BACTERIAL ENTEROTOXINS

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INTRODUCTION

Acute diarrheal diseases are an important public health problem. They cause morbidity and significant economic losses in all populations and are associated with high mortality rates in underdeveloped countries (1). Acute diarrhea is a symptom with many different causes including acute bacterial, viral, and parasitic infections, adverse reactions to antibiotics and other drugs, and diseases of non-infectious etiologies. At present, our ability to provide an exact etiological diagnosis for acute diarrheal illnesses is poor. The routine cultures and serologic tests that are available in most diagnostic laboratories fail to identify an infectious agent in the majority of cases of acute diarrhea.

Table 1 summarizes current concepts concerning the pathogenesis of bacterial diarrheas.

TABLE 1

Pathogenesis of Bacterial Diarrheas

Pathogen	Bacteria-intestinal mucosal interaction	Pathogenic mechanism	Intestinal localization
Vibrio cholerae	No invasion	Enterotoxin production	Small bowel
Escherichia coli	Penetration, intraepithelial multiplication	Mucosal inflammation with destruction	Early small bowel Later colon
Shigella *Escherichia coli* Salmonella	Complete penetration epithelial cell	Inflammation of the lamina propria	Small bowel and colon

(Ref. 1)

The initial interactions of specific enteropathogenic bacteria with the mucosa of the gastrointestinal tract vary from surface colonization without epithelial penetration to complete penetration of the epithelium with multiplication and spread of the bacteria within the intestinal wall. Each species of enteropathogenic bacteria has a predilection to localize at one or more specific sites within the gastrointestinal tract. The pathogenic mechanisms that are involved in the production of diarrhea and other signs of illness are characteristic for each type of enteropathogenic bacterium, and diarrhea may occur with or without histological evidence of inflammation in the gastrointestinal tract.

The differential diagnosis of infectious diarrheas is traditionally based on clinical, epidemiologic, and laboratory features, as outlined in Table 2, and specific etiologic diagnoses are confirmed by isolation and identification of the etiologic agent or by demonstration of significant rises in titer of specific serological tests.

TABLE 2

Clinical, Epidemiologic and Laboratory Findings in Infectious Diarrheas

	Clinical features	Epidemiology	Fecal leukocytes	Treatment
Toxigenic *E. coli* diarrhea	± Fever, watery diarrhea	Source: food or water	Absent	± Antibiotics
*Shigellosis**	Fever, colitis (urgency, tenesmus, bloody, mucoid stools)	Person-to-person	++++ Polys	Ampicillin, Tetracycline
Salmonellosis				
Typhoid	Fever, abdominal pain, constipation (occasionally diarrhea)	Source: food, water, from carrier	++ Monos	Chloramphenicol Ampicillin
Non-Typhoid	Fever, vomiting, watery diarrhea (occasionally mucoid stools)	Source: food, often associated cases	++ Polys	None
Viral Diarrhea	± Fever, vomiting, watery diarrhea of brief duration	Person-to-person with intra-family or institution spread	Absent	Symptomatic

* Some *E. coli* strains behave like virulent Shigellae.

(Ref. 1)

The presence of fecal leukocytes and their prevalence and morphology are helpful in differentiating inflammatory from non-inflammatory types of diarrhea, and direct examination of the colon by sigmoidoscopy provides additional helpful information plus an opportunity to obtain excellent specimens for cultures and for microscopic studies. Care must be taken to differentiate fecal polymorphonuclear leukocytes from amoebae.

During the past decade, several bacterial enterotoxins have been implicated in the pathogenesis of acute diarrheal syndromes, and data concerning the biology of bacterial enterotoxins has accumulated rapidly. The purposes of this review are to summarize the clinical features and the pathophysiologic mechanisms of several well studied enterotoxic enteropathies. We shall also review the current status of laboratory techniques for the diagnosis of the enterotoxic enteropathies and will consider the prospects for prevention of specific diarrheal syndromes by immunization.

CASE HISTORIES

Case 1:

The following case history is abstracted from the recent report by Weissman et al. (2).

"On August 25, 1973, a 51-year-old man from the Gulf Coast town of Port Lavaca, Texas, had precipitous onset of profuse watery diarrhea which gradually changed in color over several hours from brown to clear. The stool was neither bloody nor malodorous. Diarrhea was accompanied by nausea, a single episode of vomiting, mild abdominal pain, and incapacitating leg

cramps that began in the calves and progressed to both thighs. Four hours after the onset of symptoms the man was admitted to a local hospital in shock. His past medical history included a subtotal gastrectomy in 1972 for long-standing peptic ulcer disease, chronic low back pain, and infrequent angina pectoris.

"The admission physical examination revealed an apical pulse of 150, respiration 30 and Kussmaul in type, and no obtainable blood pressure. He was afebrile, conscious, and oriented. There was 'splotchy' cyanosis of all extremities with absent peripheral pulses. His abdomen was soft and not tender; bowel sounds were present and stools were described as being 'liquid with milk curds.' The remainder of the physical examination was unremarkable. Admission laboratory values included hemoglobin, 18.1 gm per 100 ml; white blood cell count, 13,400 per mm; sodium, 136 mEq per l; potassium, 3.1 mEq per l; carbon dioxide, 18 mEq per l; and blood urea nitrogen, 40 mg per 100 ml. A second carbon dioxide, obtained several hours after admission was 5 mEq per l.

"Initial treatment included intravenous lactated Ringer's solution and intravenous gentamicin. On the afternoon of admission the patient suffered a transient respiratory arrest while sitting in bed for a portable chest x-ray. After being resuscitated, he was examined by a consulting physician who recognized the gravity of the patient's illness and immediately gave additional intravenous fluids with supplemental potassium and bicarbonate. After a brief library review of the differential diagnosis of severe dehydrating diarrhea, the physician concluded that the patient's illness was compatible with cholera; he prescribed tetracycline and alerted the hospital bacteriology laboratory. The patient's condition improved dramatically within hours, and he was discharged in good health one week later.

"Vibrio cholerae was isolated at the Champ Traylor Memorial Hospital, Port Lavaca, and verified by the Texas State Health Laboratory from a stool culture obtained on admission. The isolate was subsequently identified as V. cholerae, biotype El Tor, serotype Inaba, at the University of Texas and the Center for Disease Control (CDC). The case was reported to the World Health Organization."

An extensive epidemiological study of this case of cholera in Texas failed to provide definitive proof of the source of infection, and no secondary transmission of V. cholerae to persons in contact with the patient was observed (2).

Case 2.

The following case history is summarized from a recent report by R. A. Finkelstein et al. (3).

"On Sunday, 13 April 1975, a 36-year-old, 52.6-kg female (P.T.) who had just returned from a cruise to Mexico, was taken from Dallas/Ft. Worth Regional Airport to Parkland Memorial Hospital, Dallas, Tex., as an emergency patient in hypovolemic shock due to diarrheal disease of less than 1-day duration. With adequate intravenous and oral fluid replacement therapy

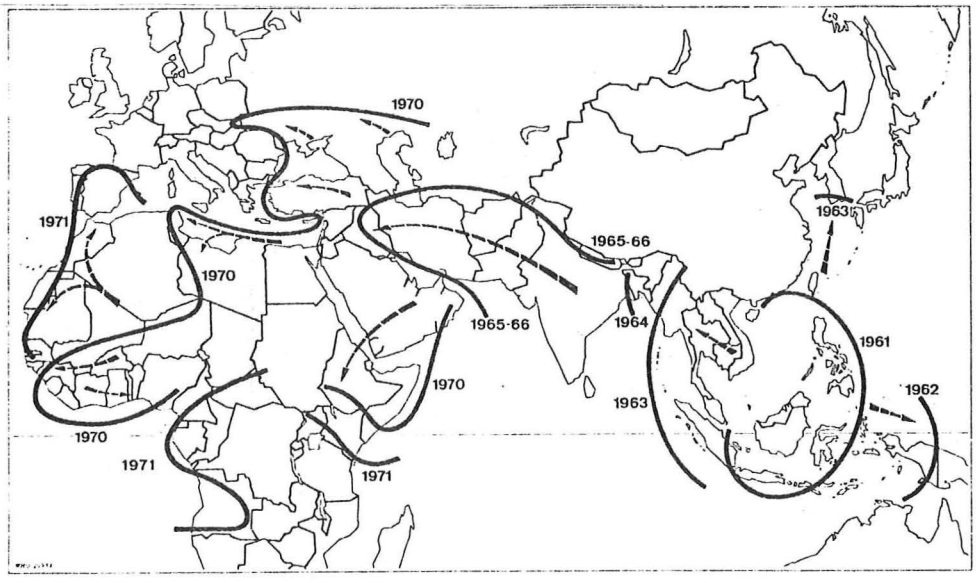
her subsequent clinical course was uneventful, although from the period between 14 April and 17 April she had discharged a total of 60.7 liters of stool (51.2) liters and urine (9.5 liters)..."

"A sample of typical 'rice-water' stool was submitted to the Department of Microbiology for examination and culture. Immediate microscopic examination of a hanging-drop preparation revealed many rod-shaped bacteria but no motile organisms, thus reducing the likelihood of Vibrio cholerae. There was no evidence of pus cells, ova, or parasites..."

Routine cultures of this patient's stool failed to reveal Salmonella, or Shigella. Ten colonies of E. coli recovered from the patient's stool were tested for serotype and for their ability to produce enterotoxins. Four isolates that were nontypable with routine typing sera for O antigens of E. coli were shown to produce an enterotoxin, and examination of acute and convalescent sera from the patient demonstrated a significant rise in the titer of agglutinating antibodies against these four isolates of E. coli. This case illustrates that some strains of Escherichia coli have the potential to produce severe dehydrating diarrhea that is clinically indistinguishable from cholera, that such bacteria may not be recognized as enteropathogenic by serotyping, that specialized techniques that are not routine for diagnistic laboratories are required to demonstrate enterotoxin production by intestinal bacteria, and that classical serologic tests for rising titers of agglutinating antibodies can help to implicate specific isolates of fecal bacteria as the etiologic agents of diarrheal illnesses in individual patients.

CHOLERA

Cholera is an acute diarrheal disease with the potential for epidemic and for pandemic spread. The seventh great pandemic of cholera began in the Celebese in Indonesia in 1961 and has persisted to the present time (4). The global spread of cholera from 1961 through 1971 is shown in Figure 1. The recent epidemics of cholera in Italy in 1973 (5) and in



Global spread of cholera, 1961–1971. (From WHO, unpublished document.)

FIGURE 1 (Ref. 4)

Portugal in 1974 (6) represent specific outbreaks in western Europe that are part of the continuation of the seventh pandemic. Although these outbreaks received broad news coverage in the United States, they represent a very small part of the world health problem of cholera (4). Since the beginning of the present pandemic, research on cholera has proceeded at an intensive rate. Recent reviews cover almost all aspects of the biology of cholera, including bacteriology, pathogenesis, pathophysiology, epidemiology, and immunology (4, 7-11). The following paragraphs will review some of the highlights of these studies.

Vibrio cholerae is a gram negative bacterium and is the etiologic agent of cholera. Cholera vibrios are divided into two main groups, or biotypes, designated classical and El Tor. Cholera vibrios of the classical and El Tor biotypes are divided into three serotypes designated Inaba, Ogawa, and Hikojima, based on somatic antigens present in their cell envelope. Fresh isolates of Vibrio cholerae from clinical specimens have characteristic motility. A presumptive diagnosis of cholera can be based on the demonstration of characteristically motile bacteria by microscopic examination of hanging drop preparations of cholera stool, and the motility of V. cholerae in fresh preparations can be inhibited by appropriate specific antisera. Confirmation of the diagnosis of cholera is based on isolation and characterization of Vibrio cholerae from appropriate clinical specimens. Until the present pandemic was shown to be caused by El Tor strains of Vibrio cholerae, the capacity of El Tor vibrios to produce epidemic or pandemic disease was questioned. It is now generally accepted that pandemic disease can be caused either by classical or by El Tor strains of Vibrio cholerae (4, 7).

The disease cholera is the result of a series of interactions between Vibrio cholerae and its human host (7). To produce cholera after ingestion of infected materials, cholera vibrios must survive the bacteriocidal effects of gastric acidity, gain access to the small bowel, resist the clearing effects of intestinal peristalsis, and colonize the mucosal surface of the small bowel. There they multiply and secrete a potent enterotoxin that intoxicates the mucosal cells and produces a secretory diarrhea. Thus, although production of enterotoxin is essential for pathogenicity, other bacterial properties including motility and the ability to produce extracellular enzymes such as mucinase, proteases, and neuraminidase, are believed to contribute to the virulence of Vibrio cholerae (7). For this reason, both antitoxic and antibacterial host-defense mechanisms appear to be involved in immunity to cholera (8).

The cardinal clinical feature of cholera is a secretory diarrhea of large volume that is associated with rapid development of dehydration, hypovolemia, severe metabolic acidosis, and potassium depletion (4). Typical electrolyte patterns in cholera stool are shown in Table 3 and typical blood chemical findings in acute cholera are shown in Table 4. The stool in cholera

TABLE 3

Electrolyte Patterns in Adult Cholera Stool (mEq/L)

	MEAN VALUES, 38 PATIENTS	
	On Admission	*48 Hours after Admission*
Sodium	116	126
Chloride	100	90
Potassium	30	16
Bicarbonate	40	50

(Ref. 4)

TABLE 4

Blood Chemical Findings in 38 Consecutive Cholera Patients Treated by the 2:1 Saline:Lactate Regimen

	MEAN VALUES ± S.D.	
	On Admission	*Four Hours after Admission*
Arterial blood pH	7.17 ± 0.06	7.40 ± 0.05
Plasma bicarbonate (mEq/L)	7 ± 4	20 ± 3
Plasma potassium (mEq/L)	5.6 ± 0.4	3.2 ± 0.3
Total plasma protein (gm/100 ml)	14.2 ± 0.8	7.5 ± 0.6

(Ref. 4)

reflects massive secretion by the small bowel of an isotonic fluid that is rich in potassium and bicarbonate. There is no disruption of the histological integrity of the gastrointestinal tract in acute cholera, and there is no loss of protein or other macromolecular components of serum in cholera stool. A more detailed presentation of the clinical features of severe cholera is presented in Table 5. The serious complications of cholera include acute

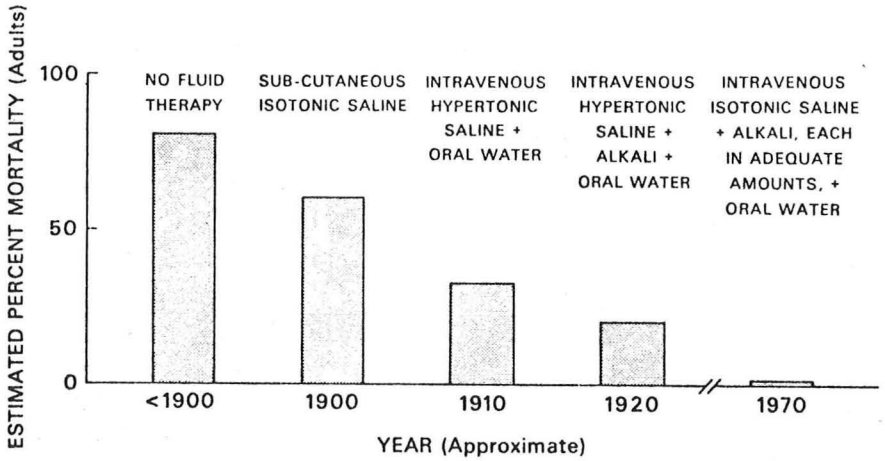
TABLE 5

TABLE 10–1. Clinical Features of Severe Cholera

SYSTEM INVOLVED	CLINICAL MANIFESTATION	PROBABLE CAUSE
Central nervous system	Stupor; coma in children	Severe hypovolemia, sometimes hypoglycemia in children, uncertain in some children.
	Grand mal seizures in children	Sometimes hypoglycemia, otherwise unknown.
Cardiovascular	Hypotension	Isotonic dehydration with hypovolemia and diminished extracellular fluid volume.
	Tachycardia	
	Diminished pulse volume or absent radial pulse	Same as above.
	Diminished skin turgor	Same as above.
	"Washer woman's hands"	Same as above.
	Peripheral cyanosis	Same as above.
	Cool, moist extremities	Same as above.
	Aphonia	Same as above.
	Sunken eyes	Same as above.
	Electrocardiographic abnormalities:	
	1. Peaked T waves	1. Hyperkalemia present before correction of acidosis.
	2. Depressed T waves, prominent U waves	2. Hypokalemia, if acidosis corrected without potassium replacement.
	3. Peaked P waves	3. Uncorrected hypovolemia.
	Cardiac arrhythmia	Hypokalemia.
Pulmonary	Hyperventilation	Compensatory response to base-deficit acidosis.
	Acute pulmonary edema during rehydration	Central redistribution of blood volume and pulmonary hypertension aasociated with uncorrected base-deficit acidosis.
Gastrointestinal	Massive "rice-water" diarrhea	Small bowel hypersecretion caused by cholera enterotoxin.
	Vomiting of fluid with neutral pH	Small bowel hypersecretion with reflux into stomach, in association with hypovolemia.
	Achlorhydria	Uncertain.
	Diminished intestinal motility	Uncertain.
	Paralytic ileus in children	Hypokalemia.
Urinary	Oliguria, anuria:	
	1. Transient (less than 24 hours)	1. Hypovolemia without tubular necrosis.
	2. Prolonged with evidence of uremia	2. Inadequate correction of hypovolemia and/or recurrence of hypovolemic shock.
Reproductive	Abortion during third trimester of pregnancy	Uncertain; probably hypovolemic shock, fetal anoxia.
Musculoskeletal	Painful muscular cramps	anoxia.
	Carpopedal spasm	Uncertain.
	Myotonia	Uncertain.
Endocrine-metabolic	Hypoglycemia in children	Uncertain.
	Interruption of growth in children	Uncertain; possibly prior undernutrition plus acute illness and withdrawal of food.
	Fever in children	Negative nitrogen balance due to diarrheal disease and withdrawal of feeding.
		Uncertain, probably related to hypovolemia.

(Ref. 4)

renal failure, acute pulmonary edema, cardiac arrhythmias, paralytic ileus, seizures, and abortion (4). Prompt replacement of fluid and electrolytes is the cornerstone of therapy for cholera. Figure 2 illustrates the improved effectiveness of fluid and electrolyte replacement in cholera during the past century as physiologically appropriate regimens for fluid and electrolyte replacement have been developed. The composition of some of the intravenous



Improvement in effectiveness of cholera therapy as fluid replacement more closely met actual needs with respect to volume, rate of delivery, electrolyte content, and alkali content. (From Pollitzer, 1959, and from Carpenter et al., 1966a.)

FIGURE 2

(Ref. 4)

solutions that have been used successfully to treat cholera is given in Table 6. The discovery that maintenance therapy following initial rehydration

TABLE 6

Composition of Intravenous Solutions Used Successfully to Treat Cholera

	Na (mEq/L)	K (mEq/L)	Cl (mEq/L)	ALKALI[a] (mEq/L)	OSMOLARITY (mOsm/L)	REFERENCE
Dacca 5/4/1[b]	133	14	99	48—Bicarbonate	294	Greenough et al., 1964.
Ringer's lactate[c]	130	4	109	28—Lactate	271	Mahalanabis et al., 1972; Gutman et al., 1969.
NAMRU-2, C.R.S.[d]	120	10	100	30—Acetate	260	Watten et al., 1969.
2:1 saline:alkali	156	0	104	52—Lactate or bicarbonate	312	Carpenter et al., 1966*a*.

[a]Acetate and lactate produce alkali in the body by metabolic conversion to bicarbonate.
[b]So named for the respective gram quantities of sodium chloride (5), sodium bicarbonate (4), and potassium chloride (1) per liter of solution.
[c]Also has Ca^{++} and Mg^{++}.
[d]Naval American Research Unit—2, cholera replacement solution.

(Ref. 4)

in cholera patients can be effectively accomplished by oral administration of electrolyte solutions containing high concentrations of glucose was a major advance in cholera therapy, particularly in underdeveloped countries where

supplies of sterile intravenous fluids are limited. The formula for an effective oral solution is given in Table 7. Administration of antibiotics

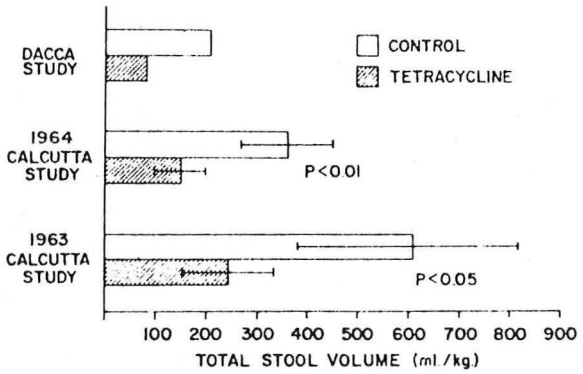
TABLE 7

Oral Solution Formula

	Drinking Water (gm/L)		Drinking Water (mEq/L or mmol/L)
Glucose	20.0	Glucose	110
Sodium chloride	4.2	Sodium	120
Sodium bicarbonate	4.0	Chloride	97
Potassium chloride	1.8	Potassium	25
		Bicarbonate	48

(Ref. 4)

to patients with cholera is important as an adjunct to adequate replacement of fluids and electrolytes. Antibiotic therapy decreases the total volume of stool, shortens the duration of illness, and reduces the shedding of cholera vibrios from patients into the environment, but antibiotics do not substiture for appropriate fluid and electrolyte therapy. (Figure 3).



Effects of tetracycline on total volume of stool in the 1963 Dacca experience, the 1963 Calcutta antibiotic trial, and the 1964 Calcutta study. Although the severity of illness, as judged by total stool volume in control patients, was different in each of these field trials, the percentage of reduction in stool volume caused by tetracycline was virtually identical in each of the studies. (Reprinted with permission of the authors from [70]).

FIGURE 3

(Ref. 4)

It is now well established that cholera enterotoxin produces the secretory diarrhea of cholera (7). Enterotoxin is an extracellular protein produced by Vibrio cholerae that has been purified to homogeneity (12, 13) and obtained in crystalline form (14). In appropriate experimental models for cholera such as the adult rabbit ligated ileal loop or the infant rabbit, intraintestinal administration of purified cholera enterotoxin produces a massive and potentially lethal secretory diarrhea that is indistinguishable from that of cholera in humans (15). In addition, nontoxinogenic (tox) mutants of V. cholerae are completely avirulent in experimental animals, although they multiply in vivo and colonize the gastrointestinal tract (16). Thus the ability to produce enterotoxin is essential for virulence of V. cholerae, and purified enterotoxin can produce the symptoms of cholera.

The structure of cholera enterotoxin has been studied in considerable detail. Enterotoxin is a protein with a molecular weight of approximately 84,000 that contains no carbohydrate, lipid, or prosthetic groups (13). The native enterotoxin, which has also been called choleragen, is an oligomeric assembly of three distinct polypeptides that will be designated A_1, A_2, and B (11, 17-22) (Figure 4). Polypeptide A_1 (MW approximately 23,000) is linked

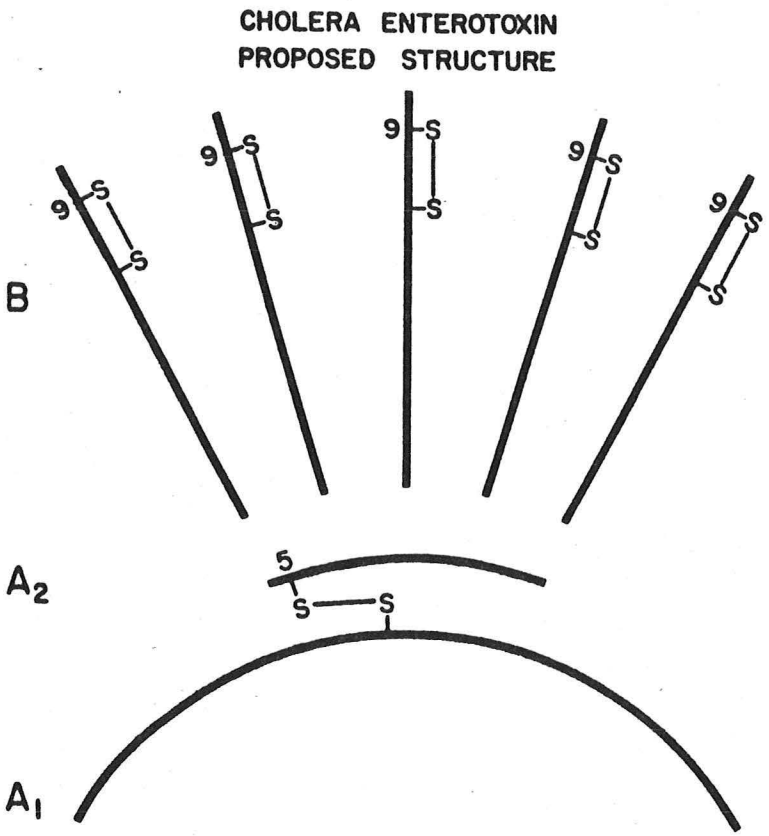


FIGURE 4

(Ref. 22)

to polypeptide A_2 (MW approximately 5,000) by a covalent disulfide bond. Enterotoxin contains several copies of polypeptide B (molecular weight about 10,000) that are not covalently linked either to each other or to polypeptides A_1 or A_2. The amino acid sequences for the amino terminal regions of polypeptides A_1, A_2, and B have been determined (21,22). At the present, the exact molecular weights of polypeptides A_1, A_2, and B are controversial, and estimates for the number of polypeptide B subunits per molecule of cholera enterotoxin vary from 4 to 7 (11, 17-22). Polypeptides A_1, A_2, and B are all essential for the full biological activity of cholera enterotoxin in intact cellular systems (23, 24). Under a variety of conditions in vitro, an oligomeric protein with molecular weight about 56,000 (choleragenoid) is formed from enterotoxin Choleragenoid contains only B polypeptides and lacks the biological activities associated with cholera enterotoxin (7, 13, 17).

Because cholera enterotoxin has been obtained in highly purified form and is antigenic, it has been possible to develop in vitro assays both for enterotoxin and for antienterotoxic antibodies. Useful immunological assays for enterotoxin have been based on precipitin reacitons (Ouchterlony (15), Oakley-Fulthorpe (25), Elek (26), and radial immunodiffusion (15) tests, etc.), passive hemagglutination inhibition tests (27), reversed passive hemagglutination tests (28), and radioimmunoassays (29). In addition, bioassays for cholera enterotoxin have been based on its many distinctive biological activities in gastrointestinal and extraintestinal tissues and in tissue cultures (7). Such assays have greatly facilitated studies of the relationships between the structure and the biological activity of cholera enterotoxin. Choleragen is now one of the best studied bacterial toxins, is a useful model for investigations on the mechanisms of intoxication of mammalian cells, and is an important biological reagent (30).

Tables 8 and 9 summarize some of the important biological activities of cholera enterotoxin. It is now well established that most of the biological effects of cholera enterotoxin are mediated by cyclic AMP (reviewed in 7). Following exposure of intestinal mucosa to enterotoxin in vivo, there is a lag period after which progressive activation of the enzyme adenylate cyclase and accumulation of intracellular cyclic AMP occurs. With rabbit ileal mucosa studied in Ussing Chambers in an in vitro preparation, the effects of cholera toxin on the short circuit current and on the unidirectional fluxes of sodium, chloride, and bicarbonate ions are similar to the effects of cyclic AMP (31). The secretory effect of cholera enterotoxin on small bowel mucosa is inhibited by cycloheximide, although the activation of adenylate cyclase and the accumulation of cyclic AMP is not blocked by cycloheximide (32). The mechanism by which cyclic AMP stimulates intestinal secretion in the small bowel has not been fully elucidated.

The mechanisms for activation of adenylate cyclase by cholera enterotoxin have been studied extensively. Enterotoxin binds with high affinity to the oligosaccharide moiety of ganglioside G_{M1} (galactosyl - N - acetylgalactosaminyl [sialosyl] lactosyl ceramide: or GGnSLC), a normal component of the plasma membranes of mammalian cells (33, 34), and incubation of mammalian cells with ganglioside G_{M1} can increase their susceptibility to enterotoxin (24, 34). There is no evidence for involvement of a protein or glycoprotein with receptor for cholera toxin (35). Thus, it is widely believed that ganglioside G_{M1} itself is the physiological receptor for cholera

TABLE 8

Biologic Activities of Choleragen and Choleragenoid

Assay	Choleragen	Choleragenoid
Choleragenesis, infant rabbit	0.25–0.5[a]	>100
Choleragenesis, adult mouse	4	>100
Lethality, I.V. adult mouse	5	>100
Rabbit ileal loops	0.2 0.03[b]	>100
Skin reaction, adult rabbit	0.00006–0.001	0.1–0.2
Lb test, Craig	0.038	not tested
Mouse (rat) foot edema	0.4	inactive
Immunizing dose vs. mouse foot edema	0.05–0.5	0.05–0.5
Fat cell lipase	0.014[a]	inactive
Lf dose, equine antitoxin	0.962	0.613

[a]Dose (μg) required, approximate, to produce readily recognizable response in indicated assay system.
[b]Data from Greenough et al.[278]

(Ref. 7)

TABLE 9

Activities of Cholera Enterotoxin in Extra-intestinal Tissues

Tissue	Response	Ref.
Skin	Permeability ↑ (erytherma, edema, induration)	201
Foot pad (mouse, rat)	Edema	177, 262
Mesenteric vessels	Permeability ↑	149
Epididymal fat cells	Lipase ↑	277, 278
Whole dog, I.V.	Hyperglycemia, hyponatremia; serum alkaline phosphatase, glutamic-oxalacetic transaminase	253
Liver	Adenyl cyclase ↑	334
Liver	Alkaline phosphatase ↑	253
Liver	Glycogenolysis ↑	335
Platelets	Glycogenolysis ↑	335
Lymphocytes	Cytotoxicity ↓	336
Leukocytes	cAMP ↑	337
Lymph node cells	Block conA, PHA; DNA synthesis ↓	D. Hart*
Immunocytes	Plaque formation ↑	340
Adrenal cells	Steroidogenesis ↑	341
Adrenal cells	Morphological changes	341
Pregnant mice	Abortion -- embryo resorption	342
Pre-pregnant	Fertility	342
Skin	Hair growth ↑	201
Turkey R.B.C.	cAMP ↑	M. Field*
Kidney	Hemodynamics and transport cAMP ↑	S. Massry*
Rat ovary	cAMP ↑	U. Zor et al.*
Spleen cells	RNA, protein synthesis ↓ DNA synthesis ↓	B. Sultzer*
Thyroid	cAMP ↑ Glucose oxidation ↑	J. Field*
? Transformed cells	→ Contact inhibition?	? (338, 339)

(Ref. 7)

enterotoxin. Since choleragenoid competes with choleragen for binding to intact cells, binding to cellular receptors is mediated by the B polypeptides of enterotoxin (36, 37). In recent studies, Gill has analyzed the activation of adenylate cyclase in intact pigeon erythrocytes and in lysed cell preparations derived from pigeon erythrocytes (24, 38). In intact erythrocytes, adenylate cyclase is activated only by native cholera enterotoxin containing the A_1, A_2, and B polypeptides. In contrast, polypeptide A_1 alone is capable of activating adenylate cyclase in the lysed cell preparations. In the lysed pigeon erythrocyte system, at least three cellular factors are required for the activation of adenylate cyclase by enterotoxin: adenosine triphosphate (ATP), nicotinamide adenine dinucleotide (NAD), and a non-dialyzable macromolecule derived from the supernatant faction. Gill has recently summarized his evidence that the A_1 subunit of cholera enterotoxin functions catalytically in the activation of adenylate cyclase, but the putative reaction catalyzed by enterotoxin has not yet been identified (38). Gill's hypothesis that enterotoxin functions catalytically in activating adenylate cyclase is not easily compatible with the models proposed by Cuatrecasas et al. that involve relatively stable direct interactions between enterotoxin and adenylate cyclase in the plasma membranes of mammalian cells (39). The details of this controversy are beyond the scope of the present review.

The literature concerning immunity to cholera has also been recently reviewed (4, 8). The classical cholera vaccines are monovalent or polyvalent suspensions of killed bacterial cells representing the different serotypes of V. cholerae. Administration of these vaccines to experimental animals or to humans elicits antibacterial antibodies but little or no antitoxic antibody. In contrast, when highly purified choleragen or choleragenoid is administered to experimental animals,or to humans (40), antitoxic antibodies can be produced in the absence of antibacterial antibodies. Challenge studies in experimental animals have established that protection against oral administration of live inocula of V. cholerae can be provided either by antibacterial immunity or by antitoxic immunity. Antibacterial immunity is most effective against V. cholerae of the same serotype as the vaccine injected. In contrast, since there is only one known antigenic type of cholera enterotoxin, antitoxic immunity appears to be effective against all strains of V. cholerae. Large scale cholera vaccine trials in humans have been carried out primarily in cholera endemic areas. Killed bacterial vaccines have been tested in East Pakistan (Bangladesh), Calcutta, and the Philippines, and a toxoid prepared by treatment of cholera enterotoxin with gluteraldehyde has recently been tested in Bangladesh. These vaccines provide only partial protection from cholera for a period of time that is limited to a few months. It is likely that their effectiveness in endemic areas is related in part to providing a booster response to individuals who have had previous clinical or subclinical infections with V. cholerae. There is a general consensus that an ideal vaccine against cholera has not yet been developed.

Several important theoretical correlations between the pathogenesis of cholera and immunity against cholera deserve consideration. Cholera is a noninvasive gastrointestinal infection, and V. cholerae colonizes the surface of the intestinal mucosa without penetrating the epithelium. Thus, immunity to cholera is probably dependent on local mechanisms that can act within the lumen of the gut. Specific immunoglobulins of the classes IgG, IgA, and IgM with activity against cholera vibrios have been demonstrated in intestinal contents, and current evidence suggests that such coproantibodies against V. cholerae may originate either as secretory IgA produced locally in the gut

or may represent serum antibodies that have gained access to the intestinal lumen (reviewed in 8). The mechanisms by which coproantibodies provide protection against cholera are currently under debate. It is unlikely that antibody and complement-dependent vibriocidal reactions or opsonization play a significant role, because A) the intestinal contents are highly anticomplementary, B) there is little evidence that Ig^A participates in complement-dependent bacteriocidal reactions, and C) phagocytic cells are not present in large numbers at the mucosal surface in the lumen of the gut. Current evidence suggests that antibacterial antibodies could interfere with colonization of the mucosal surface by V. cholerae either by reducing bacterial motility (41) or by mediating clumping and cross-linking of the bacteria within the lumen of the gut (42, 43). Freter has described an antibody dependent, complement-independent bacteriocidal activity against V. cholerae that is active at the mucosal surface of the gastrointestinal tract and appears to require viable mucosal cells (44). It seems reasonable, although it has not been directly demonstrated by experimental evidence, that the most effective local immunity in cholera would be provided by a combination of antibacterial and antitoxic mechanisms directed against the several distinct virulence factors that participate in the pathogenesis of cholera. Work in progress in several laboratories is attempting to define the optimal mixtures of components in cholera vaccines, the routes of administration (parenteral, oral, etc.), and the schedules of administration that will best accomplish these goals.

There has been considerable interest in the use of living attenuated strains of V. cholerae as vaccines (8). Candidate vaccine strains have included streptomycin-dependent derivatives of V. cholerae that fail to multiply in vivo in the absence of streptomycin (45), naturally occurring nontoxinogenic variants of V. cholerae (46), and nontoxinogenic mutants of V. cholerae isolated in the laboratory (16, 25). Studies in our laboratory have led to the isolation (25) and characterization (16, 47) of a nontoxinogenic, avirulent mutant of V. cholerae that produces only 0.1 per cent of the yield of toxin produced by by the parental strain (28) but is able to colonize the gastrointestinal tract and multiply in vivo (16). This living attenuated strain has been administered orally to volunteers by Woodward et al. (48) and was shown to be avirulent with inoculum sizes up to 10^{10} viable organisms. Because a toxinogenic isolate of V. cholerae was recovered from the stool of one asymptomatic patient after administration of the living attenuated vaccine strain, it is possible that this mutant may be genetically unstable and capable of regaining the capacity to produce enterotoxin. Thus it is not ideal as a candidate vaccine strain. Nevertheless, a significant level of complete protection (55%) against subsequent oral challenge with an homologous virulent strain of V. cholerae was demonstrated in the volunteers (48). Although an ideal vaccine candidate strain has not yet been reported, living attenuated oral bacterial vaccines have the theoretical advantages that they can colonize the gastrointestinal tract, multiply in vivo, and provide a prolonged local antigenic stimulus. From the theoretical viewpoint, the type of mutant of V. cholerae that might be optimal for use as a living attenuated vaccine should be genetically stable, should be incapable of reversion to virulence, and should be capable of producing a mutant enterotoxin that is antigenically normal but biologically nontoxic. Such a bacterium should be capable of eliciting both antibacterial and antitoxic immunity without producing disease itself. Several laboratories are currently attempting to isolate such mutants for use in future studies of virulence and immunity in cholera.

ENTEROPATHOGENIC AND ENTEROTOXIGENIC E. COLI

It has been recognized for decades that some strains of Escherichia coli are invasive enteropathogens that resemble Shigella in their pathogenic potential and produce a syndrome resembling bacillary dysentery. These invasive strains of E. coli have been termed enteropathogenic, and they belong to a somewhat limited number of serotypes, including 055, 0111, etc. Most diagnostic microbiology laboratories are equipped to detect the more commonly recognized enteropathogenic serotypes of E. coli. Although such tests are useful in certain clinical settings, such as the evaluation of hospital-associated outbreaks of diarrhea in pediatric populations, it is now clear that some strains of E. coli can produce diarrhea but do not belong to the classical enteropathogenic serotypes (49).

Following the discovery that the diarrhea of cholera is caused by an enterotoxin, isolates of E. coli from patients and from domestic animals with diarrhea were tested for enterotoxin production. Two distinct classes of enterotoxins have been associated with E. coli. These have been termed heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST) (51). The biological activity of LT is destroyed by heating at 60° C. LT resembles cholera enterotoxin in its mode of action (52). LT produces fluid accumulation in ligated ileal loops of adult rabbits (49, 50), induces changes in vascular permeability following intracutaneous injection in rabbits (53), causes morphological alterations in Chinese hamster ovary cells (54) or adrenal tumor cells (55) in tissue culture, and activates the enzyme adenylate cyclase both in intestinal mucosal cells (56) and in other susceptible cells. Ganglioside G_{M1} interacts with LT in a manner analogous to that described for cholera toxin, but there is controversy concerning the hypothesis that ganglioside G_{M1} is the physiological receptor for LT (57, 58). There is immunological cross-reactivity between cholera enterotoxin and E. coli LT that can be demonstrated by immunodiffusion tests (59) or by cross neutralization tests (60). In spite of these similarities to cholera enterotoxin, the molecular structure of E. coli LT is controversial. In various laboratories, LT activity has been associated either with large and chemically complex macromolecular aggregates or with apparently pure proteins with a wide range of molecular weights and with variable specific activities (11). The heat-stable enterotoxin (ST) of E. coli has distinctly different properties from LT (51, 61). ST retains its biological activity after heating to 100°, and its molecular structure is poorly characterized. ST appears to be a low molecular weight, non-antigenic factor that induces secretion of fluid into the lumen of the gastrointestinal tract in appropriate experimental systems (such as the infant mouse), but ST does not activate adenylate cyclase. The mode of action of ST has not been established.

The genetic control of enterotoxin production in E. coli has been carefully studied. The genetic determinants for enterotoxin production in E. coli can be located on extrachromosomal elements termed plasmids, consisting of double stranded DNA molecules that can be transferred from one bacterial cell to another (62). This phenomenon is analogous to the control of antibiotic resistance in bacteria by R factors that can also be transferred from cell to cell. Plasmids that control production of enterotoxin have been termed Ent plasmids. The Ent plasmids can be separated into two major groups. One group controls the production of both LT and ST, while the second group controls the production of ST only. Most enterotoxigenic strains of E. coli from human sources produce both LT and ST. The ST plus LT Ent plasmids are relatively

homogeneous in molecular size and have molecular weights approximately 60×10^6 (63). The ST plus LT plasmids share extensive polynucleotide sequence homologies and are related to the F1 group of plasmids in E. coli (64, 65). The molecular structure of plasmids that control production of ST only is heterogenous. They vary from 21×10^6 to 80×10^6 daltons and have no significant polynucleotide sequence homology with the ST plus LT Ent plasmids (64). Ent plasmids that code for ST only have been isolated primarily from strains of E. coli associated with diarrheal diseases in domestic animals, although recent evidence indicates that they may also be associated with diarrhea in humans (66).

Studies with enterotoxin producing strains of E. coli in domestic animals have demonstrated that production of enterotoxin is not the only bacterial property required for virulence. In addition, enterotoxigenic E. coli pathogenic for piglets produce a surface antigen called K88. Present evidence indicates that the K88 antigen is necessary for association of E. coli with the mucosal surface of the small bowel in piglets (67), and the biochemical basis for the interaction of K88 antigen with specific receptors on the mucosal surface is currently under investigation (68). The production of K88 surface antigen in porcine enteropathogenic E. coli is controlled by plasmids that are distinct from the Ent plasmids (67). Different surface antigens of E. coli appear to be essential for colonization of the small bowel in different mammals (69). Preliminary evidence indicates that an enterotoxigenic E. coli of human origin also has a plasmid determined surface antigen that is essential for colonization of the intestinal mucosa in an infant rabbit experimental model (70).

Studies of the epidemiology of enterotoxic E. coli diarrhea in humans have been limited by the expense and difficulty of the bioassays for enterotoxin and for antienterotoxic antibodies. Enterotoxin producing strains of E. coli do cause diarrhea in humans (49, 50). Rises in antibody to LT can sometimes be detected following diarrheal illnesses by LT-producing strains (71, 72). It is now clear that there is no necessary correlation between enterotoxin production and somatic antigen serotypes in E. coli (73). There is considerable controversy as to the relative importance of enterotoxigenic E. coli and other intestinal pathogens as etiologic agents of acute diarrheal diseases that occur endemically, epidemically, in travelers, and in populations of different ages. The reported proportion of pediatric patients from whom LT-producing strains of E. coli can be isolated is highly variable: approxiamtely 80 per cent in Chicago (73), 70 per cent in Brazil (74), 55 per cent in Bangladesh (75), 16 per cent among Apache children in Arizona (76), and none in Boston (77). In prospective studies of travelers'diarrhea occurring in residents of the United States during travel to Mexico or to underdeveloped countries, it has been estimated that enterotoxin producing strains of E. coli are etiologically involved in 36 to 70 per cent of cases (78A, 78B). Although strains of E. coli that produce ST only have been implicated as etiologic agents in a few cases of diarrheal disease in humans (66), there is insufficient data to estimate their prevalence.

The available evidence suggests that enterotoxin-producing strains of E. coli may be a frequent cause of acute diarrhea in humans that is presently not etiologically diagnosed. The development of rapid, inexpensive, and specific immunological assays for E. coli enterotoxins and for antienterotoxic

antibodies may be an essential prerequisite to adequate studies of this problem. The observation that individuals who are immunized with cholera toxoid develop rising titers of neutralizing antibodies against E. coli LT raises the hopeful possibility that a single immunogen might confer at least partial protection against both cholera and enterotoxic E. coli diarrhea.

OTHER BACTERIAL ENTEROTOXINS

Many different bacterial species have been reported to produce enterotoxins. In most cases, enterotoxins are detected by a secretory response following the administration of crude or purified bacterial products into ligated ileal loops in appropriate experimental animals, and most enterotoxins do no produce a secretory response in the gastrointestinal tract after parenteral administration. Many of the bacterial enterotoxins have not been purified to homogeneity, and detailed studies on their modes of action are limited. For enterotoxins that are produced by invasive enteropathogenic bacteria, the contribution of the enterotoxin to the pathogenesis of the diarrheal disease has not been clearly established. Staphylococcal enterotoxins provide an exception to these generalizations. Staphylococcal enterotoxins do not produce a secretory response after injection into ligated ileal loops, and the dose of enterotoxin required to produce vomiting and diarrhea in appropriate experimental animsls is less after intravenous injection than after intra-intestinal administration. There is little evidence to suggest that the intestinal mucosa is the primary site of action for staphylococcal enterotoxins, and it is a question of nomenclature whether the staphylococcal enterotoxins should be named enterotoxins. The prevalence of various bacterial, chemical, parasitic and viral agents in etiologically confirmed outbreaks of food-borne disease in the United States from 1972 - 1974 is summarized in Table 10 (80). Many of the bacteria on this list are potentially able to produce enterotoxins.

1. Shigella dysenteriae type 1.

The enterotoxin of S. dysenteriae type 1 is a protein with a molecular weight of approximately 55,000 to 60,000 (81). Although the protein has not been purified to homogeneity, current data suggest that the shigella enterotoxin is identical to the classical neurotoxin of S. dysenteriae (82,83,84). Enterotoxic activity is observed only after intraintestinal administration of the toxin. In tissue cultures (85) and in experimental animals (86), shigella enterotoxin produces distinctive cytotoxic effects, but it does not activate adenylate cyclase (87, 88). Production of enterotoxin in vitro has been demonstrated with Shigella dysenteriae 1 but not with other species of shigellae. Antibodies to shigella enterotoxin have been demonstrated in convalescent patients after dysentery due to S. dysenteriae 1, S dysenteriae 2, S. flexneri, and S. sonnei (89). Study of human volunteers infected with Shigella dysenteriae type 1 revealed that neutralizing antitoxic antibodies were restricted to IgM and did not appear in other antibody classes (90). The role of shigella enterotoxin in the pathogenesis of bacillary dysentery remains controversial. Studies in volunteers and in animals with laboratory derived strains of S. dysenteriae 1 that are invasive but nontoxinogenic or noninvasive but toxinogenic have established that invasiveness is essential but that toxin production is not essential for the pathogenesis of dysentery (91, 92). It is generally accepted that bacillary dysentery caused by Shigella dysenteriae 1

TABLE 10

Confirmed Foodborne Disease Outbreaks and Cases
1972--1974

	No. of Outbreaks (No. of Cases)		
	1972	1973	1974
BACTERIAL			
B. cereus	0(0)	1(2)	1(11)
Brucella	0(0)	1(4)	0(0)
C. botulinum	4(24)	10(31)	21(32)
C. perfringens	9(173)	9(1,424)	15(863)
Salmonella	36(1,880)	33(2,462)	35(5,499)
Shigella	3(86)	8(1,388)	3(212)
Staphylococcus	34(1,948)	20(1,272)	43(1,565)
Group A Streptococcus	1(35)	1(250)	1(325)
V. cholerae	0(0)	0(0)	1(6)
V. parahaemolyticus	6(701)	1(2)	0(0)
Suspect Group D Streptococcus	1(50)	0(0)	2(38)
CHEMICAL			
Heavy metals	3(8)	0(0)	4(28)
Ciguatoxin	2(27)	0(0)	26(148)
Puffer fish (tetrodotoxin)	0(0)	0(0)	1(2)
Scombrotoxin	4(15)	12(326)	10(26)
Monosodium glutamate	1(3)	2(6)	2(4)
Mushroom poison	8(20)	9(41)	6(9)
Paralytic shellfish poison	2(37)	1(3)	1(4)
Neurotoxic shellfish poison	0(0)	1(4)	1(1)
Miscellaneous chemicals	6(32)	3(12)	6(19)
PARASITIC			
T. spiralis	8(20)	10(59)	14(58)
T. gondii	0(0)	0(0)	1(4)
Anisakidae	0(0)	0(0)	1(1)
VIRAL			
Hepatitis A	5(90)	5(425)	6(282)

(Ref. 80)

tends to be more severe than dysentery caused by other species of shigellae. Although it seems likely that the cytotoxic enterotoxin of Shigella dysenteriae 1 may contribute to the pathogenesis of dysentery, this hypothesis has not been directly proven by experimental data.

2. Clostridium perfringens

The public health importance of C. perfringens has been recently reviewed (94). C. perfringens type A is commonly associated with outbreaks of food poisoning (Table 10). In most cases, the vehicle for infection is improperly cooked or preserved meat. The poisoning is characterized by a brief incubation period of 8 to 24 hours, diarrhea, abdominal pain, and sometimes nausea and vomiting. Anorexia and headache are rarely present, and spontaneous recovery within one to two days is usual. The enterotoxin of C. perfringens type A has been purified to homogeneity and is a protein with a molecular weight of 34,000 to 36,000. The toxin is heat labile and is destroyed by adequate cooking. The enterotoxin is antigenic, and specific serodiagnostic tests for this enterotoxin have been developed. The mode of action of C. perfringens enterotoxin has not been established at the biochemical level. C. perfringens enterotoxin is cytotoxic and does not activate adenylate cyclase in tissue cultures (88). Production of enterotoxin by C. perfringens type A is related to sporulation, but the role of enterotoxin in the physiology of C. perfringens is not established.

C. perfringens bype C is occasionally involved in human gastrointestinal disease. Some strains of C. perfringens type C produce an enterotoxin that is immunologically identical to that of C. perfringens type A. In addition, C. perfringens type C has been associated with a more severe inflammatory condition known as necrotizing jejunitis, enteritis necroticans, or pig-bel that has been reported in Germany, New Guinea, East Africa, and Thailand (94).

3. Staphylococcus aureus

Detailed reviews of the staphylococcal enterotoxins are available (94, 95). Five different antigenic types of staphylococcal enterotoxins designated A, B. C, D, and E have been described. Most human outbreaks of food poisoning have been associated with staphylococcal enterotoxin type A. The staphylococcus has the distinction of being one of the most common causes of food poisoning in the United States. Staphylococcal enterotoxin A is relatively heat stable and may survive heating in foods. Enterotoxins A, B, C, and E are all relatively small proteins with molecular weights between 28,000 and 35,000, and the complete amino acid sequence of staphylococcal enterotoxin B has been determined. The biological activities of staphylococcal enterotoxins are reviewed in reference 94. The mode of action of staphylococcal enterotoxins has not been established at the biochemical level.

4. Miscellaneous Enterotoxins

Enterotoxins are also produced by some strains of Vibrio parahemolyticus, nonagglutinable vibrios, Klebsiella pneumoniae, Pseudomonas aeruginosa, salmonella species, and Bacillus cereus. The hemolytic toxin of V. parahemolyticus has been highly purified, has enterotoxic activity only at high concentrations, and is cardiotoxic in experimental animals following intravenous administration (96, 97). Enterotoxins similar to cholera enterotoxin are produced by some nonagglutinable strains of vibrio (98). Klipstein et al. have described a heat-stable enterotoxin produced by strains of Klebsiella pneumoniae from patients with tropical sprue (99-101).

Progress has recently been made on the purification of this heat-stable enterotoxin, although it has not yet been obtained in pure form (102). The possible role of enterotoxin producing strains of Klebsiella pneumoniae in the pathogenesis of tropical sprue requires further investigation. Outbreaks of food poisoning associated with Bacillus cereus are uncommon (Table 10). Preliminary characterization of an enterotoxin from B. cereus has been reported, but detailed studies of its mode of action have not yet been performed (103). Studies on the enterotoxic activities associated with P. aeruginosa (104) and with salmonella (105) are too preliminary to assess the pathogenic and epidemiologic importance of these toxins.

SUMMARY AND CONCLUSIONS

The data reviewed today illustrate that enterotoxins are produced by a wide variety of bacterial species. The enterotoxins of V. cholerae and E. coli are major virulence factors that are intimately involved in the pathogenesis of diarrheal syndromes caused by these organisms. The activation of adenylate cyclase in intestinal mucosal cells appears to be a primary biochemical effect of cholera toxin and E. coli LT, but the mechanisms involved in this activation and in the stimulation of intestinal secretion by cyclic AMP require further study. The distinction between intoxications resulting from the ingestion of preformed enterotoxins and from production of enterotoxins by bacteria that are multiplying in the gastrointestinal tract is becoming blurred as the capacity of many different enteropathogenic bacteria to produce enterotoxins is recognized. The epidemiologic importance of enterotoxins as causes of acute diarrheal diseases has probably been underestimated, but improved diagnostic methods will be needed before the relative importance of bacterial, viral, and parasitic agents can be accurately estimated in different geographical areas and in various patient populations.

The traditional methods of diagnostic bacteriology are not sufficient to identify enterotoxin-producing bacteria and to distinguish them from nontoxinogenic bacteria in the intestinal flora. Current research on the structure, chemistry, and mode of action of enterotoxins should lead to the development of rapid, inexpensive, specific, and generally available diagnostic methods for detecting enterotoxinogenic bacteria. Such techniques will make it possible to carry out adequately controlled prospective studies to document the importance of the various bacterial enterotoxins in diarrheal diseases, to establish specific etiologies for many cases of acute diarrhea that are presently undiagnosed, and to test the efficacy of prophylactic and therapeutic measures for specific enterotoxic enteropathies. For diseases in which bacterial enterotoxins have an important pathogenetic role, the possibility that active antitoxic immunization may be an effective prophylactic measure deserves further study.

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