing sera. To prepare antisera for agglutination, a 2.5-kg albino rabbit was inoculated intravenously on eight occasions during a 2-week period with increasing doses (10^7 to 10^9) of living organisms of nontypable *E. coli* 214-4. On day 19, the rabbit was exsanguinated. The resultant sera when diluted 1:15 strongly agglutinated *E. coli* 214-4, but they did not agglutinate any *E. coli* colonies selected from stool cultures of six normal individuals.

Stool culture. Stool specimens or rectal swabs were inoculated on Levine eosin methylene blue agar. Fifteen colonies possessing a typical *E. coli* metallic sheen were randomly picked and subcultured to a slant of Trypticase soy agar in screw-top tubes. After 18 h of incubation, the *E. coli* were tested for agglutination with 214-4 rabbit antisera; *E. coli* 214-4 served as a positive control and saline as a negative control. The slants were overlaid with mineral oil and stored at 4°C (6 to 14 weeks) until tested for ST and LT.

Enterotoxin assay. *E. coli* from slants were inoculated into 50-ml flasks containing 5 ml of Trypticase soy broth with yeast extract, and the organisms were then incubated for 24 h at 37°C with vigorous agitation. Cultures were centrifuged, filtered through 0.22-μm-pore filters, and tested for ST by the infant mouse assay (3) and for LT by the adrenal cell assay (27); these assays are specific for ST and LT, respectively. Positive controls in the mouse assay included strains 214-4, H 10407 (LT/ST) (7), and B7A (LT/ST) (3, 5, 9); a gut-to-remaining body ratio of 0.083 or above was considered positive. Positive controls in the adrenal cell assay were B7A (5, 9) and H 10407 (7). *E. coli* HS (5), known to elaborate neither ST nor LT, served as negative control in both assays. Rice-water stools from two individuals (K. M. and V. M.) were concentrated by evaporation; liquid stool in a dialysis sac was put in front of a high-speed fan at 4°C. Undiluted and 10-fold concentrates were tested in the infant mouse assay for ST.

Serology. Preinfection and convalescent sera and jejunal fluid specimens, after absorption with unsensitized sheep erythrocytes, were tested for antibody to *E. coli* 214-4 by passive hemagglutination, using glutaraldehyde-treated sheep erythrocytes coated with *E. coli* 214-4 lipopolysaccharide antigen (13). Sera were also tested for LT antitoxin by the adrenal cell neutralization technique (4, 25).

Collection of jejunal fluid and jejunal biopsy. Volunteers ingested polyvinylchloride intestinal tubes with sampling ports at 130 cm for collection of jejunal fluid before challenge and 21 days postchallenge. From 2 to 24 h was required for tubes to pass through the pylorus, which was documented by noninvasive methods including observing the length of the tube swallowed (130 cm) and noting a change in character of aspirated material from acidic fluid, pH 1 to 4, to bile-stained fluid of pH 6 or above. Intestinal tubes were removed at least 24 h prior to inoculation. No volunteers who ingested tubes had achlorhydria. Two volunteers with fever ingested Carey capsules for jejunal biopsy (1). Localization of the capsule in the jejunum (110 cm) was confirmed nonradiographically as described above; jejunal fluid collected in this manner was streaked onto eosin methylene blue agar and inoculated into Trypticase soy broth within 30 min.

RESULTS

Clinical. No discernible illness was detected among volunteers given 10^6 organisms (Table 1). After ingestion of 10^8, three of five individuals developed mild illness; all three complained of excessively discomforting abdominal cramps. One of these volunteers vomited but had no diarrhea; one had a single voluminous watery stool (400 ml), and one had two loose stools within a 24-h period. These mild clinical illnesses began from 37 to 48 h after challenge and were quite short-lived (less than 24 h).

Four of five volunteers who ingested 10^{10} bacteria developed unequivocal diarrheal disease (Table 2). These individuals had from three to six loose stools per day and lost up to 1,500 ml within 24 h. The mean incubation period (27 h) was shorter than the mean incubation of illness after ingestion of 10^8 (43 h; Table 1). The two volunteers who experienced early onset of illness after ingestion of 10^{10} organisms (Table 2) manifested a brief cholera-like syndrome with voluminous, watery, rice-water stools. Abdominal cramps were noted in three individuals who

TABLE 1. *Response of healthy volunteers after ingestion of Escherichia coli that elaborate only heat-stable enterotoxin*

Inoculum size	No. of volunteers challenged	No. with positive stool cultures	Clinical attack rate[a]	Mean incubation period (h)	No. with rise in serum somatic antibody[b]	No. with rise in serum LT antitoxin[b]
10^6	4	3	0	–[c]	2	0
10^8	5	5	3/5 (60%)	43	4[d]	0[d]
10^{10}	5	5	4/5 (80%)	27	4	0

[a] Diarrhea or vomiting.
[b] Fourfold or greater rise.
[c] –, None.
[d] Paired specimens from one volunteer were unavailable for testing.

TABLE 2. *Clinical patterns of volunteers with illness due to ST-alone Escherichia coli*

Inoculum size	Volunteer	Incubation period (h)	Days of illness[a]	Peak no. of loose stools/24 h	Peak stool output (ml)/24 h	Nausea or vomiting	Abdominal cramps	Peak temp elevation (°F)
10^8	B.J.	37	1	1	NM[b]	+	+	98.8
	J.P.	45	1	1	400	–	+	99.2
	J.Q.	48	1	2	NM	–	–	99.0
10^{10}	K.M.	13.5	2	3	900	–	+	99.6
	V.M.	15.5	3	6	1,500	+	+	101.2
	J.C.	26	3	3	450	–	–	100.2
	M.M.	53	3	4	700	–	+	99.6

[a] Diarrhea or vomiting.
[b] NM, Not measured.

ingested 10^{10} bacteria, and low-grade fever (100.2 and 101.2°F; ca. 37.8 and 38.3°C) occurred in two volunteers. Jejunal biopsies were obtained from the two febrile volunteers and were examined by Akio Takeuchi of the Walter Reed Army Institute of Research. No sign of invasion or cellular infiltration was seen by light or electron microscopy. Fecal leukocytes were not present in any stool specimens.

Bacteriology. None of 645 randomly selected *E. coli* from prechallenge stools agglutinated with 214-4 rabbit antisera. Fifty-three of these prechallenge *E. coli* were tested for ST and LT; none was positive. After inoculation, all volunteers, except one in the low inoculum group, excreted the challenge strain. Within 2 days after challenge, *E. coli* 214-4 constituted 40% or more of the randomly picked *E. coli*. From that time and during the 4 subsequent days, 88% of 917 randomly selected *E. coli* were identified as the challenge strain. One hundred fifty-four random agglutination-positive *E. coli* (12 to 20 per volunteer) from postchallenge stools were tested for ST, and 109 (70%) were positive in the infant mouse assay; 27 of 49 isolates (55%) from volunteers without illness were ST positive compared with 82 of 105 isolates (78%) from ill volunteers. ST-positive isolates were evenly distributed among the ill volunteers. The above 154 *E. coli* isolated were also tested in the adrenal cell assay for LT; none was positive. Filtered rice-water stools from two patients were tested for ST in the mouse assay undiluted and after 10-fold concentration; ST was not detected.

Jejunal fluids collected 42 and 72 h postchallenge in the course of biopsy of two febrile individuals were cultured. Both failed to grow coliforms. One fluid was obtained while the patient was having diarrhea, whereas the other fluid was collected approximately 24 h after watery diarrhea.

Serology. Paired preinfection and convalescent sera from 13 of 14 volunteers were tested for antibody to *E. coli* 214-4 (one convalescent serum was not obtained); 10 demonstrated fourfold or greater rises in the hemagglutination titer (Table 1). The three individuals who did not develop rises were not clinically ill (two who ingested 10^6; one who ingested 10^{10}). None of the 13 paired sera showed a significant rise in LT antitoxin.

Pre- and postchallenge jejunal fluids were available for serological testing from the volunteers who ingested 10^8 organisms. Three of five showed twofold rises in antibody titer to somatic antigen by the hemagglutination technique.

DISCUSSION

These studies demonstrate that *E. coli* that produce only ST are capable of causing typical

travelers' diarrhea in adults. The clinical syndrome encountered was very similar to that seen in volunteers after ingesting 10^{10} organisms of *E. coli* strains (B7A, B_2C) that elaborate both LT and ST (5). The high-infective inoculum of the ST^+/LT^- strain necessary to cause prominent clinical illness in healthy volunteers (10^{10}) is true for other enterotoxigenic ST^+/LT^+ *E. coli* (5) and *Vibrio cholerae* (2); with the latter two pathogens, concomitant administration of $NaHCO_3$, which alters gastric function, decreases the inoculum required to cause disease by several logs (M. M. Levine, unpublished data; 2). Presumably, in nature, food serving as the vehicle of transmission would act similarly to $NaHCO_3$ and allow clinically overt infection with lower inocula.

Two individuals who exhibited a cholera-like picture had onset of illness after only 13.5 and 15.5 h. A comparably short incubation period was reported in several outbreaks in Japan in which ST-alone *E. coli* were incriminated epidemiologically (29).

It is not apparent why 30% of the agglutination-positive strains isolated by coproculture after challenge were negative in the infant mouse test. Possible explanations include cross-agglutination of strains other than *E. coli* 214-4 by the rabbit antisera and the possibility that during 6 to 14 weeks of storage some isolates lost their ST plasmids. We intend to investigate this question further as a point of interest.

The manner in which noninvasive *E. coli* 214-4 induces fever is unclear; jejunal biopsies failed to show evidence of mucosal invasion or leukocytic infiltration characteristic of shigella (14) or invasive *E. coli* infections (5), and fecal leukocytes were not present (12).

E. coli 214-4 that were excreted postchallenge remained consistently negative when tested for LT; nor were there rises in LT antitoxic antibodies. Illness, therefore, was not due to in vivo production of LT. All ill volunteers for whom acute and convalescent sera were available for serological testing developed fourfold or greater rises in antibody to *E. coli* 214-4 antigen, confirming a host immunological response to infection.

Of the two jejunal fluids cultured, only one was obtained while the patient was still purging. Failure to grow the organisms was perhaps caused by sampling when the *E. coli* were no longer in the duodenum and proximal jejunum but may have colonized in more distal areas of the jejunum or ileum. At this time, the challenge strain was the predominant stool coliform.

It has been suggested that immunological control of *E. coli* diarrheal disease may be pursued by use of a toxoid that would stimulate antitoxic antibodies to LT (18, 19, 23). Most enterotoxigenic *E. coli* elaborate ST as well as LT. If the ST of ST^+/LT^- and ST^+/LT^+ strains is identical, it would seem imprudent to approach immunological control of enterotoxigenic *E. coli* by an LT toxoid, since this would not induce protection against the ST component of ST^+/LT^+ strains that has now been shown to be a virulence property.

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