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SEASONAL GASTROENTERITIS AND MALABSORPTION AT AN AMERICAN MILITARY BASE IN THE PHILIPPINES

III. MICROBIOLOGIC INVESTIGATIONS AND VOLUNTEER EXPERIMENTS

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Dean, A. G. (Pacific Research Section, NIAID, NIH, P.O. Box 1680, Honolulu, Hawaii 96806), R. B. Couch, T. C. Jones and R. G. Douglas, Jr. Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. III. Microbiologic investigations and volunteer experiments. *Am J Epidemiol* 95: 451–463, 1972.—An epidemic of gastroenteritis, one of a series of annual seasonal epidemics, involved an estimated 5,000 of the 36,000 Americans at Clark Air Base, Republic of the Philippines, in March through July, 1969. A tropical sprue-like syndrome commonly followed the acute illness. Microbiologic studies of stools from selected acute cases included parasitologic examination, and cultures for Salmonella, Shigella, Vibrios, Clostridia, enteropathogenic serotypes of *Escherichia coli,* Mycoplasma, and *E. coli* bacteriophages. Viruses were sought by inoculation of infant and adult mice and six different cell culture systems. Tests for hemadsorption, interference, and the production of adenovirus complement fixation antigen were performed in cell cultures. Enterotoxin-producing *E. coli* could not be demonstrated, and strains of *E. coli* from individual patients appeared to be serologically diverse. Ingestion of stool suspension supernates from six patients by 34 volunteers was followed by definite gastrointestinal symptoms in only one volunteer, a man subsequently found to be prone to attacks of idiopathic diarrhea. No probable microbiologic cause for this epidemic was found, suggesting that presently known techniques are inadequate to reveal the cause of many human diarrhea cases.

diarrhea; *Escherichia coli;* gastroenteritis; malabsorption syndromes; sprue, tropical; viruses

INTRODUCTION

At Clark Air Base in central Luzon, Republic of the Philippines, epidemics of diarrheal disease of unknown cause occur annually in the months from March through

Abbreviations: CF, complement fixation; ic, intracerebrally; ip, intraperitoneally; MEM, minimal essential medium; PPLO, pleuropneumonia-like organisms; SS, Shigella-Salmonella; SGOT, serum glutamic oxaloacetic transaminase; SGPT, serum glutamic pyruvic transaminase; XLD, xylose lysine desoxycholate.

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July. The 1969 epidemic began in March and ended by late July, affecting an estimated 5,000–6,000 Americans of the 36,000 attached to the base (1). About 1,300 sought medical attention. Most patients had acute, nonbloody diarrhea, abdominal cramps and constitutional symptoms. Twelve per cent of cases had fever when seen in clinic and one-fourth reported vomiting. Twenty per cent had symptoms for more than two weeks, some for months, and malabsorption of lactose and xylose often persisted for weeks or months after the acute illness (2). An attack of acute gastroenteritis thus led in many cases to a syndrome resembling mild tropical sprue.

In the American population acute gastroenteritis cases occurred both on and off base and were distributed quite evenly by location of work or residence. No significant differences were found in date of onset in various areas on and off base. Age, sex, and race were unrelated to incidence. No connection with food, intake of beverages, recent trips, or date of arrival on base could be established. There was no evidence of person-to-person transmission, and among Americans immunity was not conferred by a previous attack of diarrhea at Clark, although local Filipinos had a much lower attack rate than Americans. The epidemiology of the "sprue" cases was similar to that of the acute diarrhea cases except that the average "sprue" patient was slightly older. Routine laboratory studies of the epidemics over a number of years have not disclosed any cause for the great majority of cases.

During the 1969 epidemic, a combined epidemiologic, clinical, and microbiologic study was carried out. The objectives were to describe the epidemic and to apply as many techniques to the discovery of a microbiologic cause as possible, including attempts to transmit the illness to human volunteers. This report describes the microbiologic investigations and human volunteer studies. Epidemiologic and clinical studies are recorded elsewhere (1, 2).

Methods

Collection of specimens and microbiologic techniques

Fifty-nine enlisted men between the ages of 19 and 43 years, seen at Clark Hospital Clinics for diarrhea from zero to five days after onset, provided specimens. Stool, serum, and throat washings were collected at the time of the first clinic visit and often several times in the next few days of illness. Additional specimens were collected three weeks or more after onset, when possible. Since administration of the patients' stool supernates to volunteers was planned, a number of patients were followed clinically and by periodic serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase (SGOT-SGPT) determinations to detect viral hepatitis.

Single stools and paired sera were collected during the middle of the epidemic from a group of healthy enlisted men working in a large repair shop on base where a high incidence of diarrhea was found, and specimens from those men who did not develop diarrhea were used as controls.

Duodeno-jejunal biopsies were obtained from 11 of the patients in the first few days

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of illness and from three of them several weeks or months later as well, using a Crosby capsule. These were fixed in formalin, sectioned and stained with hematoxylin and eosin and by Brandborg's technique for Giardia (3), and examined by Dr. Jack Welsh, Department of Medicine, University of Oklahoma, and Dr. Lloyd Brandborg, Chief of Gastroenterology, Veterans Administration Hospital, San Francisco, California. One ml of jejunal fluid, collected at the time of biopsy, was placed in 10 ml of 10 per cent glycerol-5 per cent dextrose solution, and preserved at −70 C. Two-tenths ml of this mixture was later spread on a blood agar plate and cultured aerobically at 37 C.

Portions of stools passed in the clinic by patients with acute diarrhea were submitted immediately to the Clark Hospital Laboratory where they were plated on xylose lysine desoxycholate (XLD), Shigella-Salmonella (SS), and MacConkey's agar, and inoculated into selenite enrichment broth which was then subcultured on SS agar. Identification of suspicious colonies was done by routine biochemical methods. Portions of the stool were examined for ova and parasites by the Clark Hospital Laboratory using formalin-ether concentration. Most of the stools were examined in the fresh state as well.

Aliquots of the stools were placed in Cary-Blair and also in Venkatraman-Ramakrishnan sea-salt media and sent by air-mail to the Salmonella-Shigella Unit, Epidemiology Program, Center for Disease Control (CDC), Atlanta, Georgia, where cultures were performed for enteric pathogens including Salmonella, Shigella, and Vibrios. Ten of the specimens were also screened serologically for enteropathogenic types of *E. coli* by the CDC laboratory. Portions of each stool also were placed in 10 per cent formalin and in polyvinyl alcohol fixative and subsequently examined for ova and parasites by the Helminthology and Protozoology Unit of the CDC.

A summary of the tests performed on stools, throat washings, and duodenal-jejunal fluids and biopsies is given in table 1.

The remainder of the stool was placed in sterile jars or screw-top vials, sealed with vinyl electrical tape, placed in polyethylene bags (to prevent pH changes in a CO_2 atmosphere) and frozen in solid CO_2 (dry ice), usually within a half-hour of passage. Throat washings were frozen immediately. Blood specimens were allowed to clot and after centrifugation the serum was placed in the dry ice box. The specimens remained frozen in dry ice during shipment to Honolulu and were stored in a freezer at −60 C or below until the remainder of the investigations were carried out.

Other cultures for bacteria and all viral studies were performed in Honolulu. Portions of 11 frozen stools were examined for Clostridia by growth in thioglycollate medium at 45 C. The cultures were checked at 5, 24, and 72 hours for bubbles of gas and Clostridia were sought in gram-stained smears made at 72 hours.

To search for possible new pathogenic serotypes of *E. coli*, 11 frozen stools were streaked on eosin methylene blue agar (Difco) and five colonies from each (in two cases only one or two colonies) were identified as *E. coli* by biochemical reactions. Fermentation of 15 carbohydrates was tested to further characterize these strains, and from each patient a "typical" strain was selected. Adult rabbits were immunized intravenously with suspensions of the 11 strains in 0.5 per cent formalin on days 0, 2, 7, 9, 12, 14, 16, 19, 21 and 26. Slide agglutination tests were done with the 11 rabbit antisera with each of the patients' 48 strains and with 46 strains similarly isolated from a control group of 10 healthy individuals. The strains to be tested were grown overnight on veal infusion agar plates and the confluent growth from half a plate was suspended in 1 cc of 0.45 per cent sodium chloride. Tests were performed with fresh live antigens and again with antigens boiled for one hour. All the antisera reacted with their homologous antigens by slide ag-

TABLE 1

Microbial studies performed with specimens from acute diarrhea cases in the 1969 Clark epidemic

	Laboratory	Sepcimens examined	Results
Salmonella, Shigella	Clark	44 stools	1 *Salmonella panama*
	CDC	59 stools	1 *Shigella sonnei* 1 Arizona species 3 Providence group 6 Proteus species 1 *Pseudomonas aeruginosa*
Vibrios	CDC	59 stools	Negative
Enteropathogenic serotypes of *E. coli*	CDC	10 stools	Negative
Enterotoxin-producing *E. coli*	Pacific Research	11 stools	Negative
Serologically similar strains of *E. coli* among different patients	Pacific Research	11 stools	No evidence of related strains in different patients.
Clostridia	Pacific Research	11 stools	Negative
Aerobic bacteria	Pacific Research	11 duodeno-jejunal fluids	Few *Staphylococcus epidermidis* only
Ova and parasites	Clark	43 stools	1 Ascaris and Trichuris ova
	CDC	56 stools	1 Trichuris 1 *Giardia lamblia* 4 *Dientameba fragilis* 2 Unidentified amebae
Giardia, Strongyloides	Dr. Welsh	11 acute, 3 convalescent duodeno-jejunal biopsies	Negative
Coccidia	Dr. Brandborg		
Mycoplasma	Dr. Purcell	9 stools 9 throat washings (in 2 pools)	Negative
E. coli bacteriophages	Pacific Research	10 acute stools 10 control stools	80% of both groups positive. Titers similar
Viruses	Pacific Research	See Table 2	

glutination although tube agglutination showed the titers to be low, most being from 32 to 256, but one as low as 4.

Forty-five strains of *E. coli* from 11 acute diarrhea patients were tested for enterotoxin production in infant mice by intragastric inoculation of supernates of trypticase soy broth cultures grown overnight at 37 C on a rotary shaker at 200 cycles per minute. Each strain of *E. coli* was tested in four mice one to four days old. The test in infant mice has been found in our laboratory (4) to be as sensitive as the more usual rabbit loop test and allows considerable saving in time and animal costs. Thirty strains were also tested in rabbit ileal loops with the methods described by Burrows and Musteikis (5), using positive and negative controls but making about 20 loops per animal and using single ligatures at the end of the loops. For each test, loops in two to four different rabbits were used.

For viral isolation studies, 10 or 20 per cent stool suspensions were prepared in phosphate-buffered saline with 0.5 per cent gelatin and centrifuged for one hour or more at 2200 $\times$ gravity (3500 revolutions per minute). The supernatant fluid was used to inoculate cell cultures, sometimes with the prior addition of 1 mg of streptomycin and 1000 units of penicillin per milliliter to the suspension. Four tissue culture

tubes for each type of cell were inoculated with 0.1 ml of the suspension per tube.

Cell cultures were grown in stationary racks with Eagle's Minimal Essential Medium (MEM) with Earle's salts containing 220 mg per cent bicarbonate and 10 per cent fetal calf serum and gassed with 5 per cent carbon dioxide in air before capping. All media contained 100 μg of streptomycin and 100 units of penicillin per milliliter. Maintenance medium was Eagle's MEM without added serum and containing 135 mg per cent bicarbonate—except for the HEp-2 and rabbit kidney cells to which 2.5 per cent heated (56 C, 30 minutes) chicken serum and 2 per cent heated calf serum respectively were added, and WI-38 cells, which were maintained with 110 mg per cent bicarbonate. After inoculation the tubes were placed on a roller drum making 12 revolutions per hour. The tubes were examined microscopically every other day and the maintenance medium was changed every three to five days. Passages were made as required by the condition of the cell layer. Other details are shown in table 2.

Hemadsorption was tested every seven days in the monkey kidney cultures by incubating 0.1 per cent guinea pig erythrocytes with the cell layer for 30 minutes at 4 C, and after examination, for another 30 minutes at 37 C. Tissue passages of nine stools and the two pools containing nine throat washings were challenged at the end of the observation period with 1000 tissue culture infective doses ($TCID_{50}$) of echo 11 virus to detect interfering agents. Complement fixation (CF) tests for detection of adenovirus antigen were done using known

TABLE 2

Viral isolation studies performed with specimens from acute diarrheal cases

Tissue	Temperature of incubation	Observation period (days)	No. of specimens examined	Results
Primary human fetal kidney*†	36 C	30–45	48 stools 2 pools containing 9 throat washings	Single adeno 4 virus Single ECHO 13
Primary infant rabbit kidney*	36 C	28	9 stools 2 pools containing 9 throat washings	Negative
Primary rhesus monkey kidney*‡	33 C	28	9 stools 2 pools containing 9 throat washings	Negative
Primary African green monkey kidney*‡	33 C	28	9 stools 2 pools containing 9 throat washings	Negative
WI-38 diploid human embryonic fibroblasts*‡	33 C	28	9 stools 2 pools containing 9 throat washings	Negative
Hep-2	33 C	28	9 stools 2 pools containing 9 throat washings	Negative
Adult mouse inoculation—ip and ic		28	9 stools 2 pools containing 9 throat washings	Negative
Infant mouse inoculation—ip and ic		21	48 stools 2 pools containing 9 throat washings	Single coxsackie A —not typed

* Tested for interference with 1000 $TCID_{50}$ of echovirus type 11 at the end of the observation period.

† Culture fluids tested for CF activity with adenovirus antiserum.

‡ Tested for hemadsorption with guinea pig erythrocytes every seven days.

adenovirus antiserum and fluids from the final passage in human fetal kidney. The micro adaptation of the Laboratory Branch complement fixation method of the Center for Disease Control (6) was used.

Mice less than 48 hours old were inoculated intracerebrally (ic) with 0.02 ml and intraperitoneally (ip) with 0.05 ml of stool suspension, using eight animals per test. For 11 specimens, including the two throat washing pools, separate litters were used for the two routes of injection; with 39 others, a single litter was injected by two routes. For 11 specimens, 10 adult mice each were inoculated with 0.03 ml ic and 0.1 ml ip. All mice were observed daily for 21 days—for 28 days in the case of the first 11 specimens. Mice ill or dead after the first day were used for passage to other animals, using suspensions of brain and/or carcass as indicated.

The stools of 10 patients and 10 controls were assayed for bacteriophage by mixing approximately 0.2 grams of stool with a freshly inoculated broth culture of *E. coli* and allowing this to grow overnight at 36 C. The "enriched" suspension was filtered through a Millipore (0.22 micron pore size) filter, mixed with an overnight broth culture of *E. coli* and a small amount of veal infusion agar (0.75 per cent agar) at 45 C, and poured onto the surface of a veal infusion plate containing 1.5 per cent agar. The plates were incubated overnight at 36 C, and examined for plaques. Stool specimens producing plaques or poor growth of the *E. coli* after enrichment were serially diluted and were plated directly (without enrichment) to determine the number of plaque-forming units of phage present. The strain of *E. coli* used as a detection system had been isolated from a healthy person at Clark and in preliminary experiments gave results approximately the same as several other strains of *E. coli* in detecting phage in stools from Clark.

One-tenth milliliter portions of nine of the stool suspensions used for viral studies were cultured for mycoplasmas by Dr. Robert Purcell (Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland) on PPLO (pleuropneumonia-like organisms) agar, aerobically and anerobically (in 95 per cent N_2 and 5 per cent CO_2), holding the cultures for 90 days. The cultures were examined at intervals of one to two weeks and before discarding were stained with Dienes stain and examined for minute or unusual colonies. Two pools containing aliquots of throat washings from nine patients were cultured similarly and also in thallium-free PPLO broth with 1 per cent urea, incubating for one week at 37 C.

Safety testing of inocula for volunteer studies

For volunteer inoculation, 20 per cent stool suspensions were prepared in phosphate buffered saline with 0.5 per cent gelatin using a Waring Blendor. These were centrifuged for 10 minutes at 3000 RPM and the supernates respun at 3500 RPM (2200 × gravity) for 90 minutes at 4 C. The supernate was removed, and stored in small aliquots at −70 C until used for safety testing and volunteer experiments.

The presence of known viruses and mycoplasmas was excluded by the methods outlined above. Dr. Robert Purcell performed CF tests for Australia (hepatitis-associated) antigen with the stool suspensions and acute and (three-week) convalescent sera from the donor patients, using antibody-containing human serum. Since the stool suspensions were somewhat anticomplementary they were also tested against hyperimmune guinea pig serum by agar gel diffusion.

After safety testing, suspensions from six patients passed all criteria of safety used, and came from patients with SGOT-SGPT levels known to be normal for 43 or more days after onset of diarrhea. One patient had had Ascaris and Trichuris eggs in the original whole stool, and another unidentified amebae, but these were not seen in the

supernates, and even if present would have been rendered non-infectious by freezing or, in the case of the helminths, by the lack of opportunity for extrinsic incubation.

Volunteer studies

Volunteers were healthy adult inmates of the Texas Department of Corrections. General procedures for acquiring volunteers and performing studies were similar to those previously described (7), and written informed consent was obtained prior to inoculations. Following inoculation, volunteers were examined at least once and sometimes twice daily for signs and symptoms of illness by physicians who were aware that gastrointestinal disease was possible.

Four different groups of volunteers were inoculated with stool supernates. Groups I and II were hospitalized for study and groups III and IV were housed in an isolation unit in the correctional institution. Each volunteer in group I drank 2 ml of supernate mixed in 6 oz of cold skim milk. Two weeks later, group II was given 4 ml of supernate mixed in 6 oz of cold skim milk and 6 ml of supernate via duodenal tube (Miller-Abbott). X-ray and aspiration of intestinal contents for pH and bile stain indicated in each case that tube inoculation was into the duodenum. An additional 50 ml of normal saline was used to flush the tube after inoculation.

For groups I and II, consisting of four volunteers each, all stools were collected and weighed and peripheral blood counts, liver function tests, serum amylase, and stool examination for ova, parasites, and pathogenic bacteria were performed serially. In addition, serum carotene and d-xylose absorption tests were done prior to and again 10 to 14 days after inoculation as screening tests for intestinal malabsorption.

Groups III and IV consisted of 20 volunteers each. Each volunteer was given 2 grams of $NaHCO_3$ by mouth five minutes prior to drinking a variable quantity of stool supernate or placebo mixed in 6 oz of cold skim milk. The number of stools passed daily was recorded. Complete blood counts, urinalysis and liver function tests were performed before inoculation and again four weeks later.

RESULTS

The results of stool cultures and parasitologic examinations are shown in table 1. Only in the three patients with *Shigella sonnei*, Salmonella or Arizona isolates was a probable cause of the acute illness found.

The patient with *Giardia lamblia* was the same one excreting *Shigella sonnei*. Giardia was not found in any of the other acute stools or in eight frozen specimens of jejunal fluid. The 11 acute and 14 convalescent small intestinal biopsies examined by Dr. Welsh and Dr. Brandborg did not contain Giardia or coccidia (8). None of the acute jejunal fluids contained more than a few colonies of bacteria per 0.02 ml, and these were all coagulase negative staphylococci. One convalescent specimen contained several hundred colonies of a Bacillus species.

The 11 stools from which the *E. coli* were studied serologically appeared to contain a diversity of types, although a number of patients had strains reacting with several antisera, a phenomenon occurring less frequently in specimens from the healthy control population. There appeared to be, however, no conclusive evidence for the predominance of a single serotype. None of the strains of *E. coli* from 11 patients caused dilatation of rabbit intestinal loops or of the small intestine of infant mice.

Three viruses were isolated from the 48 stools studied. One produced adenovirus-like cytopathogenic effects in human kidney cells and was identified as adenovirus type 4 by neutralization tests in KB cell culture. One stool produced illness in infant mice, and histological examination revealed generalized skeletal myositis consistent with a coxsackie A virus infection although attempts at typing by CF were unsuccessful. Unfortunately convalescent serum was not available from these two patients. The third

isolate was a strain of echo 13, isolated from a stool specimen in several cell culture systems. Hemagglutination-inhibition tests with this virus showed some inhibition by the patient's convalescent serum and not by the acute serum at dilutions of 1:10, but no inhibition at 1:20. Since the three viruses were different and 45 of 48 patients yielded no isolates, further attempts to identify and to reisolate these strains were not made. In addition to the three viruses isolated from stools, echo 13 appeared in cultures of three other stools passed for over 40 days in human kidney cell culture. These apparent isolations were not associated with antibody rises in the patients' paired sera and two separate attempts at reisolation from the original stools failed. It is thought that these three isolations must have resulted from laboratory contamination with the first isolate.

Bacteriophages affecting the strain of *E. coli* used were found in 80 per cent of the stools examined, whether from diarrheal or control patients. The average titer in positive specimens was 9×10^3 plaque forming units per 0.2 ml of stool for diarrhea cases and 4×10^3 for control specimens. The size and appearance of the plaques were similar for both groups.

No mycoplasmas were cultured from nine stools and two pools of throat washings from nine patients.

The results of the volunteer experiments are shown in table 3. In the final series of experiments, a single man given the stool supernate from patient "R" developed symptoms, beginning on the sixth day after inoculation. Although the illness was rather mild, the five soft or liquid stools on day 8 can be considered definitely abnormal. The same man however, also developed diarrhea

TABLE 3

Administration of 20 per cent stool suspension supernates to volunteers. Each dose represents one volunteer and his dose in milliliters

Volunteer group and method of inoculation	Diarrhea patient at Clark from whom inoculum derived						
	P	M	R	S	C	W	None (placebo)
I. Orally in 6 oz skim milk	2 ml*	2 ml	2 ml	2 ml			
II. Orally in 6 oz skim milk after part of inoculum given by duodenal tube	4 ml orally + 6 ml by tube (one man)	4 + 6 ml	4 + 6 ml	4 + 6 ml			
III. Orally in 6 oz skim milk five minutes after 2 gms $NaHCO_3$ in 4 oz water	7 ml†	10 ml	10 ml	10 ml	5 ml		0 ml
	7 ml	10 ml	10 ml	10 ml	7 ml		0 ml
	14 ml	20 ml	22 ml	16 ml			10 ml‡
							10 ml
							20 ml§
IV. Same as III	3 ml	10 ml	8 ml	5 ml		6 ml	10 ml, 10 ml
						8 ml	10 ml, 10 ml
	5 ml	10 ml	10 ml¶	6 ml		10 ml	10 ml, 10 ml
						10 ml	10 ml, 10 ml

Results: 31 of 34 stool-inoculated and 11 of 13 placebo-inoculated volunteers remained asymptomatic. Five others had symptoms as follows:

* Mild bloating and cramps on days 4 to 6.

† Four soft stools on day 3 without other symptoms.

‡ Four soft stools per day on days 3 and 4 without other symptoms.

§ Afebrile upper respiratory infection on days 3 to 6 without other symptoms.

¶ Four soft stools on day 6 and three on day 7 without other symptoms; five stools (three liquid) on day 8 with mild cramps; two normal stools on day 9; no fever at any time

while participating in a subsequent and unrelated experiment on influenza virus, suggesting that he may be an individual prone to attacks of idiopathic or psychogenic diarrhea. Six other volunteers ingested the same specimen without symptoms. Attempts to "pass" the illness by feeding the ill volunteer's stool to others have not been made.

Two volunteers who ingested specimen "P" in the first and third experiments had mild gastrointestinal symptoms but definite diarrhea did not occur and uninoculated or placebo-inoculated men in the same setting had a similar incidence of symptoms. No changes in clinical laboratory tests or d-xylose absorption were noted in the eight patients for whom these were performed.

Discussion

Routine culture and microscopy identified only four organisms in more than a single stool: Providence and Proteus species, *Dientameba fragilis*, and *Trichuris trichiura*. In one reported epidemic of food poisoning (9) Providencia were isolated from 13 of 16 patients, and *Proteus rettgeri* or *mirabilis* from 12 of 16, but most authors seem to feel that these organisms have not been definitely shown to cause diarrhea (10, 11). In any case the epidemiologic pattern at Clark does not suggest food poisoning of the type described for Proteus and Providence species (9).

D. fragilis has been found in up to 42 per cent of asymptomatic people in some studies (12) and it is therefore not surprising that 7 per cent of the patients at Clark harbored this organism. Trichuris is commonly found in the tropics and is not likely to have been responsible for the patients' diarrheal illness.

A number of infectious causes of diarrhea are described in the literature which are not detected by careful routine bacteriologic and parasitologic examination of stools from cases. Recently an epidemic of diarrhea in British Army troops newly arrived in Aden was attributed to a new type of enteropathogenic *E. coli* (13), and Gorbach et al. (14) and Sack et al. (17) found enterotoxin-producing *E. coli* in a high percentage of idiopathic adult diarrheas in India, using the rabbit ileal loop as a detection system.

The recent volunteer experiments of DuPont et al. (15) with enteropathogenic *E. coli* in adults demonstrated a pattern of clinical illness similar to the syndrome seen at Clark in volunteers given large inocula of an enterotoxin-producing strain. In contrast to the "penetrating" strains which produced a febrile, shigella-like disease with bloody diarrhea, the enterotoxic strains in their experiments caused watery, nonbloody diarrhea without fever, lasting in some cases for several weeks. We were unable however to demonstrate exterotoxin production by strains of *E. coli* from the Clark material. This, the lack of antigenic homogeneity among strains, and the failure of the CDC laboratory to find the classical pathogenic serotypes in samples from 10 patients suggest that *E. coli* are not responsible for the Clark epidemics.

The epidemiologic and clinical pattern of the Clark epidemic does not resemble that of staphylococcal food poisoning. *Vibrio parahemolyticus*, *Vibrio cholerae*, and *Clostridium welchii* are essentially ruled out by the negative findings on culture.

The studies of Cohen et al. (16) and Gorbach et al. (14) have disclosed abnormal numbers of bacteria, principally coliforms, in the upper small intestine of acute diarrhea patients. Whether their presence there is causative or merely secondary to an abnormal intestinal motility pattern has not yet been determined. The latter is at least possible, since experimental induction of diarrhea by infusion of fluid into the small intestine of volunteers (18) causes coliforms to appear in the upper small bowel within a few hours.

In duodeno-jejunal fluids from eight acute patients at Clark, significant numbers of bacteria were not found by aerobic culture on blood agar. Although the specimens

had been frozen, they were placed in 10 per cent glycerol-5 per cent dextrose prior to freezing, and it has been reported (19) that 10 per cent glycerol will allow essentially quantitative recovery of the intestinal flora after freezing at −70 C. Our results therefore probably represent the actual state of the intestinal contents when collected.

Cultures for fungi and for anaerobic bacteria (other than Clostridia) were not done on the whole stools. Fungi, if present in large numbers, probably would have been seen in the fecal smears or in the biopsy material. Anaerobic bacteria should be studied in cases at Clark in the future, although their usual absence from the small bowel and tendency to decline in number in the stools during experimentally induced diarrhea (18), make the anaerobes seem less likely to cause diarrhea than the aerobes.

Epidemics of diarrhea in children have been associated with a reasonable degree of certainty with infection by echo 1, 11, 14, and 18, coxsackie B2 and B3, adenovirus 3, and perhaps adenoviruses 6 and 31 (20–28). Numerous studies comparing viral isolations from children with diarrhea to those in a control group without gastrointestinal disease have been done (29–37). Taking the studies as a whole, the control children have yielded virus isolates with the same frequency as the diarrhea cases.

In adults the situation with regard to viruses is somewhat simpler, since virus isolations from stools are relatively infrequent (16). An epidemic of diarrhea in adults due to a known virus has apparently not been reported, with the exception of three, possibly laboratory acquired, infections with echo virus type 11 (38) and cases in two families with adenovirus type 3 infection and gastrointestinal symptoms but also respiratory and conjunctival manifestations (39).

Our negative results in 45 of 48 stools studied for known viruses and the miscellaneous nature of the three virus isolations made suggest that viruses at Clark were merely coincidental with the presence of diarrhea and are most unlikely to have caused the epidemic.

A great deal of attention has been given to bacteriophages as a possible defense against disease (40, 41), but it is also conceivable that by lysing toxin-containing bacteria in the intestine they might cause illness. For this reason cultures for phage were made. Because of the high incidence of phages in both diarrhea and control populations, and their similar titers and plaque morphology this part of the study was not carried further, although much more could be done to characterize the phage(s) present if desired.

Although mycoplasmas have not been reported to cause diarrhea in humans, one author (42) reported finding cold and streptococcus MG agglutinins in the sera of most of the patients in an epidemic of infantile diarrhea. However, no mycoplasmas were found in the nine patients in whom they were sought in the Clark epidemic.

Transmission of diarrheal disease to volunteers by inhalation of filtered stool suspensions and throat washings was first reported by Reimann in 1945 (43). Gordon and his associates thoroughly established the existence of filtrable agents of gastroenteritis infectious by the oral route, using material from an epidemic in a New York state mental institution in 1946–1947 (44, 45) and from the Cleveland family study in 1951 (46). In 1947–1948 an epidemic of nonbacterial gastroenteritis swept over Japan and a number of groups were able to establish the serial transmissibility of this and later epidemics to volunteers by feeding stool filtrates (47–49).

Attempts were made to grow the causative agents in embryonated chicken eggs (44), in liver, intestine, lung, kidney, brain, heart, muscle, and cartilage cultures from human embryos (50) and to produce disease in rabbits, mice, muskrats, cats, chickens (49), one monkey, guinea pigs, and dogs (47). No consistent success was attained, and further volunteer experiments were not

reported until 1971 when Dolin et al. (51) described gastrointestinal symptoms in volunteers fed stool suspensions from one of six cases of "winter vomiting disease." The minimum infectious dose for volunteers varied from 0.005 ml (48) to several milliliters (44) of undiluted stool.

The agents in the earlier experiments provoked immunity in the volunteers, and Gordon's "Marcy" strain and the strain from Japan were shown to protect against one another and were therefore presumed to be immunologically related or identical (52). In general the infectious material was preserved by freezing at −70 C, and destroyed by heat (70 C for 30 minutes) (44, 48). The agents passed through various types of bacteria-tight filters, although "sizing" experiments were not done. At least seven serial passages in volunteers were carried out with the Marcy agent (45).

Except for the prolonged course of some of the cases at Clark, their clinical symptoms are quite similar to those encountered in the experiments of Gordon and of the Japanese workers. This and the lack of other identifiable causes for the Clark epidemic led us to believe that it might be another example of transmissible nonbacterial diarrhea, and that by volunteer experiments material might be obtained for renewed attempts at isolation of an agent in the laboratory. The single illness which occurred among 34 volunteers in our experiments was probably psychogenic, since the same man developed diarrhea in a subsequent and unrelated experiment. Attempts at further passage in volunteers might resolve this question. Even if illness appeared in further passages however, it would be extremely difficult to work with a transmissible agent of this nature because of the large number of volunteers required. If 34 volunteers were necessary to obtain a single isolate from an epidemic, better methods would clearly have to be devised before epidemiologic work could proceed.

The epidemiologic and clinical evidence in the present study indicated that at Clark acute gastroenteritis and the tropical sprue-like syndrome were manifestations of the same illness and probably share the same etiology (2). If this is true, the rather thorough virologic and volunteer experiments done may have some bearing on tropical sprue. It has long been suggested from epidemiologic evidence that tropical sprue may be a viral illness (53), but apparently only one virologic study has been reported. Bayliss et al. (54) did viral cultures of the stools of 50 sprue patients in Puerto Rico, with rectal swab specimens taken three weeks or more after onset. Only one virus —a strain of coxsackie B-3—was isolated, and the control group of 48 persons also yielded a single virus isolate. At Clark, one can say with assurance that in the patients studied, viruses easily detectable by present-day methods were absent from the stools in 45 of 48 patients during the first few days of illness. This and the negative results of the volunteer experiments give no support to the hypothesis that viruses cause tropical sprue.

Essentially all the known infectious causes of diarrhea epidemics have been looked for in this study, and none found to be responsible for the epidemic. There is no epidemiologic evidence for staphylococcal or clostridial food poisoning or for organic or inorganic chemical poisoning (1). The possibility remains that a causative microorganism is either producing a toxin or multiplying in the host intestine or both. Apparently however if an infectious form is excreted in the stools, it is removed in large part by freezing and/or centrifugation, or not readily detected by our methods. Since the volunteer experiments were designed primarily to detect viral rather than bacterial agents, it is possible that a pathogenic strain of bacteria not yet described could be responsible. In any case, it appears that the assignment of a given case or epidemic of diarrhea to the "viral", or even "*E. coli*" category merely by ruling out other known causes is too facile an assumption. Much remains to be done before it can be said

that careful search will reveal the cause of every case or epidemic of diarrheal disease.

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