

# Enteric Bacterial Toxins: Mechanisms of Action and Linkage to Intestinal Secretion

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## INTRODUCTION

Bacteria that colonize the intestine, invade intestinal epithelial cells, and/or produce one or more toxins are important etiologies of diarrheal disease in both industrialized and developing countries. The primary focus of this review will be to discuss the toxins produced by human enteric pathogens and how the mechanisms of action of these toxins have been linked to intestinal secretion. To accomplish these goals, the range of criteria utilized to demonstrate pathogenicity of an enteric bacterial toxin and potential mechanisms stimulating net intestinal secretion will be reviewed (Table 1). This will be followed by a discussion of the criteria used to classify a given bacterial enteric toxin. Because of the numerous, often overlapping systems by which bacterial toxins can be classified on the basis of mechanisms, we will first discuss potential enteric toxins grouped by organism. Following this initial recitation, the toxins will be discussed on the basis of potential mechanisms yielding secretion in the intestinal epithelium. Lastly, enteric bacterial toxins acting by more than one potential mechanism to cause intestinal secretion will be discussed. This growing area of investigation, which suggests that a bacterial toxin may subvert multiple cellular processes to yield intestinal epithelial cell secretion, is particularly intriguing. In each case, an attempt has been made to be explicit about the limitations of the data demonstrating pathogenicity and the ability to classify the toxin. Throughout this review, emphasis has been given to data

derived from studies of toxins in potentially relevant models of disease, namely, intestinal mucosa in vivo and/or intestinal epithelial cells in vitro.

## CRITERIA FOR ESTABLISHING THE PATHOGENICITY OF AN ENTERIC BACTERIAL TOXIN

### Human Experimental Data

**Volunteer challenges.** The most convincing criterion to determine whether an enteric toxin contributes to the pathogenicity of a human pathogen is to demonstrate that the purified toxin causes diarrhea when ingested by human volunteers. This stringent criterion is met for only two enteric bacterial toxins, cholera toxin (CT) and the *Clostridium perfringens* enterotoxin (CPE). In addition, preliminary data indicate that *Escherichia coli* heat-labile enterotoxin (LT-I) is secretory in volunteers (487). Ingestion of as little as 5 µg of CT resulted in diarrhea in four of five subjects with a mean stool volume of 2.5 liters, and ingestion of 25 µg resulted in diarrhea in two of two subjects with a mean stool output of 21.9 liters over a mean duration of 93 h (308). These data unequivocally establish the potent secretory activity of CT in the human intestine but do not identify the mechanism(s) resulting in the secretory response. In contrast, the CPE was not very potent in stimulating intestinal secretion. Volunteers who ingested 8 to 12 mg of CPE passed only two to three diarrheal stools beginning 1 to

TABLE 1. Criteria for establishing the pathogenicity of an enteric bacterial toxin

Type of data	Criterion
Human experimental data	
Volunteer studies <sup>a</sup>	Toxin ingestion <sup>b</sup> Ingestion of isogenic bacterial strains
Clinical studies associating an enteric toxin with disease	Toxin detection in stool specimens Identification of an intestinal or serum antibody response to the toxin Epidemiologic association of the toxin-producing bacteria with disease
Nonhuman experimental data	
In vivo animal models of disease <sup>c</sup>	Ligated intestinal segments Perfusion of ligated intestinal segments Oral inoculation of toxin or bacteria Reversible ileal tie adult rabbit diarrheal disease model (RITARD model)
In vitro assays	Ussing chambers <sup>d</sup> In vitro tissue culture assays

<sup>a</sup> See Table 5.

<sup>b</sup> Only purified CT and *C. perfringens* CPE have been studied in volunteers.

<sup>c</sup> The most commonly used animal species for enteric toxin assessment are rabbits, rats, and suckling mice.

<sup>d</sup> See the text and Table 2.

2.5 h after toxin ingestion and ending 5 h at the latest after toxin administration (506).

An alternative strategy for establishing the significance of a toxin in volunteer trials is to feed subjects isogenic bacterial strains specifically engineered so that the only difference between the strains is expression of an active toxin. Studies with strains of *Vibrio cholerae* specifically mutated in genes encoding CT (*ctx*) demonstrated that volunteers ingesting the wild-type strain expressing CT experienced the severe diarrhea characteristic of cholera while individuals ingesting the same strain mutated in the *ctx* genes did not experience clinical cholera (310). However, ca. 50% of the volunteers in the latter group did experience mild to moderate diarrhea of low volume, indicating that although CT is responsible for the severe diarrhea of cholera, *V. cholerae* produces additional secretogenic factors that can cause mild diarrhea.

**Other criteria.** Short of volunteer trials, other criteria for associating an enteric toxin with human disease may be used. Supporting evidence for such an association includes (i) detection of a toxin directly in stool, (ii) measurement of an intestinal or serum antibody response to the toxin, or (iii) epidemiologic association of bacteria producing the toxin with clinical disease. Fulfillment of either of the first two criteria indicates in vivo production of the toxin. However, because enteric bacteria most often produce numerous virulence factors, such as attachment and/or invasion factors, in addition to an enteric toxin, the detection of toxin in stool, the demonstration of an antitoxin response, or an epidemiologic association is only supportive and not definitive in determining that the toxin contributes to the intestinal secretion observed in the infection.

Examples of toxins that have been detected directly in stools from infected patients include *Clostridium difficile* toxin A (reviewed in reference 271), Shiga toxin (324), CT (reviewed in reference 383), and the heat-labile (LT-I) and heat-stable (STa) toxins from enterotoxigenic *E. coli* (ETEC) (365). How-

ever, failure to detect toxins in stools may be due to the insensitivity of the detection assay, the toxin-binding effects of free gangliosides present in mucin found in stools, proteolytic degradation of the toxin in the intestinal tract, or other reasons.

The production of an immune response against a toxin can also be taken as evidence of in vivo toxin production, but failure to detect an immune response does not rule out in vivo toxin production. Although serum and intestinal antibody responses against CT and LT-I are seen, an antibody response against STa has not been demonstrated (reviewed in reference 308). The lack of an immune response to STa is probably because STa is nonimmunogenic as a result of its small size, unless it is experimentally conjugated to a larger carrier protein. The poor immune response against Shiga toxin in patients infected with *Shigella dysenteriae* 1 is probably due to quite different reasons. In the mid-1970s, Keusch et al. (277) reported that volunteers ingesting this pathogen developed no serum immunoglobulin G (IgG) responses against Shiga toxin but did develop low levels of IgM antibodies. Similarly, the majority of patients infected with enterohemorrhagic *E. coli* (EHEC), which also expresses Shiga toxin, do not develop an IgG antibody response to this toxin (266). It is now recognized that the globotriaosylceramide (Gb<sub>3</sub>) receptor for Shiga toxin is a B-cell differentiation antigen known as CD77. Shiga toxin selectively kills IgG- and IgA-committed lymphocytes, whereas most IgM-producing cells are resistant to Shiga toxin (75). Thus, an enteric toxin may actively suppress an immune response, although other possible explanations for the lack of antibodies against Shiga toxin cannot be definitely ruled out.

Many examples of an epidemiologic association of toxin production with human disease can be cited, but such studies have been particularly useful for establishing the significance of LT-I and STa-producing ETEC and enterotoxigenic *Bacteroides fragilis* (ETBF). *E. coli* and *B. fragilis* are both members of the normal intestinal flora, but the strains isolated from healthy individuals usually do not produce enterotoxins. Epidemiologic studies have demonstrated that *E. coli* strains producing LT-I and STa (197, 364) or ETBF strains (470, 472, 474) are found significantly more often in individuals with diarrheal disease than in healthy individuals.

### Nonhuman Experimental Data

Additional approaches to studying the pathogenicity of enteric bacterial toxins involve one or more nonhuman experimental approaches including in vivo assays in animal models and in vitro assays such as Ussing chambers and tissue culture assays. Most data on the pathogenicity of bacterial enteric toxins are based on these approaches, and then, by analogy, the toxin is proposed to be important in human diarrheal disease.

**In vivo assays. (i) Ligated intestinal loops or segments.** Although only gross intestinal secretion is typically measured, a classic technique to determine whether an enteric toxin stimulates secretion is the ligated intestinal segment or loop assay as originally described by De, using sterile culture filtrates of *V. cholerae* inoculated into rabbit ileal loops (89). For this experimental approach, small intestinal (jejunal or ileal) or colonic ligated intestinal segments are inoculated with toxin preparations or bacterial cultures and the subsequent presence or absence of secretion is assessed at time points up to 18 h. As discussed above, since bacteria usually produce multiple virulence factors, inoculation of whole bacterial cultures is less definitive in establishing the importance of the toxin to disease pathogenesis unless the experiments are conducted with isogenic mutant strains of bacteria. Isogenic strains of enteric bacteria differing only in the presence or absence of a toxin

gene are a potent tool for establishing the pathogenicity of a toxin in the animal model. For example, isogenic strains of *Vibrio parahaemolyticus* differing only in production of the thermostable direct hemolysin (TDH) were tested in ligated segments to demonstrate the importance of TDH in causing the secretion (390).

**(ii) In vivo perfusion studies.** In vivo perfusion, a modification of the ligated intestinal loop technique, allows a more precise measurement of net fluid movement across the intestinal epithelium as well as measurements of the net change in specific ions in the intestinal fluid over time (7). For this experimental approach, a ligated intestinal segment is cannulated with a multiperforated tube and cleansed by flushing prior to initiation of the experiment. Either polyethylene glycol (7) or phenolsulfonphthalein (571) may be used as a volume marker, and sequential samples can be analyzed for ionic content or osmolarity to measure net changes over time. One limitation of this approach is that measurements are typically made over only a few hours. However, one advantage of this technique is that its sensitivity for identifying changes in water and ion transport exceeds that of ligated intestinal loops.

**(iii) Oral inoculation.** An alternative approach to establishing the relevance to secretion of an enteric bacterial toxin is oral inoculation of an animal with the toxin with subsequent demonstration of either diarrhea or intestinal fluid accumulation. Examples of common animal models in which this approach has proved valuable include suckling mice in which oral inoculation of *E. coli* STa stimulates fluid accumulation (224); infant rabbits used to study the secretory response to CT (456); and the sealed adult mouse model, which has been used to study host genetics modulating the secretory response to CT, among other purposes (457). Alternatively, oral inoculation of animal models with isogenic strains can aid in establishing the importance of a toxin to the disease. For example, isogenic strains of *Yersinia enterocolitica* have been used to establish that the heat-stable enterotoxin produced by most pathogenic strains contributes to the severity of disease in a rabbit animal model (93).

**(iv) RITARD model.** The reversible ileal tie adult rabbit diarrheal disease model (RITARD model) is useful in studying the pathophysiologic sequelae of enteric bacteria in an uninterrupted intestine, an approach which more closely mimics native disease than do some of the above approaches (513). In this model, the cecum is ligated permanently and the bacteria are injected into the intestine at a site proximal to a temporary ileal ligature. After a short incubation period (ca. 2 h) to allow the infection to become established, the ligature is removed and the animals are observed for the development of diarrhea, usually over several days. This model has been particularly useful in studying the immune responses to ETEC (471) and *V. cholerae* (434) and in showing the secretory potential of adherent bacterial strains without other defined virulence factors (566). Another potential use of the RITARD model is to study the pathogenicity of isogenic strains of bacteria differing only in the presence or absence of a toxin gene. Recently, a modification of this technique was used to establish an adult rabbit model of dysenteric infection with *Shigella flexneri* by direct colonic inoculation of the bacteria after ligation of the cecum (448).

**In vitro assays. (i) Ussing chambers.** The Ussing chamber is a valuable in vitro experimental approach used extensively to identify specific changes in active (energy-dependent) ion transport stimulated by enteric bacterial toxins (149, 484, 550). For this technique, either native intestinal epithelium or polarized monolayers of cultured intestinal epithelial cells are mounted between Lucite chambers under conditions of ionic,

osmotic, and electrical ("voltage-clamped") equilibrium. The ability of an enteric toxin to stimulate anion (usually chloride) secretion and/or to inhibit NaCl absorption, both potentially contributing to net intestinal secretion, can be measured under these conditions. Three measurements related by Ohm's law ( $V = IR$ ) are made in Ussing chambers: (i) short-circuit current ( $I_{sc}$  or  $I$ ); (ii) potential difference (PD or  $V$ ); and (iii) resistance ( $R$ ). Increases in  $I_{sc}$  and PD in monolayers of intestinal epithelial cells indicate the secretion of chloride. In native intestinal tissue, increases in  $I_{sc}$  and PD in combination with secretion in a ligated intestinal segment are also consistent with net intestinal anionic secretion.

**(ii) Tissue culture assays.** Another experimental approach to identify activities of enteric bacterial toxins is the use of a wide variety of nonintestinal cell lines. Most often, the activity of a particular toxin is identified by a change in the shape of the cells or by cytotoxicity in response to treatment with the enteric toxin. For example, Chinese hamster ovary (CHO) and Y-1 adrenal cells have proven useful for identifying toxins that increase intracellular cyclic AMP levels, such as CT (reviewed in references 262 and 263), the *Salmonella* toxin (421, 424), and the heat-labile enterotoxins of *E. coli* (reviewed in reference 510). In response to an increase in intracellular cyclic AMP levels, CHO cells elongate (195) and Y-1 adrenal cells become round (117). Recently, however, the toxin of *Vibrio hollisae* (HT) and a 70-kDa heat-stable secretory toxin produced by *Aeromonas* spp. have been shown to cause elongation of CHO cells in the absence of increased cyclic AMP levels (293, 347), thus demonstrating that morphological changes in nonintestinal cell lines cannot be assumed to identify the biochemical mediator by which the enteric toxin acts. Another example of the use of nonintestinal cell lines to study enteric toxins is the use of HeLa or Vero cells for demonstrating cytotoxicity due to inhibition of protein synthesis by Shiga and Shiga-like toxins (401). Although the use of these nonintestinal cell lines has been very valuable in identifying one or more activities of enteric toxins, these experimental data do not aid in demonstrating whether a toxin stimulates secretion, nor can the mechanism by which the toxin stimulates secretion be established in these cell lines. Thus, data from nonintestinal cell lines must be interpreted with caution when discussing the importance of the results to pathogenicity (322).

## POTENTIAL MECHANISMS OF INTESTINAL SECRETION

The purpose of this section is to provide an overview of potential mechanisms which stimulate intestinal secretion. A detailed discussion of each potential mechanism is not possible. However, an attempt has been made to identify and reference areas of controversy or complexity to guide the reader to more complete discussions of these issues. The mechanisms have been divided into those which are considered established and those which are more speculative. In most instances, the mechanism has been implicated in intestinal secretion through basic studies of mediators and, in some cases, by using an enteric toxin as a probe. In the section on functional classification of enteric bacterial toxins by genus and species (below), the data linking each enteric pathogen and its toxin(s) to one or more of these secretory mechanisms are reviewed in detail.

### Components of the Intestinal Epithelium Potentially Contributing to Secretion

The intestinal epithelium is a complex structure containing multiple cell types that can contribute to net intestinal secre-



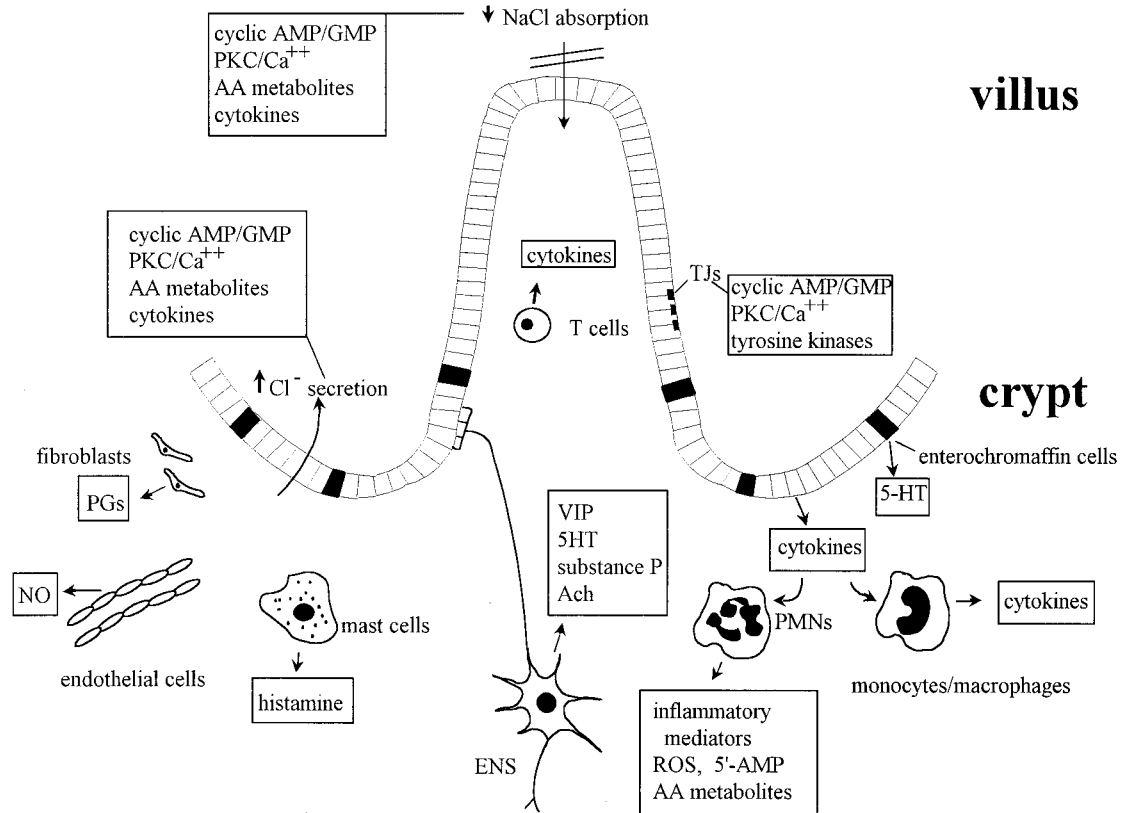


FIG. 1. Components of the intestinal epithelium potentially contributing to intestinal ion transport. Enteric bacterial toxins stimulate one or more components of the intestinal epithelium to yield net intestinal secretion. This figure depicts a wide range of possible mechanisms by which an enteric toxin might stimulate intestinal secretion. However, not all of the pathways depicted have been implicated as potential mechanisms of toxin action at this time. First, the intestinal epithelial cell layer contains multiple cell types, including villus cells, crypt cells, and enterochromaffin or endocrine cells, which can contribute to intestinal secretion. Under basal conditions, the villus cells absorb NaCl, a process which is inhibited by serine/threonine protein kinases regulated by increases in the levels of intracellular cyclic nucleotides (A or G kinases) or calcium (PKC). Production of arachidonic acid (AA) metabolites (e.g., prostaglandins [PGs], leukotrienes, and/or PAF) and/or cytokines by intestinal epithelial cells and/or submucosal cells also inhibits NaCl absorption. The crypt cells secrete increased levels of chloride in response to the same signal transduction mechanisms. Enterochromaffin cells may release secretory hormones such as 5-HT (serotonin). Cells of the intestinal epithelial cell layer are joined near the apical membrane by specific morphologic structures called tight junctions, which define, in part, the polarity of these cells. Tight junctional permeability is regulated by cyclic nucleotides, calcium, activation of tyrosine kinases, and possibly nitric oxide (released by endothelial cells in the lamina propria). Increased intestinal permeability may contribute to intestinal secretion by allowing increased solvent drag. The role of NO as a secretory agonist is controversial. Second, numerous cell types such as T cells, mast cells, PMNs, and monocytes/macrophages in the lamina propria of the intestinal epithelium may contribute to secretion by production of inflammatory mediators and/or cytokines. For example, PMNs release reactive oxygen species (ROS), prostaglandins, leukotrienes, PAF, and/or 5'-AMP and mast cells release histamine. Cytokines may be released by the intestinal epithelial cells and/or immune system cells in the lamina propria. Cytokine release may augment secretion by stimulating further recruitment of inflammatory cells or by directly stimulating ion secretion. In addition, fibroblasts and endothelial cells in the lamina propria can be stimulated to release prostaglandins and NO, respectively. Third, cells of the enteric nervous system (ENS; presented only schematically as a single neuron in the figure) can release a variety of secretory peptides such as VIP, 5-HT, substance P, and acetylcholine. Some nerves of the enteric nervous system end directly on the intestinal epithelial cells, enabling direct stimulation of secretion. Although the relationship between the enteric nervous system and enterochromaffin cells is incompletely defined, mediator crosstalk between these two components of the intestinal epithelium is likely. Fourth, changes in the activity of intestinal smooth muscle (not depicted) may contribute to the development of diarrhea. See the section of text on potential mechanisms of intestinal secretion for detailed discussions and references.

tion (Fig. 1). First, polarized villus and crypt epithelial cells exhibit specific ion-transporting properties. In general, crypt cells secrete chloride and have been best studied by using human colonic epithelial cell lines *in vitro* (T84, Caco-2, and HT29 cells) (reviewed in reference 101). A stable *in vitro* model of the NaCl-absorbing villus cells does not exist, and thus, data on their function have been derived largely from studies of intestinal epithelium *in vivo*. Second, the gut epithelium contains enterochromaffin cells, which secrete hormones, such as 5-hydroxytryptamine (5-HT or serotonin), capable of stimulating intestinal secretion (113). Third, cells in the lamina propria of the intestinal epithelium contribute to secretion through production of secretory mediators. These cells include immune cells such as polymorphonuclear leukocytes (PMNs), monocytes, macrophages, and lymphocytes, as well as fibroblasts and endothelial cells. Fourth, the submucosa contains

elements of the enteric nervous system. Lastly, changes in the activity of intestinal smooth muscle may contribute to the production of diarrhea.

#### Established Mechanisms of Intestinal Secretion

**Cyclic nucleotides.** Cyclic nucleotides stimulate intestinal secretion by three potential mechanisms: (i) altered activity and/or upregulation of ion transporters; (ii) modulation of the host cell cytoskeleton and/or tight junctional function in the intestinal epithelial cell; and (iii) increases in intracellular calcium levels. Substantial data support the first two mechanisms, while the third is considerably more tentative.

First, elevation of either cyclic AMP or cyclic GMP levels in intestinal epithelial cells stimulates active chloride secretion and/or inhibits electroneutral NaCl absorption in the intestinal

epithelium (73, 149, 150). Cyclic AMP acts via stimulation of A kinase with direct phosphorylation and activation of the major chloride channel identified in intestinal epithelial cells, the cystic fibrosis transmembrane conductance regulator (CFTR) (63). On the basis of studies of intestinal epithelial cell lines in vitro, the action of cyclic GMP is more controversial, with roles proposed for both A-kinase and G-kinase in its activation of CFTR (57, 162, 317, 534). In addition, cyclic AMP activates an outwardly rectifying chloride channel (200) located in the apical membrane of the intestinal epithelial cell. Cyclic nucleotides also activate a barium-sensitive potassium channel and the Na/K/2Cl cotransporter located on the basolateral membrane of polarized T84 cells (342–344, 361).

Second, cyclic AMP (and perhaps by analogy cyclic GMP) regulates intestinal epithelial cell secretion via effects on cytoskeletal proteins. Inhibition of microtubule polymerization in T84 cells and preparations of rat colon reduces the secretory response to a cyclic AMP-dependent (but not a calcium-dependent) agonist, suggesting that the chloride secretory response to cyclic AMP is dependent on microtubules (171). Subsequent work has revealed that apical membrane recruitment of CFTR in T84 cells in response to cyclic AMP is dependent on microtubules but not calcium or F (filamentous) actin (536). Additional data indicate that immobilization of F actin in intestinal epithelial cells by phalloidin, a mushroom toxin, also diminishes cyclic AMP-dependent chloride secretion (342, 496). Cyclic AMP appears to increase either directly or indirectly both the activity and number of Na/K/2Cl cotransporters in the basolateral membrane (344). Furthermore, phalloidin appears to inhibit ion translocation by the cotransporter but not the number of cyclic AMP-elicited membrane cotransporters (344). Thus, remodeling or dissociation of intestinal epithelial-cell F actin appears to contribute to cyclic AMP-stimulated chloride secretion in intestinal epithelial cells. Consistent with these observations, elevation of the levels of intracellular cyclic nucleotides (either cyclic AMP or cyclic GMP) alters the structure of F actin at the basolateral pole of polarized intestinal epithelial cells (343, 496). The mechanism by which elevation of intracellular cyclic nucleotides reorganizes F actin is unknown. In addition to activation of ion transporters, data from nonintestinal systems (summarized in reference 483) and animal tissues treated with CT (see the section on *V. cholerae*, below) (62, 149, 332, 540) suggest that cyclic AMP also regulates tight junction function, potentially contributing to intestinal secretion.

Third, in HT29 but not T84 intestinal epithelial cells, cyclic AMP-dependent agonists stimulate increases in intracellular calcium by a pathway distinct from that mediated by inositol trisphosphate (see the section on protein kinase C [PKC], below) (18, 95, 227). While cyclic AMP agonists have been shown to stimulate calcium-dependent responses in several nonintestinal cell types, this response in intestinal epithelial cells appears to be cell line dependent and its relevance to secretion in intestinal epithelium in vivo is unknown.

**Intracellular calcium.** There are several potential mechanisms by which an elevation in intracellular calcium levels may stimulate a net intestinal secretory response. First, elevation of intracellular calcium levels alters the regulation of several ion-transporting proteins. Thus, elevation of intracellular calcium levels activates calmodulin-dependent protein kinases and potentially protein kinase C (also see below), which can directly phosphorylate and activate CFTR (74, 112, 428, 580). These kinases can also inhibit the activity of the apical membrane sodium-hydrogen exchanger (NHE3), resulting in diminished sodium absorption (542). In addition, an increase in intracellular calcium levels can activate a basolateral potassium chan-

nel, which is separate from the cyclic nucleotide-activated potassium channel (102, 361). However, the mechanism of activation is unknown. Notably, calcium alone is a sufficient signal to stimulate chloride secretion in some intestinal epithelial cells in vitro in the absence of additional second messengers derived from membrane phospholipid turnover (see the section on protein kinase C, below) (259). Data examining the secretory action of different calcium-dependent agonists further suggest the existence of multiple effector mechanisms that augment or attenuate the action of intracellular calcium (100). Second, calcium has been demonstrated to regulate tight junctions (reviewed in reference 483), suggesting that changes in intracellular calcium levels may modulate intestinal permeability (see below) and contribute to intestinal secretion. However, when F actin was immobilized in intestinal epithelial cell monolayers by using phalloidin, chloride secretion stimulated by a calcium-dependent agonist was unaltered (496).

**Protein kinase C.** The PKC family consists of multiple isoenzymes that differ in activation requirements (reviewed in reference 397). However, the specific isoforms of PKC either present in various intestinal cell types or responsible for changes in intestinal ion transport are not yet known. In the most frequently cited scenario believed to apply in the intestine, metabolism of phosphatidylinositol by phospholipase C (PLC) results in release of inositol trisphosphate and diacylglycerol. Inositol triphosphate releases intracellular calcium, and diacylglycerol activates PKC. Notably, certain PKC isoforms can be activated in the presence of basal levels of intracellular calcium and other isoforms do not require calcium for activation (397). The role of PKC activation in stimulating intestinal secretion has been examined both in vivo and in vitro and is complex (18, 54, 114, 115, 341, 552, 571). By using phorbol esters as direct activators of PKC, net intestinal secretion has been observed in ligated intestinal loops and stimulation of chloride secretion and inhibition of electroneutral NaCl absorption have been demonstrated in Ussing chambers (54, 114, 571). Studies with intestinal epithelial cells in vitro have demonstrated that PKC activation has sequential stimulatory and inhibitory effects on chloride secretion (18, 260, 341, 552). Furthermore, PKC activation may modulate the secretory and biochemical responses to both cyclic AMP- and cyclic GMP-dependent agonists (84, 85, 268, 313, 567, 572). PKC activation also leads to reversible disassembly of the intestinal epithelial cell cytoskeleton and thus modulates tight junctional function (208). Together, these data suggest that PKC activation may contribute to intestinal secretion through direct effects on one or more ion transporters as well as through regulation of the paracellular transport pathway.

**Activation of phospholipases.** Activation of several phospholipases can lead to changes in intracellular mediators that regulate ion transport. The best-studied examples are (i) release of arachidonic acid metabolites, presumably through activation of epithelial cell PLA<sub>2</sub> (32), and (ii) release of intracellular calcium and diacylglycerol through activation of PLC. Activation of PLA<sub>2</sub> leads to the release of secretory metabolites of the cyclooxygenase (i.e., prostaglandins) and lipoxygenase (i.e., leukotrienes) pathways, as well as the release of platelet-activating factor (PAF) (reviewed in reference 72). Furthermore, certain prostaglandins and leukotrienes may serve to amplify the secretory response; for example, prostaglandin I<sub>2</sub> (PGI<sub>2</sub>) can activate the enteric nervous system and leukotriene B<sub>4</sub> stimulates the recruitment of PMNs, leading to an inflammatory response in the intestine. Activation of PLC regulates ion transport through the release of calcium and PKC activators, as summarized above. A notable and novel aspect of PLC activation in the intestinal epithelial cell has

recently been described; namely, treatment of rabbit ileum with the acetylcholine analog carbachol, which acts via basolateral receptors, rapidly increases the activity and amount of the phosphatidylinositol-dependent PLC ( $\gamma$ -1 isoform) in the apical membrane (80, 279). The mechanism by which carbachol rapidly transduces transcellular activation of PLC is not known. Recently, additional signalling pathways that involve the activation of PLD and phosphatidylcholine-specific PLC have been identified (reviewed in references 106 and 397). Each of these novel pathways leads to the release of unique but overlapping lipid metabolites (e.g., diacylglycerol and phosphatidic acid) that regulate specific cell functions. There are no specific data on how these new pathways modulate intestinal ion transport, but it is likely that a regulatory role exists (see the section on protein kinase C, above) (555).

**Inflammatory mediators.** The mechanisms by which invasive organisms such as *Shigella* spp., *Salmonella* spp., and *Yersinia enterocolitica* stimulate intestinal secretion are poorly understood. Recent insight into this issue has been gained through studies demonstrating intestinal epithelial cell production of proinflammatory cytokines in response to invasive pathogens (128, 354). The best-studied example is interleukin-8 (IL-8), produced and secreted in a polarized fashion across the basolateral membrane of the epithelial cell after attachment of *Salmonella typhimurium* to intestinal epithelial cell monolayers (354). This directed delivery of IL-8 to the subepithelial compartment recruits PMNs and generates an inflammatory response which can involve the release, for example, of reactive oxygen species, prostaglandins, or leukotrienes, which can stimulate intestinal secretion (reviewed in references 72, 419, 444, 445, and 477). Overall, the subepithelial compartment, not the intestinal epithelial cells, is the major site of production of arachidonic acid metabolites in the intestinal epithelium (300). Both inflammatory cells in the submucosa and fibroblasts may release arachidonic acid metabolites such as prostaglandins (28). In addition, the activation of subepithelial immune system cells can lead to the production of additional cytokines such as IL-1 or IL-3, which can directly stimulate secretion (53, 65, 527), or gamma interferon, which regulates intestinal epithelial cell tight junctional permeability (331). Furthermore, PMNs release an epithelial chloride secretagogue, 5'-AMP, which acts on the apical epithelial cell membrane by activating adenosine receptors (330, 384, 538). Although intestinal mucosal mast cells are not typically noted to be a prominent component of the inflammatory response to bacterial enteric pathogens, their activation also leads to the release of numerous potentially secretogenic mediators (reviewed in references 18, 72, and 419).

**Enteric nervous system.** Considerable evidence implicates activation of one or more components of the enteric nervous system in the secretory responses to enteric bacterial toxins (reviewed in references 247 and 419). The neural pathways involved are complex and incompletely delineated. These pathways appear to involve villus to crypt neural reflexes and sensory spinal reflexes innervating the same, as well as distal, segments of the intestine exposed to the toxin. Specifically, peptidenergic neurons such as those secreting substance P, 5-HT, and vasoactive intestinal polypeptide (VIP) have been implicated in the secretory responses to *C. difficile* toxin A (substance P) (436), CT (5-HT, VIP) (246, 426), and the *E. coli* heat-stable enterotoxins (STa and STb) (5-HT) (205, 426). However, no specific data implicate neurons as the source for the enhanced release of 5-HT. Alternatively, and probably more likely, enterochromaffin cells may release 5-HT after stimulation by CT or *E. coli* STa or STb. Despite the rapid expansion of information available on the enteric nervous sys-

tem, the studies linking these pathways to specific enteric pathogens are fragmentary, indicating that considerably more research is necessary to develop a better understanding of the interface between this area of intestinal physiology and infectious diarrheal diseases. Lastly, several enteric bacterial pathogens and/or their toxins have been shown to alter intestinal myoelectric patterns. These include CT (338), *E. coli* STa (339), wild-type and recombinant *V. cholerae* strains (318, 338), and various classes of *E. coli* (e.g., enteropathogenic *E. coli* [EPEC], ETEC, and enteroinvasive *E. coli* [EIEC]) (40, 41, 503).

### Postulated Mechanisms of Intestinal Secretion

**Changes in intestinal permeability.** Changes in intestinal permeability have long been postulated to contribute to intestinal secretion associated with, for example, postprandial transport changes or infectious diarrhea (reviewed in references 329 and 483). The mechanism by which a change in intestinal permeability would lead to intestinal secretion is unclear. One hypothesis is that the actin-myosin ring which inserts into the cell membrane at the zonula adherens (located just below the tight junction or zonula occludens) contracts, thereby pulling open the tight junction. Another is that dissolution of F-actin filaments which insert into both the zonula occludens and zonula adherens increases the permeability of the epithelium. Either of these mechanisms may allow the back diffusion of ions and water into the lumen (i.e., development of a "leaky" epithelium), a process potentially augmented by tissue hydrostatic pressure and/or increased intraluminal osmotic pressure.

Over the past decade, the tight junctional complex of the intestinal epithelium has been recognized to be a dynamic structure that is under physiologic regulation by intracellular mediators such as cyclic nucleotides, calcium, PKC, tyrosine kinases and possibly nitric oxide (473, 561; reviewed in reference 483). Furthermore, specific ion transporters, including the Na/K/2Cl cotransporter and sodium-hydrogen exchangers (NHEs) (342, 542), have been demonstrated to be functionally linked to the cytoskeleton. Another possible mechanism for regulation of paracellular intestinal permeability by a bacterial toxin or pathogen may involve stimulating the production of immune system mediators in the intestinal epithelium (280, 359, 454). For example, gamma interferon experimentally decreases the resistance of the intestinal epithelium (331). These data, combined with the growing recognition that the intestinal epithelial cell F-actin structure is altered by certain enteric bacterial toxins (e.g., *Clostridium difficile* toxins A and B, *Bacteroides fragilis* toxin, *E. coli* cytotoxic necrotizing factors 1 and 2, *V. cholerae* Zot toxin), invasive enteric pathogens (e.g., *Shigella* spp., *Salmonella typhimurium*), and EPEC (through the intimate attaching-and-effacing [AE] lesion), strongly suggest that alteration of intestinal permeability may be important in the pathogenesis of diarrheal disease (see the section on functional classification of enteric bacterial toxins by genus and species [below] for references).

**Adherence.** Several investigators have proposed that attachment of organisms without recognized virulence factors in a nonintimate manner (i.e., lacking the AE lesion associated with EPEC infection) to the intestinal mucosa may be sufficient to stimulate intestinal secretion (482, 507, 566). The secretory response in some studies occurs in association with diminished brush border digestive enzymes, suggesting a malabsorptive osmotic mechanism. Alternatively, the diminished activity of brush border enzymes may be a marker for a change in the function of apical membrane transporters (e.g., NHEs and CFTR), but this possibility has not been specifically investi-

TABLE 2. Definitions for enteric bacterial toxin classification<sup>a</sup>

Type of toxin	Definition
Enterotoxin	Produces net secretion in ligated intestinal segments without either histologic evidence of intestinal damage or evidence of injury to nonerythrocytic cells in <i>in vitro</i> assays <sup>b</sup> . Stimulates an increase in $I_{sc}$ and PD in Ussing chambers without evidence of intestinal injury; this result implies electrogenic (active) anion secretion <sup>c</sup> . In addition, a toxin may impair electrically neutral NaCl absorption, which also results in net ion secretion.
Cytoskeleton-altering toxin <sup>d</sup>	Produces an alteration in cell shape, most often demonstrated to be due to rearrangement of F actin. The toxin may produce limited cell injury but is not lethal to cells and may or may not be associated with evidence of net secretion in <i>in vivo</i> or <i>in vitro</i> intestinal epithelial cell models of disease.
Cytotoxin <sup>d</sup>	Produces cell or tissue damage, usually culminating in cell death. The toxin may or may not be associated with net secretion in <i>in vivo</i> or <i>in vitro</i> intestinal epithelial cell models of disease.
Toxins with neural activity	Involves release of one or more neurotransmitters from the enteric nervous system. Alters smooth muscle activity in the intestine.

<sup>a</sup> To be classified within a particular toxin group, only one of the listed criteria is necessary. Toxins may be classified in more than one category (see Table 3). The text discussing the toxins by genus and species, as well as Tables 3 and 4, attempts to delineate the extent of the intestinal data available for each enteric toxin.

<sup>b</sup> Specific data on intestinal histology or *in vitro* cell assays are not consistently available (see the text and Tables 3 and 4).

<sup>c</sup> Stimulation of an increase in  $I_{sc}$  in intestinal epithelial cell monolayers (e.g., Caco-2 and T84 cells) indicates chloride secretion; an increase in  $I_{sc}$  in native intestinal tissue combined with net secretion in ligated intestinal segments implies anion secretion (chloride and/or bicarbonate).

<sup>d</sup> Data are not always available regarding the specific determination of whether these toxins injure tissues or cells.

gated. Another possibility is that adherence alone is capable of triggering the intestinal epithelial cell to secrete cytokines, thereby potentially amplifying the secretory response. In fact, nonintimate attachment of *S. typhimurium* has been shown to stimulate intestinal epithelial cells to release IL-8 (354). However, given the continual recognition of new virulence factors for enteric pathogens (e.g., the heat-stable enterotoxin [EAST 1] of enteroaggregative *E. coli* [EAEC] [481]), it is unclear whether the strains used in these studies possess unrecognized virulence factors that contribute to the observed intestinal secretion. Recent results with recombinant attenuated *V. cholerae* strains that are adherent without causing diarrhea indicate that at least for this species, adherence alone does not necessarily lead to diarrhea (82, 309, 520).

**Tyrosine kinases.** Activation of tyrosine kinases, rather than serine/threonine kinases (e.g., A or G kinase, PKC), represents a potential mechanism of intestinal secretion. The studies to date evaluating the involvement of one or more tyrosine kinases in the regulation of ion transport in intestinal epithelial cells suggest that tyrosine kinase activity serves primarily to limit rather than stimulate chloride secretion and also stimulates the absorption of NaCl (116, 231, 489). However, tyrosine kinases have also been implicated in regulating ion transport proteins potentially leading to intestinal secretion (116). These studies have all used inhibitors of tyrosine kinase activity to identify these transport changes; thus, the specific tyrosine kinases present in intestinal epithelial cells and their substrates remain to be identified.

**Pore formation.** One proposed mechanism of action for bacterial toxins, particularly amphipathic molecules, involves formation of multimeric units that can insert into membranes, thereby creating a functional ion channel. Although several enteric bacterial toxins have been proposed to act by this mechanism (e.g., *Staphylococcus aureus* delta toxin [264, 363], *C. perfringens* beta toxin [226], and *V. cholerae* Ace [541]), no definitive data exist to implicate this as the mechanism by which any enteric bacterial toxin intoxicates the intestinal epithelial cell and causes secretion.

**Phosphatase inhibition.** Treatment of airway epithelial cells with phosphatase inhibitors stimulates chloride secretion through the activation of CFTR (22). Whether this is through

a direct effect on the CFTR protein or indirect through an action on a closely associated protein is unclear. Similar data for intestinal systems are not yet available, but phosphatase inhibition represents a potential mechanism of intestinal secretion.

## CLASSIFICATION OF ENTERIC BACTERIAL TOXINS

A strict classification of enteric bacterial toxins is often problematic (Table 2). In some instances, this difficulty is because the available data are incomplete; in other cases, it is because of the complexity of the mode of action. For example, recent data suggest that chloride secretion stimulated by CT, considered a "classic" secretory enterotoxin (see the definitions below and Table 2) acting via the second messenger, cyclic AMP, may also involve stimulation of the enteric nervous system and formation of PAF (196) and/or prostaglandins (423, 425, 426), as well as possible remodeling of intestinal epithelial cell F actin (496). While the term "enterotoxin" is sometimes used in the broadest sense to include a toxin with any effect on intestinal tissue, we will use more narrowly drawn definitions in the present discussion. Similarly, toxins classified as cytotoxic in earlier publications (276) are classified as either enterotoxins or cytoskeleton-altering toxins. Thus, for the purposes of this review, four classes of toxins will be defined: (i) enterotoxins, (ii) cytoskeleton-altering toxins, (iii) cytotoxins, and (iv) toxins with neural activity. To be included within a particular toxin class, only one of the listed criteria (see below and Table 2) must be fulfilled. As is evident in Table 3, which lists the enteric bacterial toxins by genus and species, several of the toxins can be placed in more than one category. Table 4 classifies the enteric toxins based solely on data available from *in vivo* and *in vitro* intestinal models of disease. Notably, several enteric toxins have been classified as cytoskeleton-altering toxins or as cytotoxins without specific data being available on the ability of the toxin to induce cell injury.

A toxin is classified as an enterotoxin if (i) the toxin stimulates net secretion in one or more intestinal models without either histologic evidence of intestinal damage in light-microscopy studies or evidence of injury to nonerythrocytic cells in *in*



TABLE 3. Functional classification of enteric bacterial toxins by genus and species<sup>a</sup>

Bacterium and toxin	Condition of toxin <sup>b</sup>	Toxin gene cloned?	Type of toxin <sup>c</sup>			
			Enterotoxin	Cytoskeleton altering	Cytotoxin	Neural activity
<i>Aeromonas hydrophila</i> <sup>d</sup>						
Aerolysin <sup>e</sup>	Purified	Yes	+		+ (s)	
Other toxins <sup>f</sup>	Purified/crude <sup>g</sup>	Yes <sup>g</sup>	+	+		
<i>Bacillus cereus</i>						
Emetic toxin	Crude	No				+ (?)
Diarrheal toxin (HBL)	Purified	Yes <sup>t</sup>			+ (s)	
<i>Bacteroides fragilis</i>						
BFT	Purified	Yes		+ (i, s) <sup>i</sup>	+ (s) <sup>i</sup>	
<i>Campylobacter jejuni</i> <sup>d</sup>						
LT-like	Partially purified	No	+	+		
CLDT	Crude	No		+	+ (s)	
Cytotoxin	Crude	No			+	
<i>Clostridium botulinum</i>						
C2 toxin	Purified	No		+ <sup>j</sup>	+ (s)	
<i>Clostridium difficile</i>						
Toxin A	Purified	Yes		+ (i, s)	+ (s) <sup>k</sup>	+
Toxin B	Purified	Yes		+ (i)	+ <sup>k</sup>	
<i>Clostridium perfringens</i> type A						
CPE	Purified	Yes			+ (s)	
<i>Clostridium perfringens</i> type C						
Beta toxin	Purified	Yes			+	
Alpha toxin <sup>l</sup>	Purified	Yes	+ (?)		+	
Enterotoxin (CPE)	Purified	No <sup>m</sup>			+ (s)	
EAEC <sup>d</sup>						
EAST 1	Crude <sup>n</sup>	Yes	+			
120-kDa heat-labile toxin	Crude	No	+ (?) <sup>o</sup>		+ (?) <sup>o</sup>	
108-kDa heat-labile toxin	Partially purified	No	+		+	
EHEC						
Shiga-like toxins I and II	Purified	Yes			+	
EIEC <sup>d</sup>						
ShET 2	Purified	Yes	+			
Cytotoxin	Crude	No			+	
EPEC <sup>d,p</sup>	Crude <sup>q</sup>	Yes <sup>q</sup>		+ <sup>p</sup>		
ETEC <sup>d</sup>						
STa	Purified	Yes	+	+		+
STb	Purified	Yes	+ <sup>r</sup>			
LT-I	Purified	Yes	+	+		
LT-II	Purified	Yes	+	+		
Other <i>E. coli</i> toxins						
CLDT	Crude	Yes		+ <sup>j</sup>	+	
CNF 1	Partially purified	Yes		+	+	
CNF 2	Crude	Yes		+	+ (s)	
<i>Plesiomonas shigelloides</i> <sup>d</sup>						
Heat-labile enterotoxin	Crude	No	+	+		
Heat-stable enterotoxin	Crude	No	+			
<i>Salmonella typhi</i>						
LT-like enterotoxin	Crude	Yes <sup>s</sup>	+	+		
<i>Salmonella</i> spp. (non- <i>typhi</i> ) <sup>d</sup>						
Enterotoxin (Stn)	Partially purified	Yes	+	+		
<i>Shigella dysenteriae</i> <sup>d</sup>						
Shiga toxin	Purified	Yes			+ (s)	

Continued on following page

TABLE 3—Continued

Bacterium and toxin	Condition of toxin <sup>b</sup>	Toxin gene cloned?	Type of toxin <sup>c</sup>			
			Enterotoxin	Cytoskeleton altering	Cytotoxin	Neural activity
<i>Other Shigella</i> spp. <sup>d</sup>						
ShET 1 <sup>e</sup>	Purified	Yes	+			
ShET 2 <sup>e</sup>	Purified	Yes	+			
Shiga-like toxin	Crude	No			+	
CLDT	Crude	Yes		+ <sup>j</sup>	+	
<i>Staphylococcus aureus</i>						
Enterotoxins A to E	Purified <sup>h</sup>	Yes <sup>h</sup>	+			+
Delta toxin	Purified	Yes	+		+ <sup>v</sup>	
<i>Vibrio cholerae</i> 01/0139 <sup>d,w</sup>						
CT	Purified	Yes	+	+		+
Zot	Crude	Yes		+		
Ace	Crude	Yes	+			
Hemolysin	Purified	Yes			+ (s)	
<i>Vibrio cholerae</i> non-O1, non-O139 <sup>d,x</sup>						
Hemolysin	Purified	Yes			+ (s)	
ST-like	Purified	Yes	+			
<i>Vibrio fluvialis</i> <sup>y</sup>						
CHO cell cytotoxin	Partially purified	No	+ <sup>y</sup>		+ (s) <sup>y</sup>	
CHO cell-rounding toxin	Crude	No	+ <sup>y</sup>	+	+ (s)(?) <sup>y</sup>	
CHO cell elongation factor	Partially purified	No	+ <sup>y</sup>	+		
<i>Vibrio hollisae</i>						
HT <sup>z</sup>	Purified	No	+			
TDH <sup>aa</sup>	NA	Yes	+			
<i>Vibrio metschnikovii</i>						
Cytolysin	Purified	No			+ (s)	
<i>Vibrio mimicus</i> <sup>bb</sup>						
Hemolysin	NA	No			+	
<i>Vibrio parahaemolyticus</i> <sup>cc</sup>						
TDH	Purified	Yes	+			
<i>Yersinia enterocolitica</i>						
Heat-stable enterotoxin I	Purified	Yes	+			
Heat-stable enterotoxin II	Crude	No	+			

<sup>a</sup> Table 3 categorizes the enteric bacterial toxins on the basis of the range of biologic activities described in experimental intestinal and nonintestinal systems. A question mark indicates that the data are not considered definitive. Adapted in part from reference 490.

<sup>b</sup> Although the toxin may have been purified, not all data for a particular toxin are based on studies with purified toxin. This approach was taken to permit the table to reflect the full range of data available. In instances when activity is based on crude toxin but a putative structural gene has been cloned, it cannot be stated with absolute assurance that the gene encodes the stated toxin activity. "Cloned" indicates that a putative structural gene with estimated toxin size has been identified. NA, not available.

<sup>c</sup> (s), cytotoxin which is secretory in animal intestine and/or Ussing chambers in vitro; (i), alteration of the cytoskeleton or change in morphology in intestinal epithelial cells in vivo or in vitro; (i, s), alteration of the cytoskeleton plus evidence for secretion in intestinal tissue or monolayers of intestinal epithelial cells.

<sup>d</sup> The organism has been tested in volunteers (see Table 5).

<sup>e</sup> Other names for the aerolysin of *A. hydrophila* are beta-hemolysin, cytotoxic enterotoxin, and CT-cross-reactive cytolytic enterotoxin (CTC-cytolysin).

<sup>f</sup> At least four additional nonhemolytic toxins have been described for *Aeromonas* spp. All cause either elongation of CHO cells or rounding of Y-1 adrenal cells; one is cross-reactive with CT antiserum; and three have been demonstrated to stimulate secretion in suckling mice or ligated intestinal segments in rats or rabbits. No intestinal histopathology is available for these toxins.

<sup>g</sup> *Aeromonas* toxins of 15 and 70 kDa have been purified; in contrast, the *Aeromonas* toxin which cross-reacts with anti-CT antiserum has been studied only in the crude form. Although two clones encoding CHO cell elongation activity have been isolated (51, 70), specific structural toxin genes have not been identified.

<sup>h</sup> Only the gene for the B or binding component of the HBL toxin has been cloned (209).

<sup>i</sup> BFT stimulates secretion in rabbit, rat, lamb, and calf ligated small intestinal and/or colonic segments. Histopathology of lamb, rabbit, or rat intestine after exposure to enterotoxigenic *B. fragilis* organisms and/or purified BFT reveals intestinal epithelial cell rounding and detachment from the lamina propria, as well as an inflammatory infiltrate (400, 493). Treatment of intestinal mucosa with high concentrations of BFT sometimes results in hemorrhage (400). However, studies of intestinal epithelial cells in vitro to date have not revealed cytotoxic activity (52, 292). Thus, similar to *C. difficile* toxin A, BFT is best described as a cytoskeleton-altering toxin in vitro and as a secretory cytotoxin in vivo.

<sup>j</sup> Cells display altered morphology for up to three days with lethality (cytotoxicity) occurring later.

<sup>k</sup> In vivo studies with *C. difficile* toxin A demonstrate that secretion is consistently associated with inflammation and damage to the intestinal epithelium. However, in vitro studies with intestinal epithelial cells have not revealed cytotoxic activity with either *C. difficile* toxin A or B. Thus, although commonly called an enterotoxin, this toxin is best described as a cytoskeleton-altering toxin in vitro and as a secretory cytotoxin in vivo.

<sup>l</sup> The alpha toxin of *C. perfringens* type C is also a PLC.

<sup>m</sup> The *C. perfringens* type C CPE gene has not been specifically cloned; by all other available assays, this toxin is thought to be identical to *C. perfringens* type A CPE.

<sup>n</sup> EAST 1 (8–29) has been synthesized and has activity similar to crude EAST 1.

<sup>o</sup> From data obtained with HEp-2 cells (12), the 120-kDa heat-labile toxin of EAEC is postulated to act as an enterotoxin by increasing intracellular calcium levels and/or to act as a cytotoxin on the basis of its cross-reactivity with *E. coli* hemolysin antiserum. The relationship of this 120-kDa toxin to the 108- and 116-kDa proteins identified by Eslava et al. (135) is unknown.

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<sup>7</sup> EPEC strains cause secretory diarrhea in infants and volunteers, but the mechanisms and virulence factors causing diarrhea are incompletely defined. However, development of the AE lesion, which alters the intestinal epithelial cell cytoskeleton, is crucial to disease pathogenesis.

<sup>8</sup> Multiple potential virulence genes have been identified for EPEC. However, the protein products of most of these genes have not yet been purified or characterized.

<sup>9</sup> In detailed studies, alterations in villous cell morphology have been identified in rat intestine after treatment with *E. coli* STb.

<sup>10</sup> Cloned DNA expressing the *S. typhi* LT-like activity has been reported (148), but the structural gene has not been identified.

<sup>11</sup> The gene for ShET1 (*set1*) is rarely found outside of *S. flexneri* 2a. In contrast, the gene for ShET2 has been found in 75% of EIEC strains and 79% of *Shigella* strains examined, including members of all four *Shigella* species.

<sup>12</sup> All the staphylococcal enterotoxins have been purified to homogeneity, and structural genes have been identified. The structural genes can be localized on the chromosome, a plasmid, or on a bacteriophage.

<sup>13</sup> Delayed histologic damage is seen in vivo after treatment of the intestine with delta toxin. However, delta toxin rapidly (within minutes) alters intestinal transport by increasing PD and decreasing resistance in guinea pig ileum studied in Ussing chambers.

<sup>14</sup> O1/O139 refers to the O1 and O139 serogroups of *V. cholerae*. These serogroups form the epidemic strains of *V. cholerae*, which produce CT and produce clinical cholera.

<sup>15</sup> No toxins unique to *V. cholerae* non-O1, non-O139 serogroups have been reported. However, strains of these serogroups may produce one or more toxins that have been previously characterized in *V. cholerae* O1 or other species (218). Most *V. cholerae* non-O1 strains produce a cytotoxic protein apparently identical to the El Tor hemolysin or hemolysin-cytolysin of *V. cholerae* O1 (583). A minority of strains also produce an *E. coli* ST-like enterotoxin (9). A few strains produce CT (83) and also possess genes encoding Ace and Zot toxins of *V. cholerae* O1/O139 (249). Genes encoding a *V. parahaemolyticus* TDH-like toxin have also been found in a few *V. cholerae* non-O1, non-O139 strains (220).

<sup>16</sup> Crude concentrates of each *V. fluvialis* toxin stimulate intestinal secretion in infant mice, but no histopathologic studies are available. In contrast, only the CHO cell-rounding toxin stimulates secretion (sometimes hemorrhage) in ligated rabbit ileal loops. Thus, for example, although the CHO cell cytotoxin is secretory in mice, it is unclear if the in vivo response includes cellular damage.

<sup>17</sup> HT has been reported to stimulate intestinal secretion in suckling mice. However, no intestinal histopathology is yet available for this toxin.

<sup>18</sup> All strains of *V. hollisae* possess genes encoding the TDH enterotoxin of *V. parahaemolyticus* (391), but TDH from *V. hollisae* has not been studied for intestinal activity.

<sup>19</sup> *V. mimicus* produces a cytotoxic protein apparently identical to the El Tor hemolysin or hemolysin-cytolysin of *V. cholerae* O1 (452), but activity of *V. mimicus* hemolysin in an intestinal model has not been shown. A few strains also produce a TDH-like toxin and an ST-like toxin (452, 512).

<sup>20</sup> *V. parahaemolyticus* produces other potential toxins, but data are very limited (see the text).

vitro assays or (ii) the toxin stimulates ion secretion when studied in Ussing chambers.

The second class of enteric toxins is the cytoskeleton-altering toxins. These toxins produce an alteration in cell shape without inducing significant cell injury. When data are available, toxin-induced changes in cell shape have most often been demonstrated to be due to rearrangement of F actin in the affected cells. As delineated in Tables 3 and 4, cytoskeleton-altering toxins may or may not stimulate secretion in vivo and in vitro intestinal models of disease.

The third class of enteric toxins contains the cytotoxins. These toxins produce cellular damage documented by gross findings (e.g., intestinal hemorrhage), light-microscopic evidence of intestinal damage, or studies demonstrating cellular injury. The criteria used to define cellular injury and thus a cytotoxin vary from study to study. Less stringent criteria indicating cell toxicity include cell uptake of trypan blue, cell detachment, and evident loss of cells, for example, from monolayers in tissue culture (presumably by cell lysis). More stringent criteria indicating cell toxicity include measurements of specific cell metabolic processes or identification of changes in plasma membrane function. Examples of these more specific toxicity assays include inhibition of protein synthesis, release of the intracellular enzyme lactic dehydrogenase, and changes in intracellular ion concentrations (i.e., decreases in intracellular potassium levels and increases in intracellular sodium levels). Similar to the cytoskeleton-altering toxins, the activity of enteric cytotoxins may or may not be associated with secretion in vivo or in vitro intestinal models of disease.

Lastly, enteric bacterial toxins may have neural activity. A toxin is classified in this group if available data suggest that at least part of the secretory activity of the toxin is attributable to the release of one or more neurotransmitters from the enteric nervous system or that the toxin alters smooth muscle activity in the intestine. Although several enteric toxins with neural activity have been identified, no enteric toxin has yet been clearly established as stimulating secretion solely through potential neural mechanisms, nor has any enteric toxin been identified to act by binding to and directly altering neuron function.

## FUNCTIONAL CLASSIFICATION OF ENTERIC BACTERIAL TOXINS BY GENUS AND SPECIES

### Introduction

In this section, the various enteric toxins are discussed in detail by genus and species. Table 3 is a compendium of the enteric bacteria and the toxins they produce. These enteric bacterial toxins are categorized by the definitions described in the text above and in Table 2. The data in Table 3 reflect the range of biologic activities described for these toxins in intestinal and nonintestinal experimental systems. Question marks indicate toxins for which the data are too limited to definitively classify the activity of the toxin. In limited instances, toxin-producing bacteria that are not known to play a significant role in human disease or have only a weak association with disease are listed in Table 3. A pertinent example is the C2 toxin produced by *Clostridium botulinum*, whose role in human disease is unknown. Similarly, *Vibrio metschnikovii* and strains of *E. coli* producing the cytolethal distending toxin (CLDT) and the cytotoxic necrotizing factors 1 and 2 (CNF1 and CNF2) are associated with human diarrheal disease only by limited anecdotal reports at present (34, 38, 44, 97, 252, 370). Table 4 specifically lists toxins by bacterial genus and species for which experimental data are available, indicating activity in intestinal models of disease, and it indicates which toxins stimulate an intestinal secretory response. These bacteria have been linked to human illnesses predominantly through epidemiologic studies and, in many cases, volunteer trials (Table 5) (see below).

### *Aeromonas* Species

*Aeromonas* species have been implicated in a variety of intestinal and extraintestinal infections. Although volunteer studies showed that only 2 of 57 subjects experienced diarrhea after ingestion of up to  $10^{10}$  CFU of *Aeromonas hydrophila* (376), there are several epidemiological studies showing an association of *Aeromonas* with diarrheal disease (6, 239). A wide spectrum of gastrointestinal symptoms have been reported with *Aeromonas* infections, but the most common presentation is an acute, self-limited watery diarrhea; dysenteric disease is less commonly seen. There is considerable confusion

TABLE 4. Enteric bacterial toxins with demonstrated activity on native intestine or intestinal epithelial cell lines<sup>a</sup>

Bacterium and toxin	Native intestinal activity <sup>b</sup>	Activity in intestinal epithelial cell lines	Secretion
<i>A. hydrophila</i>			
Aerolysin	Enterotoxin <sup>c</sup>		+
Other toxins	+ <sup>c</sup>		+
<i>B. cereus</i>			
Emetic toxin	+ <sup>d</sup>		
Diarrheal toxin (HBL)	Cytotoxin		+
<i>B. fragilis</i>			
BFT	+ <sup>e</sup>	Cytoskeleton altering	+
<i>C. jejuni</i>			
LT-like	+ <sup>f</sup>		+
Cytotoxin		Cytotoxin	
CLDT	Cytotoxin		+
<i>C. botulinum</i> C <sub>2</sub> toxin	Cytotoxin		+
<i>C. difficile</i>			
Toxin A	Cytotoxin	Cytoskeleton altering	+
Toxin B	Cytotoxin <sup>g</sup>	Cytoskeleton altering	-
<i>C. perfringens</i> type A			
CPE	Cytotoxin		+
<i>C. perfringens</i> type C			
Beta toxin	Cytotoxin		
Alpha toxin (PLC)	+ <sup>h</sup>		+ <sup>h</sup>
CPE	Cytotoxin		+
EAEC			
EAST 1	Enterotoxin		+
108-kDa heat-labile toxin	Enterotoxin <sup>i</sup>		+
EHEC			
Shiga-like toxins I and II	Cytotoxins		+
EIEC			
ShET 2	Enterotoxin		+
ETEC			
STa	Enterotoxin, neural activity	Enterotoxin, cytoskeleton altering	+
STb	Enterotoxin <sup>j</sup>		+
LT-I	Enterotoxin	Cytoskeleton altering <sup>k</sup>	+
LT-II	+ <sup>l</sup>	Cytoskeleton altering <sup>k</sup>	+
<i>E. coli</i>			
CLDT	- <sup>m</sup>		
CNF1	- <sup>m</sup>		-
CNF2	+ <sup>n</sup>		+
<i>P. shigelloides</i>			
Heat-labile enterotoxin	+ <sup>o</sup>		+
Heat-stable enterotoxin	+ <sup>o</sup>		+
<i>S. typhi</i>			
LT-like enterotoxin	+ <sup>n</sup>		+
<i>Salmonella</i> spp. (non-typhi)			
Enterotoxin (Stn)	Enterotoxin <sup>i</sup>	Cytoskeleton altering <sup>k</sup>	+
<i>S. dysenteriae</i>			
Shiga toxin	Cytotoxin		+
Other <i>Shigella</i> spp.			
ShET1	Enterotoxin		+
ShET2	+ <sup>n</sup>		+
<i>S. aureus</i>			
Enterotoxins A-E	+ <sup>d,p</sup>		+
Delta toxin	Enterotoxin <sup>q</sup>		+

Continued on following page



TABLE 4—Continued

Bacterium and toxin	Native intestinal activity <sup>b</sup>	Activity in intestinal epithelial cell lines	Secretion
<i>V. cholerae</i> O1/O139			
CT	Enterotoxin	Cytoskeleton altering <sup>f</sup>	+
Ace	Enterotoxin		+
Zot	Cytoskeleton altering	Cytoskeleton altering	
Hemolysin	Cytotoxin		+
<i>V. cholerae</i> non-O1, non-O139			
ST-like	+ <sup>n</sup>		+
Hemolysin	Cytotoxin		+
<i>V. fluvialis</i>			
CHO cell cytotoxin	+ <sup>n</sup>		+
CHO cell rounding toxin	+ <sup>n</sup>		+
CHO cell elongation factor	+ <sup>n</sup>		+
<i>V. hollisae</i>			
HT	+ <sup>n</sup>		+
<i>V. metschnikovii</i>			
Cytolysin	+ <sup>n</sup>		+
<i>V. parahaemolyticus</i>			
TDH	Enterotoxin		+
<i>Y. enterocolitica</i>			
Heat-stable enterotoxin I	+ <sup>n</sup>		+
Heat-stable enterotoxin II	+ <sup>n</sup>		+

<sup>a</sup> Adapted in part from reference 490.

<sup>b</sup> Where possible, the activity exhibited in native intestine is categorized by using the definitions of enteric toxins in Table 2. Each + is accompanied by a footnote; the reader should refer to the section of text on specific bacteria for additional details.

<sup>c</sup> The aerolysin of *A. hydrophila* is cytotoxic to nonintestinal cells but causes secretion without histologic changes in perfused rat jejunum (368, 463). Data on intestinal secretory activity exist for three of the four reported toxins of *A. hydrophila*, but no intestinal histology is available.

<sup>d</sup> Toxin elicits vomiting in monkeys.

<sup>e</sup> Histologic examination of lamb, rabbit, or rat intestine exposed to ETBF strains or purified BFT has revealed rounded intestinal epithelial cells with detachment of some cells, as well as an inflammatory infiltrate (400, 493). Treatment of intestinal mucosa with high concentrations of purified BFT sometimes results in hemorrhage (400).

<sup>f</sup> Culture supernatants of *C. jejuni* have elicited hemorrhagic fluid in rat ileal loops, which was neutralized with cholera antitoxin (468). Rabbit ileal loop and suckling mice assays were negative in this study. Because these assays were done only with culture supernatants, it is unclear whether the secretory activity observed was due to the heat-labile enterotoxin, the heat-labile cytotoxin, the heat-labile cytolethal distending toxin, and/or another toxin potentially produced by *C. jejuni* strains.

<sup>g</sup> Toxin B alone is inactive on animal intestine in vivo. However, recent studies examining the activity of toxin B on human colonic tissue in vitro demonstrated focal epithelial cell damage, altered F-actin staining, and a loss of epithelial resistance (458).

<sup>h</sup> The alpha toxin (PLC) of *C. perfringens* type C has been reported to increase  $I_{sc}$  in Ussing chambers only when added to the serosal side of rat colonic mucosa. Histologic results are not available.

<sup>i</sup> The partially purified 108-kDa heat-labile toxin produced by EAEC stimulates an inflammatory response in ligated rat intestinal loops in vivo and an increase in  $I_{sc}$  and PD (without a change in  $R$ ) in rat intestinal tissue examined in Ussing chambers in vitro.

<sup>j</sup> Mild histologic changes have been reported.

<sup>k</sup> Intestinal epithelial cell chloride secretion stimulated by increases in intracellular cyclic AMP concentrations has been shown to be dependent, in part, on rearrangement of F actin (342, 344, 496). Thus, by analogy, it is postulated that enteric bacterial toxins which increase cyclic AMP levels may also be cytoskeleton-altering toxins in intestinal epithelial cells.

<sup>l</sup> *E. coli* LT-II, unlike *E. coli* LT-I, does not stimulate secretion in rabbit ileal segments. However, *E. coli* LT-II does stimulate secretion in the sealed adult mouse model. Histologic studies of intestinal tissue are not available.

<sup>m</sup> These toxins did not yield secretion when tested in rabbit ileal segments.

<sup>n</sup> Results of histologic examination of intestinal tissue have not been reported.

<sup>o</sup> Culture filtrates of *P. shigelloides* may contain heat-labile or heat-stable secretory factors detectable in rabbit ileal loops, which do not alter the histology of the intestine (340, 476).

<sup>p</sup> The staphylococcal enterotoxins are also potent T-cell activators. In the stomach and/or small bowel of humans and monkeys, these toxins stimulate an influx of PMNs associated with short-lived histologic damage.

<sup>q</sup> Delta toxin stimulates a rapid PD change in intestinal tissue with delayed damage by histopathology.

in the literature about enterotoxins produced by *Aeromonas* species. Part of the confusion results from the changing taxonomy of this genus, in which strains that were previously grouped in only one species, *A. hydrophila*, can now be subdivided into 15 species on the basis of DNA hybridization data. More commonly, however, a simpler scheme of three species, *A. sobria*, *A. caviae*, and *A. hydrophila*, is employed (239). Confusion also results from the different toxin purification and assay methods used by the several different groups working in this field. No definitive mechanisms of secretion have been

identified for any of the *Aeromonas* toxins, but limited suggestive evidence is available for some of the candidate toxins.

**Aerolysin.** A heat-labile 50- to 52-kDa protein known as aerolysin, cytotoxic enterotoxin, beta-hemolysin, or CT-cross-reactive cytolytic enterotoxin has been purified and shown to have enterotoxin activity (10, 222, 462). This toxin (to be called aerolysin in this review) lyses a wide variety of nonintestinal cells, including CHO cells and rabbit erythrocytes, and kills mice within 2 min after intravenous injection (10, 463). The purified toxin causes fluid accumulation in rabbit ligated ileal

TABLE 5. Enteric bacteria and toxins studied in volunteers<sup>a</sup>

Bacterium or toxin	Inoculum <sup>b</sup> (CFU)	Outcome	Reference
<b>Bacteria</b>			
<i>Vibrio cholerae</i> <sup>c</sup>			
O1 (CT <sup>+</sup> )	10 <sup>3*</sup> (10 <sup>3</sup> )	Diarrhea	307
O139	10 <sup>4*</sup> (10 <sup>4</sup> )	Diarrhea	378
Non-O1/O139 (ST <sup>+</sup> )	10 <sup>6</sup> (10 <sup>6</sup> )	Diarrhea	379
Non-O1/O139 (ST <sup>-</sup> )	10 <sup>9</sup>	No diarrhea	379
<i>Shigella dysenteriae</i>	10 <sup>1*</sup> (2 × 10 <sup>2</sup> )	Diarrhea/dysentery	126
<i>S. flexneri</i>	10 <sup>2*</sup> (10 <sup>4</sup> )	Diarrhea/dysentery	126
<i>S. sonnei</i>	5 × 10 <sup>2*</sup> (5 × 10 <sup>2</sup> )	Diarrhea/dysentery	126
EPEC	10 <sup>6*</sup> (10 <sup>6</sup> )	Diarrhea	311
EPEC	10 <sup>6*</sup> (10 <sup>10</sup> )	Diarrhea	305
EIEC	10 <sup>8</sup> (10 <sup>8</sup> )	Diarrhea/dysentery	125
EAEC	7 × 10 <sup>8</sup> (-)	Diarrhea	337
Diffusely adherent <i>E. coli</i>	10 <sup>10</sup>	No diarrhea	522
<i>Campylobacter jejuni</i>	8 × 10 <sup>2*</sup> (10 <sup>8</sup> )	Diarrhea	33
<i>Vibrio parahaemolyticus</i>	3 × 10 <sup>7</sup> (10 <sup>8</sup> )	Diarrhea	475
<i>Salmonella</i> spp. (nontyphoidal) <sup>d</sup>	10 <sup>5</sup> (10 <sup>7</sup> )	Diarrhea	35
<i>Aeromonas hydrophila</i> <sup>e</sup>	10 <sup>10</sup>	No diarrhea	376
<i>Plesiomonas shigelloides</i>	4 × 10 <sup>9</sup>	No diarrhea	211
<b>Toxins</b>			
CT	2.5 µg	No diarrhea	308
	5.0 µg	Diarrhea in 4 of 5 subjects, mean stool volume of 2.5 liters	308
	25 µg	Diarrhea in 2 of 2 subjects, mean stool volume of 21.9 liters	308
<i>C. perfringens</i> enterotoxin (CPE)	6 mg	No diarrhea	506
	8 mg	Diarrhea in 1 of 2 subjects	506
	10 mg	Diarrhea in 1 of 1 subjects	506
	12 mg	Diarrhea in 2 of 2 subjects	506

<sup>a</sup> Adapted in part from reference 490.

<sup>b</sup> The lowest inoculum giving diarrhea in one or more subjects is shown. \*, this inoculum was the lowest one studied. Inocula in parentheses show the lowest dose causing diarrhea in 50% or more of subjects receiving that inoculum; (-) indicates that no inoculum tested gave diarrhea in 50% or more of subjects. In most but not all studies, sodium bicarbonate was administered prior to ingestion of the inoculum to neutralize stomach acidity.

<sup>c</sup> Naturally occurring *V. cholerae* O1 strains lacking both CT and the TCP intestinal colonization factor have been fed to volunteers and did not cause diarrhea at inocula of 10<sup>8</sup> (306). Genetically engineered *V. cholerae* O1 strains deleted of CT have caused diarrhea in volunteers when administered at inocula of 10<sup>4</sup> (310). *V. cholerae* non-O1/O139 indicates those strains belonging to serogroups other than O1 or O139; one such strain expressed a toxin similar to *E. coli* ST.

<sup>d</sup> The lowest dose capable of causing diarrhea in volunteers ranges from 10<sup>5</sup> to 10<sup>10</sup>, depending on the strain and the vehicle. An extensive review of outbreak reports suggests that the actual minimum dose necessary for infection is <10<sup>3</sup> (reviewed in reference 35).

<sup>e</sup> Ingestion of up to 10<sup>10</sup> *A. hydrophila* cells resulted in diarrhea in only 2 of 57 subjects (376).

loops and infant mouse intestines (10, 463). Studies by Chopra et al. (67) demonstrated that the product of the cloned aerolysin gene has all the biological activities described above. Although the toxin has been reported to cross-react with antibodies against CT, there is no obvious sequence homology between the two toxins and antiserum against CT does not neutralize the cytotoxic or hemolytic activities of aerolysin (463). This toxin has been purified and studied from strains identified as *A. hydrophila* (10) and *A. sobria* (187), and genetic studies indicate that the *aerA* gene encoding the structural subunit is present in all environmental and clinical *Aeromonas* isolates tested (229). However, the *aerA* gene of one species, *A. trota*, is genetically distinct from that of other *Aeromonas* species and shares only 77% nucleotide homology with *aerA* from *A. hydrophila* (10), a level resembling the 80% homology observed between the genes encoding CT and ETEC LT-I (262).

The mode of action of aerolysin has been extensively studied by using primarily erythrocytes rather than intestinal cells. The translocation of the inactive proaerolysin across the bacterial outer membrane, the binding of the inactive toxin to the erythrocytic receptor glycoprotein, and toxin activation by proteolytic cleavage at the C terminus by bacterial or host proteases have recently been reviewed (557). Planar lipid bilayer studies have shown that oligomers of the toxin form a transmembrane channel which is slightly anion selective and which shares structural and functional characteristics with other channel-forming proteins such as *Staphylococcus aureus* alpha-toxin and the C9

complement component (557). However, the data for intestinal cells are fragmentary. Cytotoxicity for nonerythrocytic cells such as CHO cells has been demonstrated by chromium release techniques (463), but histological studies with purified aerolysin in rat jejunum showed no striking pathological change (368). One study indicated that antiserum against this cytotoxin neutralized secretion in rabbit ileal loops stimulated by *A. hydrophila* culture filtrates (515), and another study of 147 *Aeromonas* strains reported that only production of aerolysin correlated with the ability to cause fluid accumulation in rabbit ileal loops (501). Culture filtrates of *A. sobria* produce enterotoxic activity in a rat jejunal perfusion system that was neutralized by monoclonal antibody against aerolysin (368). In this system, the culture filtrates induced net water, potassium, and sodium loss with a rapid onset (less than 5 min) that was readily differentiated from the effects of CT. Thus, while the ability of this toxin to form an ion channel presents a potential mode of action in the intestine, there are no direct data reporting the ability of aerolysin to form ion channels in intestinal cells and no data linking this property to secretory diarrhea.

**Other *Aeromonas* toxins.** Although aerolysin appears to account for most of the secretory activity found in culture filtrates of *Aeromonas* species, at least four other noncytotoxic factors have been described that also have secretory activity (222). These toxins cause elongation of CHO cells and/or rounding of Y-1 adrenal cells but are not cytotoxic. Thus, they would be termed cytoskeleton-altering toxins by our classifica-

tion. Results of intestinal histopathology have not been reported for these four noncytotoxic factors. Ljungh et al. (319) described one toxin of 15 kDa that was heat labile (60°C for 20 min). This partially purified toxin caused fluid accumulation in rabbit ileal loops, rounding of Y-1 cells, and increased levels of cyclic AMP in Y-1 and rabbit intestinal epithelial cells. A toxin of similar size and activities was recently purified from *A. sobria* (187). A distinctly different cytoskeleton-altering toxin has been purified by Chopra et al. (66, 71). This toxin is ca. 44 kDa in size and heat labile (56°C for 20 min) and causes fluid accumulation in rabbit ileal loops. This toxin also causes elongation of CHO cells and increases cyclic AMP and prostaglandin E<sub>2</sub> levels in CHO cells. Another toxin which also elongates CHO cells and stimulates secretion in infant mice but is heat stable and has a molecular mass of 70 kDa has recently been purified (347). Although this toxin elongates CHO cells, no increase in cellular cyclic AMP, cyclic GMP or PGE<sub>2</sub> levels is detectable in these cells. In addition to these three toxins, which do not react with antisera against CT, another heat-stable toxin capable of causing rounding of Y-1 cells and fluid accumulation in infant mice and rat ileal loops has been isolated from *A. sobria*, *A. hydrophila*, and *A. caviae* on the basis of cross-reactivity with anti-CT antiserum (442). Significant work remains in characterizing these toxins and establishing the contribution of each toxin in secretory diarrhea due to *Aeromonas* species. One approach which would greatly aid this investigation is the construction of isogenic strains specifically mutated in genes encoding each of these toxins. Chakraborty et al. (51) and Chopra et al. (70) have reported the isolation from *A. hydrophila* of multiple clones that encode CHO cell elongation activity. The former study reports a single clone encoding a toxin of unknown size and heat stability that induces fluid accumulation in rabbit ileal loops and suckling mice. The latter study reports two genetically distinct clones, one encoding a heat-labile toxin of ca. 35 kDa and the other encoding a heat-stable toxin of unknown size that caused marginal fluid secretion in the rat ileal loop assay. However, the relationship of these different cloned gene products to the toxins previously described is not clear.

#### *Bacillus cereus*

*Bacillus cereus* is associated with separate emetic and diarrheal food poisoning syndromes, which are thought to be caused by distinct toxins produced by the organism (525, 545, 546). The emetic syndrome is strongly (but not exclusively) associated with ingestion of rice. The conditions necessary for production of the emetic toxin by *B. cereus* are poorly defined. The emetic toxin (molecular mass, <10 kDa) survives autoclaving, pH extremes, and protease treatment, making it unlikely that this toxin is a protein (545, 546). A crude preparation of emetic toxin stimulates emesis in approximately 50% of monkeys inoculated (545). Although some *B. cereus* strains may produce both the emetic and diarrheal toxins, there is no consistent relationship between these two factors. The diarrheal toxin appears to be a heat-labile, trypsin-sensitive protein complex with three components (designated B, L<sub>1</sub>, and L<sub>2</sub>, for proposed binding and cell lysis properties [23–26]), all with estimated molecular masses between 34 and 45 kDa (24, 26, 209, 533, 545). Hence, the toxin has been called hemolysin BL or HBL (23, 24, 26). Several biological activities have been attributed to HBL, including secretion and necrosis in rabbit ligated ileal segments, a vascular permeability reaction in guinea pig and rabbit skin, cytotoxicity to Vero and CHO cells, and death of mice (24, 26, 545). In particular, the response in rabbit ileal segments is highly correlated with the vascular

permeability and cytotoxicity assays. Although limited studies suggested that the intestinal secretory activity might be due to only one of the three proteins (188, 497), recent data examining the biologic activity of highly purified component proteins indicate that all three proteins are necessary to produce hemolysis, increased vascular permeability, and secretion in ligated rabbit intestinal loops (25). The cellular mechanism of action of HBL is unknown, and only inconclusive data on the role of cyclic AMP in the secretion observed in rabbit intestine have been reported (544, 545). Of note, although HBL is frequently referred to as an enterotoxin in the literature, it stimulates secretion and villus necrosis in ligated rabbit intestinal loops, indicating that it is, in fact, a secretory cytotoxin. The necrotic intestinal response in animals is at odds with the observation that this organism appears to stimulate secretory diarrhea (not inflammatory diarrhea) in humans.

#### Enterotoxigenic *Bacteroides fragilis*

ETBF strains have been associated with watery diarrhea in children between the ages of 1 and 5 years in Bangladesh and in the United States (470, 472, 474; reviewed in reference 493). These strains secrete a heat-labile protein toxin of ca. 20 kDa called *B. fragilis* toxin (BFT) (381, 558). A preliminary report of a partial sequence of the toxin gene indicates homology to a family of bacterial proteins known as zinc metalloproteases (281, 372). Although crude toxin has been reported to stimulate secretion in lamb and calf ligated intestinal segments but not in rabbit ligated ileal loops (381, 382), purified toxin stimulates proteinaceous secretion which is sometimes hemorrhagic in ligated rat, rabbit, and lamb ileal or colonic intestinal segments (400, 559). Secretion stimulated by purified BFT is accompanied by histopathologic changes including exfoliation and necrosis of villus epithelial cells with villus shortening and crypt elongation associated with the presence of increased numbers of neutrophils in the lamina propria (400). Preliminary data in experiments in lamb intestine suggest that epithelial cell changes precede the detection of fluid secretion (400). These findings are similar to the histopathology of infant and adult rabbit, lamb, and gnotobiotic piglet intestine after infection with toxigenic strains of *B. fragilis* (reviewed in reference 493), suggesting a role for BFT in the pathogenesis of disease. Crude and purified toxins also alter the morphology of intestinal epithelial cells (HT29/C1, Caco-2, and T84) with F-actin rearrangement demonstrated by phalloidin staining (52, 292, 569). Although application of BFT to the basolateral cell membrane stimulates a self-limited increase in short circuit current ( $I_{sc}$ ) in T84 monolayers mounted in Ussing chambers, the predominant response to this toxin in vitro is diminished monolayer resistance without evidence of cellular toxicity by viability assays (52). It is postulated that BFT is a virulence factor of ETBF strains, potentially contributing to diarrhea by altering the barrier function of the intestinal epithelium and by stimulating chloride secretion (493). In animal models in vivo, BFT (similar to *C. difficile* toxin A [see below]) also appears to recruit an inflammatory response that probably contributes to the intestinal damage and proteinaceous secretion observed (400). Thus, at present, BFT is best described as a cytoskeleton-altering toxin (which is potentially secretory) on the basis of studies of intestinal epithelial cells in vitro (52, 292, 569) and as a secretory cytotoxin on the basis of studies of animal intestinal responses (400). Similar to the discrepancy in human and experimental data described for the HBL toxin of *B. cereus*, ETBF strains have been associated largely with watery diarrhea in young children (382, 470, 472, 474). However, because rapid diagnosis of ETBF infections is not yet possible,

results of studies carefully examining children infected with ETBF for an intestinal inflammatory response are not available.

### *Campylobacter jejuni*

*Campylobacter jejuni* causes enteric disease ranging from watery diarrhea to dysentery. In addition to the invasive potential of some strains, three major toxins have been reported to be produced by at least some *C. jejuni* strains associated with diarrheal disease. However, consistent associations between one or more of these toxins and clinical disease are lacking (37, 137, 157, 284, 285). Similarly, available data on human antibody responses to either the heat-labile enterotoxin or the cytotoxin are negative or variable (219, 420). None of the toxin genes have been identified, and only the heat-labile enterotoxin has been even partially purified (88). The vast majority of studies have examined culture supernatants of *C. jejuni* strains rather than pure toxin preparations for toxin activity.

***C. jejuni* heat-labile enterotoxin (LT-like).** The first and the most often reported *C. jejuni* toxin is a 60- to 70-kDa iron-regulated heat-labile enterotoxin that stimulates secretion in rat and, in some cases, rabbit ileal segments, increases rabbit skin permeability, and causes elongation and rounding, respectively, of CHO and Y-1 adrenal cells (88, 147, 282, 348, 468, 564). Intestinal histopathology has not been reported, but, in one paper, *C. jejuni* culture supernatants yielded hemorrhagic fluid in rat ileal loops, a response that was inhibited by cholera antitoxin (468). Whether this hemorrhagic secretion in the rat was due to the heat-labile enterotoxin, the cytotoxin, the *C. jejuni* CLDT, and/or another toxin produced by these strains cannot be discerned from the available data. Thus, this toxin is presently classified as an enterotoxin with cytoskeleton-altering properties on the basis of its ability to alter the shape of CHO and Y-1 adrenal cells. Crude *C. jejuni* heat-labile enterotoxin also increases cyclic AMP levels in tissue culture cells, binds GM<sub>1</sub> ganglioside, and is neutralized by anti-CT antibody (250, 282, 468, 564). All of these biologic activities are reminiscent of CT or *E. coli* LT (see below for discussions of these toxins). However, homology between genes encoding CT or *E. coli* LT and *C. jejuni* DNA, as determined by DNA hybridization studies, appears to be limited to the B subunit of the *C. jejuni* enterotoxin (42, 283).

**Other toxins.** *C. jejuni* strains have also been reported to produce a heat-labile protein cytotoxin that is not neutralized by anti-CT, anti-Shiga, or anti-*Clostridium difficile* antisera (198, 250, 333). This cytotoxin is lethal for several cell types including an intestinal cell line (Int 407), but its role in disease is unknown (420). In addition, it was not secretory in 6- or 18-h rabbit ileal loops or in suckling mice (198). A third potential toxin is a CLDT-like factor that has been identified in some *C. jejuni* strains (253). In vitro, the *C. jejuni* CLDT causes progressive cell distension and eventually death. This CLDT is reported to cause a hemorrhagic response in rat ligated intestinal segments in vivo but is negative in rabbit ligated ileal segments, suckling mice, and the rabbit skin permeability test. The role of the *C. jejuni* CLDT in disease is unknown. A fourth potential toxin is a Shiga-like toxin. Low levels of a cell-associated cytotoxic factor that is neutralized by anti-Shiga toxin serum have been reported from some *C. jejuni* strains (374). However, no genetic homology has been found between the *E. coli* Shiga-like toxin genes and *C. jejuni*. Lastly, cell-free filtrates of *C. jejuni* have been reported to contain a heat-stable substance that alters intestinal myoelectric activity in rabbit ileum, but no further characterization of this factor is available (508).

**Other potential secretory mechanisms.** Although the mechanisms for secretion in *C. jejuni* disease are unclear, rabbit ligated ileal segments infected with *C. jejuni* strains (with or without the ability to secrete the heat-labile enterotoxin) reveal inflammatory infiltrates and villous damage in association with fluid accumulation (138). Analysis of loop fluids and ileal tissues from these animals revealed elevated levels of cyclic AMP, PGE<sub>2</sub>, and leukotriene B<sub>4</sub>, suggesting that mediators released secondary to the inflammation caused by *C. jejuni* infection may be crucial for secretion in this disease (136). Indeed, antibodies against PGE<sub>2</sub> inhibited cyclic AMP elevation in Caco-2 cells treated with the secreted fluid from ligated intestinal loops infected with *C. jejuni* (136). In addition, loop fluids from these animals did not contain detectable heat-labile *C. jejuni* enterotoxin, suggesting that production of this toxin was not important in the secretion and tissue damage observed in this model.

### *Clostridium botulinum*

Although *C. botulinum* is not a primary enteric pathogen, some strains of *C. botulinum* types C and D produce a unique two-component (binary) toxin, the C2 toxin, that is secretory in mouse intestinal loops after a lag period of 2 h (405). This toxin differs from other *C. botulinum* toxins in two regards. First, the components of the C2 toxin that mediate binding to cells (component II, 105 kDa) and biologic activity (component I, 55 kDa) are not covalently or noncovalently linked, as are the toxins produced by *C. botulinum* types A through F; and second, the C2 toxin is not a neurotoxin (406). The C2 toxin is a member of a family of clostridial bacterial toxins that act by ADP-ribosylation of eukaryotic cell actin (1, 257). Notably, the C2 toxin exhibits substrate specificity. This toxin ADP-ribosylates β/γ-nonmuscle cytoplasmic and γ-smooth muscle G (monomeric) actin, but not α-muscle actins, at Arg-177, leading to loss of the ATPase activity of actin (345, 556). Interestingly, because the α-muscle and γ-smooth muscle actin isoforms differ only in three amino acids located at the N terminus, this region of the actin monomer may be crucial to defining the substrate specificity of this toxin (345). ADP-ribosylation of monomeric actin effectively traps the molecule in its unpolymerized form and, over time, results in the depolymerization of the cellular microfilament network. This results in cell rounding after about 1 h and lysis after 24 to 48 h (407). Consistent with these observations, the C2 toxin causes degenerative and necrotic changes over time in the mouse intestinal mucosa. The onset of the morphologic changes precedes the detection of fluid secretion, suggesting that the intestinal secretion results from the epithelial cell damage (408).

### *Clostridium difficile*

Strains of *C. difficile* associated with antibiotic-associated diarrhea or, in its most severe clinical expression, pseudomembranous colitis produce two toxins, enterotoxin (toxin A) and cytotoxin (toxin B). Both toxin genes have been cloned and sequenced, revealing that they encode proteins with estimated molecular masses of 308 kDa for toxin A and 270 kDa for toxin B (19, 119, 427; reviewed in reference 581). Of interest, nearly one-third of the carboxy terminus of the toxin A gene consists of a series of repeating units which appear to encode the portion of the toxin molecule that binds to its glycoprotein receptor (119, 562). The carboxy terminus of the toxin B gene also encodes repeating units related to those identified in the toxin A gene (581). Because toxins A and B are cytotoxic or alter the cytoskeleton of many cell types, numerous studies have used these toxins to study alterations in the biology and



biochemistry of nonintestinal cells (see references 156, 326, and 435 for reviews). The following discussion, however, will focus on data available from intestinal disease models or intestinal epithelial cell lines with partially purified or purified preparations of toxins A or B. Figure 2 summarizes the complexity of the biologic activities of toxin A, which are likely to contribute to the stimulation of intestinal secretion as detailed below.

The bulk of data from *in vivo* animal intestinal disease models indicates that toxin A is of primary importance in disease pathogenesis. Toxin A binds to a G-protein-coupled glycoprotein or to sucrase-isomaltase in rabbit ileal brush border membranes (438, 440). However, the relationship between these two potential receptors and whether these receptors are functional at the primary site of disease, the colon, are presently unclear. Similar to the age-specific clinical presentation of *C. difficile* intestinal disease (which increases with age, despite asymptomatic colonization with toxigenic strains in neonates), newborn rabbits lack brush border receptors for toxin A (326). Although often described as an enterotoxin, toxin A causes rounding of many nonintestinal cell types, which usually precedes evidence of cell membrane injury and is lethal for mice (156, 543). In addition, *in vivo* studies indicate that toxin A induces hemorrhagic fluid secretion and markedly damages, even erodes, ileal and colonic epithelium (315, 316, 327, 328, 369, 539, 540). In guinea pig and/or rabbit ileal tissues mounted in Ussing chambers, direct addition of toxin A (i.e., in the absence of recruited PMNs and blood flow) is reported to stimulate chloride secretion, increase intestinal epithelial cell permeability, and alter epithelial cell structure (369, 375). The mechanism by which toxin A stimulates secretion is unlikely to involve an increase in intracellular calcium levels because, although very high concentrations of toxin A increase intracellular calcium levels in PMNs (441), increased calcium levels in intestinal epithelial cells have not been identified and inhibitors of calcium or calmodulin do not modify the morphologic response of rat intestinal crypt cells to toxin A (154, 492). Interestingly, however, toxin A treatment of CHO cells transfected with sucrase-isomaltase (a potential toxin A intestinal receptor) stimulated an increase in intracellular calcium levels (438).

In contrast, in monolayers of human colonic epithelial cells (T84 cells) studied *in vitro*, toxin A does not increase *I<sub>sc</sub>* but markedly diminishes monolayer resistance over time without evidence of cellular damage (207). After toxin A treatment, staining of F actin at the tight junctional ring is strikingly diminished in T84 cells with no disruption of the tight junctions being evident by transmission electron microscopy (207). The mechanism by which toxin A disrupts F actin in intestinal epithelial cells has not been specifically investigated. However, in several nonepithelial cell lines, toxin A leads to covalent modification of Rho, small GTP-binding proteins involved in the regulation of actin filament assembly. The covalent modification of Rho precedes the toxin-induced loss of F actin in these cell lines and prevents its ADP-ribosylation by the *C. botulinum* toxin (exoenzyme) C3 (105, 182, 255). Recent work indicates that in intact nonintestinal cells, toxin A catalyzes the O-glucosylation of Rho subtype proteins at threonine 37, thereby functionally inactivating Rho and resulting in depolymerization of actin filaments (258).

The contrast between the *in vivo* and *in vitro* intestinal epithelial cell responses to toxin A indicates that in *in vivo* experimental systems containing both intestinal epithelial cells and submucosal elements, cytotoxic effects in response to toxin A are observed. This cytotoxic activity of toxin A is probably explained by several observations, indicating the importance of

nonepithelial cell responses, including neutrophil migration, in the pathophysiology of this disease. For example, toxin A may stimulate neutrophil recruitment *in vivo*, possibly through a G-protein-linked neutrophil toxin A receptor (270). Binding of toxin A to a neutrophil receptor would most probably require endocytosis of this large protein by the intestinal epithelial cell with delivery to the submucosal compartment. Consistent with this potential mechanism, data from nonintestinal cells indicate that both toxins A and B are internalized via receptor-mediated endocytosis (158, 210, 529). Alternatively, toxin A can cause injury to the intestinal epithelium (369, 375, 458), potentially allowing toxin A to gain access to the subepithelial compartment, where it could activate inflammatory cells and amplify its toxic effects (270, 369, 375). The importance of neutrophil migration in the secretory response and extensive epithelial damage after toxin A treatment is shown by several lines of evidence. First, pretreatment of rabbits subsequently exposed to toxin A with a monoclonal antibody blocking the leukocytic adhesion molecule CD18 results in inhibition of fluid secretion (ca. 65%), mannitol permeability (ca. 65%), and histologic damage (only residual focal enterocyte damage at some villus tips) (270). Second, treatment of the intact intestinal epithelium with toxin A stimulates an inflammatory response with release of numerous inflammatory mediators (e.g., leukotrienes, PGE<sub>2</sub>, PAF, and histamine) which are potentially secretory and/or may modify intestinal permeability (439, 540). Similarly, PLA<sub>2</sub>, cyclooxygenase, or PAF antagonists or a mast cell stabilizer, ketotifen, inhibits the secretory response to and histological damage caused by toxin A in ligated rabbit or rat ileal segments (141, 160, 439). Both the epithelium and intestinal submucosal compartment (e.g., neutrophils) could contribute to this release of inflammatory mediators. Third, the inflammatory and secretory effects of toxin A are reduced in rats by neuronal antagonists including blockers of afferent sensory neurons and ganglia (50) and an antagonist of the peptide substance P (436). Substance P is a transmitter of submucosal afferent sensory neurons known to participate in inflammatory responses. In addition, toxin A treatment of rat ileum upregulates substance P epithelial cell receptors within 15 min and stimulates new synthesis of substance P in the intestinal mucosa within 3 h (437). These studies suggest rapid (within 15 min) activation of mucosal mast cells and neuronal circuits contributing to the inflammatory response, as well as changes in intestinal epithelial cells, which potentially contribute to the secretory response to toxin A. Lastly, recent data suggest that toxin A activates the transcriptional factor NF- $\kappa$ B in rat intestinal epithelial cells *in vitro* by 1 h, stimulating the cells to produce IL-1, IL-8, and PGE<sub>2</sub> (214). This response was blocked by pertussis toxin, consistent with signaling dependent on toxin A binding to a G-protein-coupled receptor. These data provide the first link between membrane events and the transcriptional activation of inflammatory mediators by toxin A. Together, these data indicate that toxin A stimulates secretion and inflammation most probably by direct receptor-mediated effects on intestinal epithelial cells and indirect effects via activation of submucosal immune effector cells and the enteric nervous system. Furthermore, the Ussing chamber experiments cited above (369, 375) indicate that *in vivo* recruitment of neutrophils is not essential for all of the pathophysiologic response to toxin A. Thus, although toxin A is commonly referred to as an enterotoxin, by our definitions this toxin acts as a secretory cytotoxin *in vivo* and as a nonlethal cytoskeleton-altering toxin on intestinal epithelial cell monolayers *in vitro*.

In contrast to the extensive animal data available for toxin A, until recently (see below) only very limited data linked toxin B to the pathogenesis of animal intestinal disease due to *C.*

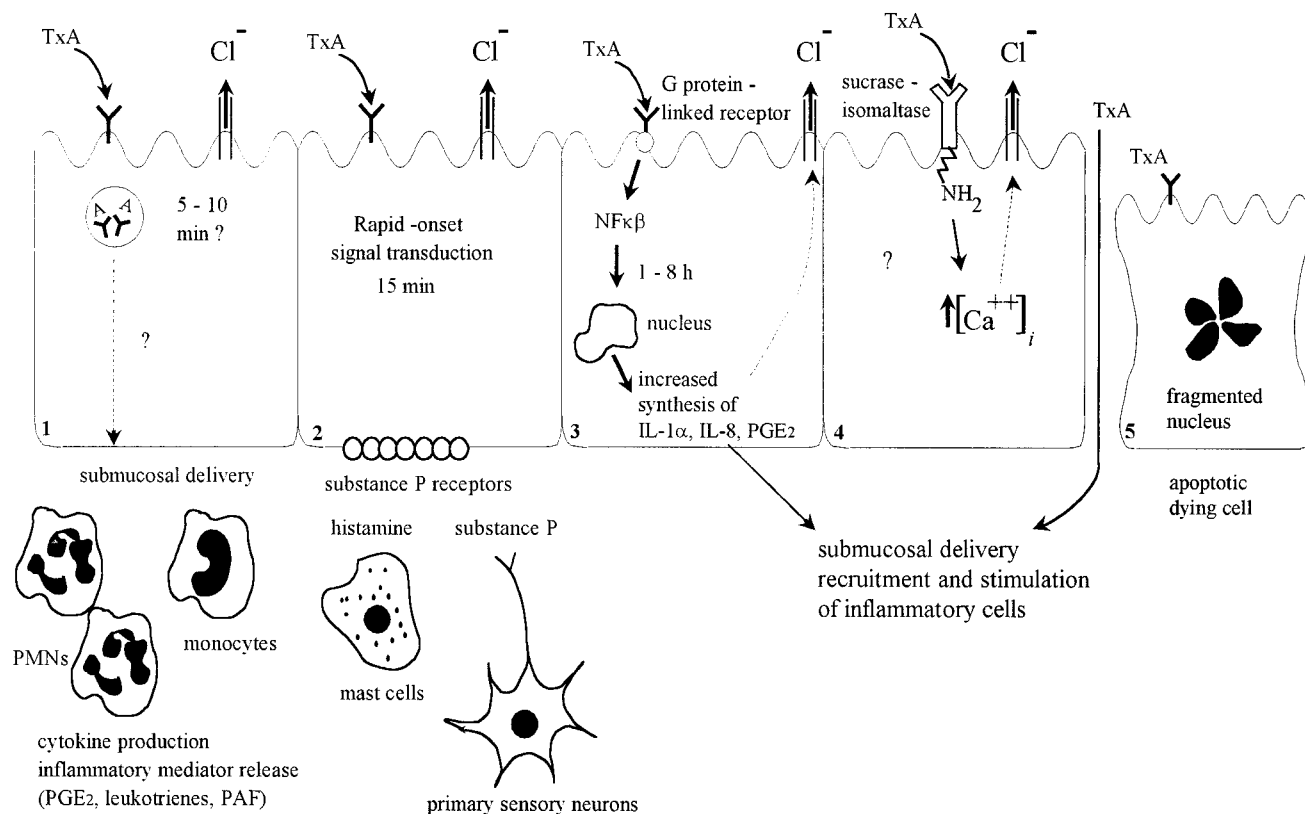


FIG. 2. Model of the pathogenesis of inflammatory diarrhea for *C. difficile* toxin A. This figure presents potential mechanisms by which *C. difficile* toxin A may act to stimulate an inflammatory and secretory response in the intestine. Although schematically presented in five intestinal epithelial cells, it is possible that all of the depicted events occur in a single intestinal epithelial cell. In cell 1, after binding to one or more host cell receptors, toxin A is rapidly endocytosed, an event which precedes detection of cytoskeletal changes induced by the toxin. Recent data indicate that toxin A monoglucosylates small GTP-binding Rho proteins on threonine 37, resulting in the depolymerization of F actin in the cell. It is unknown whether toxin A can transcytose the polarized intestinal epithelial cell and be delivered to the submucosal compartment of the epithelium. However, toxin A acts as a chemoattractant for PMNs in vitro and stimulates these cells to express CD18, the leukocyte adhesion molecule. In addition, toxin A stimulates PMNs and monocytes to produce IL-8, a proinflammatory cytokine. If toxin A is delivered to the submucosal compartment, recruitment and stimulation of PMNs and monocytes may promote secretion via cytokine production and release of inflammatory mediators. In cell 2, following treatment of intestinal mucosa in vivo, toxin A leads to rapid intracellular and transepithelial signalling by as yet unknown mechanisms. Within 15 min, submucosal mast cell degranulation occurs, releasing histamine, which is proinflammatory; primary sensory neurons are activated, which release substance P, mediating neurogenic inflammation; and increased expression of substance P receptors can be detected on intestinal epithelial cells. Both histamine and substance P may stimulate the intestinal epithelial cell to secrete chloride. Over the course of 2 to 4 h, substance P synthesis also increases in the intestinal submucosal compartment. In cells 3 and 4, two host cell membrane receptors have been described for toxin A. The first receptor is a pertussis toxin-sensitive G-protein-linked glycoprotein which has been identified on rabbit ileal cells and is developmentally regulated. Neither the distribution of this receptor throughout the intestine nor its relevance to human disease is known. Recent data indicate that activation of this receptor stimulates translocation of the transcriptional activator, NF- $\kappa$ B, to the intestinal epithelial cell nucleus, resulting in the synthesis over 1 to 8 h of inflammatory mediators such as IL-1, IL-8, and PGE<sub>2</sub>. Production of these mediators could directly stimulate chloride secretion in the cell, or their release to the submucosal compartment could augment the inflammatory response. The second potential toxin A receptor identified on rabbit ileal cells is sucrase-isomaltase. Transfection of CHO cells with this receptor followed by treatment with toxin A stimulates the release of intracellular calcium. If similar signalling occurs in intestinal epithelial cells, release of intracellular calcium could contribute to the stimulation of chloride secretion by toxin A. Both the relevance of this receptor (found predominantly in the small bowel) to the pathogenesis of *C. difficile* disease (predominantly a colonic disease) and the relationship of this receptor to the G-protein-linked receptor are unclear. In cell 5, the histopathology of intestinal mucosa (including human colonic tissue) treated with toxin A reveals focal loss of apoptotic intestinal epithelial cells. These breaks in the integrity of the intestinal epithelial barrier may permit direct submucosal delivery of toxin A, with stimulation and recruitment of host inflammatory cells as described for cell 1 in this figure. In summary, toxin A most probably stimulates secretion and inflammation by direct receptor-mediated effects on intestinal epithelial cells and indirect and possible direct effects via activation of submucosal immune effector cells and the enteric nervous system. See the section of text on *C. difficile* for detailed discussion and references.

*difficile*. In hamster intestine, toxin B alone is inactive, although its systemic absorption is lethal and appears to be facilitated by subthreshold concentrations (for intestinal damage) of toxin A (328). Consistent with this observation, toxin B (unlike toxin A) fails to specifically bind to hamster small intestinal brush border membranes (460). Despite acting as a potent cytotoxin on many nonepithelial cell lines, toxin B decreases monolayer resistance and alters the F-actin architecture of intestinal epithelial cell monolayers (T84 cells) with no cellular damage (206). However, the time course of this response and the appearance of the F-actin changes are delayed and distinct from those seen in response to toxin A. Similar to toxin A, toxin B

also does not change  $I_{sc}$  in T84 monolayers. However, a recent study examining the activity of toxins A and B on human colonic tissue mounted in Ussing chambers demonstrated that human colonic mucosa was ca. 10 times more sensitive to the damaging effects of toxin B than to toxin A (458). Similar to the data obtained with T84 monolayers, both toxins A and B diminished the resistance of human colonic tissue without stimulating an increase in  $I_{sc}$ . Both toxins A and B also caused focal damage to superficial (rather than crypt) epithelial cells, with disorganization of the F-actin staining pattern in the cells. These results suggested, for the first time, that toxin B may be more important than toxin A, at least in the initiation of hu-

man *C. difficile* disease. A potential hypothesis to tie together the various reports on the activities of toxins A and B is that toxin B-mediated injury of the human colon allows the entry of toxin A, which stimulates secretion through proinflammatory and/or neural mechanisms. Alternatively, this recent study (458) may indicate that species differences are very important in the actions of toxin A and B, raising questions about the relevance to human disease of the published animal experiments.

Lastly, the precise molecular mechanisms by which toxin B alters the cytoskeletal structure of cells have now also been identified (256). Similar to data on toxin A, initial data from nonepithelial cell lines suggested that toxin B inhibits directly or indirectly the ADP-ribosylation of Rho proteins by *C. botulinum* toxin (exoenzyme) C3 (105, 254). Subsequent data obtained with several nonintestinal cell lines indicate that toxin B, like toxin A, catalyzes the glucosylation of threonine 37 of Rho (256). Furthermore, microinjection of monoglucosylated Rho is sufficient to cause actin disruption in a kidney epithelial cell monolayer, an effect which is indistinguishable from the effect of toxin B. However, similar to the situation with toxin A, studies with intestinal epithelial cells are not yet available. Thus, despite being distinct toxins by genetic and protein analysis, toxins A and B stimulate the identical molecular modification of Rho in all cell lines tested to date and have similar effects on human colonic mucosa in vitro (458). However, these toxins have distinct effects on the physiology and F-actin arrangement in T84 monolayers (206, 207) and in animal intestine in vivo. A potential explanation for the experimental discrepancies described includes different receptors for toxins A and B, resulting in altered processing or compartmentalization of the toxins by the intestinal epithelial cells and/or altered signaling mechanisms in response to the toxins. In fact, preliminary data suggest that toxins A and B are processed differently by human lung fibroblasts (158, 210).

#### *Clostridium perfringens* Type A

*C. perfringens* type A is a common cause of food-borne disease in the United States. Noninflammatory diarrhea results from ingestion of contaminated meat or poultry products and is associated with in vivo production of a heat-labile 35-kDa single polypeptide toxin during sporulation of the organism in the small intestine (20, 349). Less than 5% of *C. perfringens* isolates contain the chromosomal *cpe* gene encoding this toxin. CPE expression occurs in type A, C, and D strains, although the reason(s) why types C and D infrequently cause foodborne disease is unknown (87, 349). As previously discussed, when human volunteers ingest 8 to 12 mg of purified CPE, short-lived mild diarrhea results (506).

CPE is a membrane-active cytotoxin with a proposed multistep mechanism of action (202, 349, 352, 353). Specific functional domains of CPE have been defined, with the receptor-binding region being localized to the extreme carboxy terminus and the region associated with cytotoxicity being closer to the amino terminus of the protein (202–204). In vitro studies with nonintestinal cell lines or rabbit small intestinal brush border membranes have identified separate calcium-independent and calcium-dependent steps in the mode of action of CPE (353). The initial steps of the action of CPE are calcium independent. First, CPE binds specifically and irreversibly to an as yet incompletely characterized protein receptor in the intestinal brush border membrane (202, 353). CPE remains localized to the plasma membrane after binding. The results of kinetic studies, the progressive insensitivity of the toxin to release by protease treatment, and the temperature dependence of CPE

cellular intoxication support the hypothesis that CPE next inserts into the brush border membrane (202, 349, 352, 353). However, in contrast to pore-forming toxins that create an ion channel by oligomerization of toxin molecules, insertion of CPE into the cellular membrane is followed by formation of a 160-kDa protein complex including two eukaryotic membrane proteins of 50 and 70 kDa (352, 579). Formation of this protein complex results in membrane permeability changes with loss of intracellular  $K^+$  and amino acids within minutes of toxin treatment (349, 352, 353). Disruption of protein, RNA, and DNA synthesis soon occurs because defined intracellular  $K^+$  concentrations are required for synthesis of these macromolecules. These latter steps of CPE action are calcium dependent, resulting in increased permeability of cells to larger molecules (e.g., nucleotides) with cellular morphologic changes including, for example, membrane blebbing (349, 351–353). Ultimately, CPE is lethal to eukaryotic cells. Interestingly, CPE creates a high-conductance, linear, ion-permeable channel in lipid bilayers, consistent with a direct membrane insertion step (518). However, recent data indicate that CPE is not amphipathic in the absence of eukaryotic membrane proteins, suggesting that pore formation in the experimentally artificial lipid bilayer system is not physiologically relevant for this toxin (349).

In vivo crude or purified CPE stimulates the rapid onset (within 15 min) of  $Na^+$  and  $Cl^-$  secretion in ligated ileal segments of several animal species, including rats and rabbits, without altering mucosal cyclic AMP levels (349, 357). Consistent with its proposed mechanism of action as a cytotoxin, CPE inhibits glucose absorption and causes histologic damage to the intestinal epithelium with desquamation of villous tip cells and blebbing of brush border membranes (357, 358). Time course and concentration dependence studies indicate a close correlation between the onset of CPE-induced tissue damage and intestinal secretion (349). In vitro treatment of CPE with trypsin activates the toxin (two- to threefold increase in biologic activity), an event which could theoretically occur in the intestine during food poisoning (204). Direct evidence for in vivo proteolytic activation of CPE is currently lacking. A resolution of the discrepancy between the cytotoxic action of CPE and the human clinical sequelae of rapidly resolving noninflammatory diarrhea is not obvious at present.

#### *Clostridium perfringens* Type C

*C. perfringens* type C organisms are the causative agents of the destructive intestinal disease called enteritis necroticans. Alternative names are pig bel in New Guinea and Darmbrand for a similar syndrome associated with malnourishment during World War II. These strains produce three toxins (alpha toxin, beta toxin, and an enterotoxin) of potential pathogenetic significance. The ca. 40-kDa, trypsin-sensitive beta toxin is thought to be crucial to the coagulative transmural intestinal necrosis that occurs when beta toxin-producing organisms are ingested in conjunction with trypsin inhibitors (i.e., undercooked pork with sweet potatoes in pig bel) or are ingested by individuals with protein deficiency due to malnourishment (i.e., Darmbrand) (298). Toxic filtrates of *C. perfringens* type C, but not type A, reproduced intestinal necrosis in guinea pigs only in the presence of protease inhibitors (298). Furthermore, active immunization against the beta toxin has proven to be effective in preventing enteritis necroticans (pig bel) in children in Papua, New Guinea (299). The beta toxin gene (*cpb*), found only in type B and C strains of *C. perfringens*, encodes a 34-kDa extracellularly secreted protein (226). The predicted sequence of the *cpb* product reveals homology to the alpha toxin, gamma toxin, and leukocidin of *Staphylococcus aureus*

(226). Reported biological activities of the crude and/or purified beta toxin include mouse and guinea pig death, dermo-necrosis, hemorrhagic necrosis of guinea pig intestinal loops, and cytotoxicity for CHO cells (226, 298). The mechanism of action of beta toxin is unknown. The apparent ability of beta toxin to form multimers and the amino acid homology to the pore-forming cytolysins of *S. aureus* (alpha and gamma toxins) suggest pore formation as a potential mechanism of action for the beta toxin (226). Of note, this toxin is cytotoxic, not lytic, for CHO cells and, in limited analysis, is not hemolytic (226).

Similar to the beta toxin, numerous biological activities have been ascribed to the alpha toxin (or PLC), a 43- to 46-kDa protein encoded by the *plc* gene (267). This toxin is produced by all types of *C. perfringens* including type A. Although it is regarded as the major factor responsible for gas gangrene, its role in other disease processes associated with *C. perfringens* infection is unclear, and some data indicate little correlation between the virulence of *C. perfringens* strains and production of alpha toxin (371). The alpha toxin is a multifunctional protein with one domain containing PLC activity and another domain being responsible for other toxicities (535). Serosal addition of purified *C. perfringens* PLC to rat colon mounted in Ussing chambers is reported to stimulate a biphasic increase in  $I_{sc}$  consistent with the stimulation of  $Cl^-$ -dependent secretion (104). Experiments with inhibitors and/or removal of serosal calcium from the bathing medium suggest that the biphasic  $I_{sc}$  response to PLC is dependent on prostaglandins and intracellular/extracellular calcium, respectively. However, PLC did not stimulate an increase in intracellular calcium levels when measured in isolated intact rat crypts (104). Notably, mucosal application of PLC had no effect on rat colon mounted in Ussing chambers; therefore, the contribution of *C. perfringens* PLC to intestinal disease is unclear. The histopathology of rat colonic tissue after application of PLC is not available from this study.

*C. perfringens* type C strains also produce an enterotoxin which by immunologic, physical, and functional assays appears to be largely identical to the enterotoxin (CPE) produced by type A organisms (504, 505). Whether or not this enterotoxin contributes to the pathogenesis of enteritis necroticans is unknown.

#### Enterotoxigenic *Escherichia coli*

EAEC strains are associated with persistent diarrhea in young children primarily in the developing world. EAEC strains have been reported to produce three toxins that are potentially able to stimulate intestinal secretion. EAEC heat-stable enterotoxin (EAST 1), the first recognized and best-characterized toxin produced by these strains, is a 4.1-kDa protein (38 amino acids) encoded by the *astA* plasmid-associated gene (480). Although approximately 50% homologous at the protein level to *E. coli* STa (see below for a discussion of this toxin), EAST 1 is distinct genetically and antigenically from *E. coli* STa. In rabbit ileum, EAST 1 stimulates both a rapid increase in anion-dependent  $I_{sc}$  and the level of mucosal cyclic GMP without any histologic changes in the intestinal mucosa being evident by light or electron microscopy (479, 480). However, these responses are smaller than those observed with *E. coli* STa, suggesting differences in the mechanism of action or potency of these toxins. Interestingly, EAST 1 has four cysteine residues, the same number as in guanylin, the endogenous peptide activator of intestinal particulate guanylate cyclase, and fewer than the six cysteines present in *E. coli* STa. Recent examination of the distribution and prevalence of the EAST 1 gene in different classes of bacterial enteropathogens indicated the *astA* gene was widespread in

certain diarrheagenic *E. coli* strains (e.g., 41% of EAEC, 41% of ETEC, 89% of EHEC, and 22% of EPEC strains) but was not detected in EIEC, *Y. enterocolitica*, or *V. cholerae* non-O1 strains (481). In most instances, the presence of the gene correlated with stimulation of an increase in rabbit ileal  $I_{sc}$  by culture filtrates of the *E. coli* strains studied. Of note, however, 38% of *E. coli* strains isolated from children without diarrhea were also positive for the EAST 1 gene (481). It is unknown whether these strains express the EAST 1 protein.

The second toxin produced by EAEC is proposed to be a heat-labile protein with an approximate molecular mass of 120 kDa, which is immunologically cross-reactive with *E. coli* hemolysin (12). In the nonintestinal epithelial cell line HEP-2, crude culture filtrates of EAEC stimulate an increase in intracellular calcium levels that is dependent on extracellular calcium and inhibitable by antihemolysin antiserum; culture filtrates also stimulate calcium-dependent phosphorylation. It is postulated that the 120-kDa protein is responsible for this activity and could contribute to the pathogenesis of diarrhea either by affecting intracellular calcium levels or through causing membrane pore formation as shown for the *E. coli* hemolysin. However, the relevance of this heat-labile toxin to intestinal pathophysiology is unknown.

A recent report suggests that EAEC may secrete yet another toxin capable of stimulating a severe acute intestinal inflammatory response in ligated rat intestinal loops and in infected children (135). During an outbreak of EAEC infection, the serum of 61.5% of infected infants revealed an antibody response to 108- and 116-kDa proteins produced by EAEC strains. Testing of these two proteins separately in ligated rat intestinal loops yielded an acute inflammatory response and shortening of the microvilli only with the 108-kDa protein. These results suggest that this 108-kDa protein, probably distinct from the 120-kDa heat-labile protein discussed above, may be a virulence factor of EAEC. The relationship of the 116- and 120-kDa proteins is uncertain. The heat-labile 108-kDa toxin has been partially purified and shown to increase PD and  $I_{sc}$  with no change in  $R$  in rat intestinal tissue mounted in Ussing chambers (387). Antiserum prepared against the 108-kDa protein completely inhibited the Ussing chamber activity, and antisera against CT partially inhibited Ussing chamber activity. Because the 108-kDa toxin is only partially purified, it is unclear whether the inflammatory response noted in rat intestine and the  $I_{sc}$  response in rat tissue mounted in Ussing chambers are due to the same protein. From the available data, this 108-kDa heat-labile toxin has been classified as a secretory cytotoxin in Tables 3, 4, and 6. Thus, EAEC produces at least two toxins capable of increasing  $I_{sc}$  in Ussing chambers.

#### Enterohemorrhagic *Escherichia coli*

EHEC, also known as Shiga-like toxin-producing *E. coli* or Vero cytotoxin-producing *E. coli*, produces a variety of clinical syndromes including bloody and nonbloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. The primary site of histopathology in human disease is the colon. In animal models, EHEC strains produce the same AE histopathology that is seen with EPEC strains (166, 360, 547, 548). Like EPEC, EHEC contains the 35-kb chromosomal locus of enterocyte effacement (LEE) region that is necessary to produce this histopathology (see the section on EPEC, below) (587). Therefore, the same potential secretogenic mechanisms seen with EPEC (see below) may also play a role in diarrhea due to EHEC. However, in addition to the AE lesion, EHEC strains produce one or more of a family of cytotoxins known as the Shiga toxin family, which are among the most potent of all



bacterial toxins. These toxins are thought to account for the unique clinical features of EHEC disease including hemorrhagic colitis and hemolytic uremic syndrome, in part through local and systemic absorption of the toxins, resulting in damaged vascular endothelium. In addition, recent data suggest that most EHEC O157:H7 strains produce EAST 1, which may contribute to intestinal secretion in these infections (481).

The Shiga toxin family contains two groups called Shiga-like toxin I (SLT-I) or verocytotoxin I (VTI) and Shiga-like toxin II (SLT-II) or verocytotoxin 2 (VT2); both toxins are encoded by bacteriophages (404, 516). Most EHEC O157:H7 strains (the most common EHEC serotype in North America) isolated from human disease produce SLT-II only or a combination of SLT-I and SLT-II (486). The SLT-I toxin is essentially identical to Shiga toxin produced by *Shigella dysenteriae* type I. The Shiga holotoxin contains a single A subunit of approximately 32 kDa associated with a pentamer of 7.7-kDa B subunits (526). The B subunit binds to a membrane glycolipid, globotriaosylceramide or Gb<sub>3</sub> (235). The A subunit has *N*-glycosidase activity, which cleaves a single adenine residue from the 28S rRNA component of eukaryotic ribosomes. The resulting inhibition of protein synthesis leads to the death of the intoxicated cell. Interestingly, the rRNA *N*-glycosidase activity of the Shiga toxin family is identical to the mode of action of the plant toxin ricin (134). The A and B subunits of SLT-I and SLT-II are 55 and 57% identical, respectively, in their predicted amino acid sequences (237). Sequence variation exists within SLT-II, and one subtype, SLT-IIv, binds with greater affinity to Gb<sub>4</sub> than to Gb<sub>3</sub> (90). Shiga toxin is cytotoxic for a variety of endothelial and epithelial cells including human colonic and ileal epithelial cells (380, 401), as well as being cytotoxic to B lymphocytes expressing CD77 (see the section on other criteria, above) (75).

The contribution of Shiga-like toxins to the nonbloody and bloody diarrhea seen with EHEC infection is not certain, although the ability of purified Shiga toxin (purified from *S. dysenteriae*) and SLT (purified from an *E. coli* O157:H7 strain) to cause fluid accumulation and histologic damage (but not an inflammatory response) in ligated rabbit ileal loops was established some time ago (261, 269; reviewed in reference 401). In contrast, purified SLT fed orally to infant rabbits often resulted in grossly bloody diarrhea and histologic changes primarily in the colon (e.g., apoptotic surface epithelial cells), with heavy neutrophilic infiltration (415). In studies of rabbit ileum, Shiga toxin and SLT appear to act directly on the absorptive villus epithelial cell because of the greater content of Gb<sub>3</sub> in villus cells than in crypt cells, thereby making the villus cells much more susceptible to the toxin-induced inhibition of protein synthesis (261, 269). Furthermore, recent data demonstrate that expression of the SLT-I receptor is coregulated with cell marker enzymes for intestinal epithelial cell differentiation (236). The effect of Shiga toxin on rabbit jejunal water and electrolyte transport was studied by Kandel et al. (261), using in vivo perfusion studies and Ussing chamber voltage-clamp techniques. Consistent with the data on the cellular expression of the toxin receptor, Shiga toxin had no effect on anion secretion but did decrease active basal NaCl absorption. These results suggest that Shiga toxin induces fluid accumulation by selectively killing the absorptive villus cells, thereby decreasing fluid absorption and unmasking basal crypt anion secretion. These changes shift the usual balance of intestinal absorption and secretion toward net secretion. Although an earlier report had indicated that Shiga toxin increased adenylate cyclase activity (58), the *I*<sub>sc</sub> response to theophylline in rabbit jejunum exposed to Shiga toxin is unaltered (261), thereby suggesting that cyclic AMP-dependent anion secretion was not affected by

Shiga toxin and that adenylate cyclase is not involved in the effects of Shiga toxin on transport. Notably, parallel studies of Shiga toxin activity on colonic transport are lacking and no studies to date have examined the activity of SLT-I and/or SLT-II on small bowel or colonic transport. However, it is presumed that the activity of SLT-I on rabbit jejunum is identical to that of Shiga toxin, because these are essentially identical proteins. In contrast, as summarized below, available studies on the pathogenesis of EHEC infection have focused on the cecum or colon, where colonization by SLT-positive or -negative organisms is demonstrable.

One approach to studying the role of SLT in enteric disease due to EHEC is to add genes encoding SLT to an *E. coli* strain capable of colonizing rabbit intestine. Sjogren et al. (502) used the rabbit pathogen RDEC-1, which produces the AE lesion primarily in the cecum and proximal colon and causes diarrhea in infant rabbits. When RDEC-1 expressing SLT-I was compared with RDEC-1 without SLT-I in rabbits, the SLT-I-producing variant caused more serious histologic lesions (including vascular changes, edema, and more severe inflammation) and higher mucosal levels of IL-1 and PAF. Notably, vascular lesions and marked submucosal edema were confined to infection with the SLT-I-producing *E. coli* strain. Furthermore, the development of diarrhea was more rapid and more common with the SLT-I-producing *E. coli* strain than with RDEC-1 alone.

However, even in the absence of SLTs, infection with EHEC can lead to changes in intestinal ion transport, suggesting that the pathogenesis of intestinal secretion during this infection is likely to involve additional EHEC virulence proteins. Using an infant rabbit model, Li et al. (314) showed that infection with an EHEC O157:H7 strain lacking SLTs inhibited Na<sup>+</sup> absorption and stimulated Cl<sup>-</sup> secretion to the same extent as did the SLT-positive *E. coli* O157:H7 strain by day 4 or 5 of colonic infection. In addition, intestinal mucosal infiltration with PMNs was similar with the SLT-positive and -negative strains. These changes in ion transport were accompanied by necrosis of surface colonocytes and the development of diarrhea. No effect on the small intestine was identified. However, infection with a nonpathogenic *E. coli* K-12 strain containing the SLT-I genes but lacking the LEE region resulted in less severe transport changes, suggesting that SLT-I contributes to transport changes but that development of the AE phenotype is crucial to disease pathogenesis. In a subsequent study by Elliott et al. (132), further examining this disease model, infection with an *E. coli* O157:H7 (SLT-positive) strain induced diarrhea and inhibition of Na<sup>+</sup> absorption by day 3 after inoculation. However, Cl<sup>-</sup> secretion occurred on day 5 and was associated with infiltration of the intestinal tissue with PMNs. Pretreatment of the animals with a monoclonal antibody directed against the leukocyte adhesion molecule CD18 prevented histologic damage and tissue infiltration with PMNs. In addition, pretreatment of the rabbits with anti-CD18 antibody prevented the changes in Na<sup>+</sup> and Cl<sup>-</sup> transport. Thus, the development of an inflammatory response may play a key role in the pathogenesis of secretory diarrhea in EHEC disease, as discussed above for *C. difficile* (see the section on potential mechanisms of intestinal secretion, above).

The likelihood that pathogenesis of intestinal secretion with EHEC strains involves expression of additional unique virulence proteins by these organisms is further supported by data directly contrasting EHEC and EPEC infections in intestinal models of disease. Although EHEC contains the LEE chromosomal region, similar to EPEC, initial data have identified both similarities and differences in the pathogenesis of these infections in in vitro and in vivo intestinal models of disease.

First, similar to EPEC (373), development of diarrhea in a gnotobiotic-piglet model of EHEC disease is strongly associated with the development of AE intestinal lesions (360, 547). Development of this AE lesion is dependent on the expression of a bacterial protein called intimin (product of the *eaeA* gene contained in the LEE region; see also the discussion of EPEC, below) (360). However, expression of the EPEC *eaeA* gene in an *eaeA*-mutated EHEC strain alters the distribution of colonization from the colon (with the wild-type EHEC strain) to both the distal small intestine and the colon (547), a result partially consistent with the known distribution of intestinal lesions in EPEC and EHEC human disease. Second, while EPEC strains readily colonize human intestinal epithelial cells (T84), forming AE lesions (233, 491, 514), EHEC strains colonize these cells intimately but with little AE lesion formation (233). In addition, EPEC but not EHEC infection of T84 cells stimulated host cell tyrosine phosphorylation. However, both EPEC and EHEC organisms stimulated inositol triphosphate release or an increase in intracellular calcium levels in nonintestinal (HEp-2) and T84 cells, respectively. Thus, EHEC, unlike EPEC, stimulated an increase in intracellular calcium levels in the absence of cellular tyrosine phosphorylation, suggesting that these organisms may employ different signal transduction mechanisms to stimulate intestinal secretion (233). Release of intracellular calcium may trigger vesiculation of host cell microvilli and stimulate chloride secretion (see the section on intracellular calcium, above), contributing to the development of diarrheal disease.

Together, these data suggest that at least two, and possibly four, major virulence factors play a role in EHEC disease pathogenesis: the SLTs, the bacterial products responsible for the AE lesion (see the discussion of EPEC below), EAST 1, and an as yet undefined bacterial product which increases intracellular calcium levels in the absence of host cell tyrosine phosphorylation and AE lesion formation. The precise mechanisms and virulence factors accounting for the striking inflammatory response observed in experimental infection and the contributions of SLT-I and SLT-II production to disease are only a few of the questions requiring further investigation to better understand the pathogenesis of secretion and intestinal damage in EHEC disease. At present, available data, although conflicting, suggest that the inflammatory response observed in EHEC disease models does not require the production of SLTs by the organisms but, rather, is due to signals potentially stimulated by the AE lesion or another bacterial virulence mechanism. Alternatively, the variable reports of inflammation in response to the purified toxins versus organisms expressing SLTs or Shiga toxin (see the discussion of *S. dysenteriae*, below) may be due to the different animal species studied. Additional studies examining the effects of Shiga toxin, SLT-I, and/or SLT-II on colonic transport would help discern the impact of the toxin(s) alone on colonic transport and histology with respect to the contribution of additional virulence factors of the organism. Although SLTs are detectable in the stools of infected patients, there are no data indicating where in the bowel these toxins are produced. Thus, the spectrum of human disease expression (e.g., watery diarrhea and/or bloody diarrhea) may relate to regional differences (i.e., small bowel versus colon) in response to these toxins.

#### Enteroinvasive *Escherichia coli*

Although infections with EIEC strains can result in a dysenteric syndrome, most often these strains cause watery diarrhea. The ability of these strains to invade cells is due to virulence genes present on a 140-MDa plasmid (*pInv*), and

invasion probably contributes to dysenteric disease. The pathogenesis of the watery diarrhea associated with these isolates has long been unexplained, but recent studies offer new insights into this issue. Fasano et al. reported that culture filtrates of EIEC stimulate moderate secretion without histologic damage in 18-h ligated rabbit ileal segments and also increase  $I_{sc}$  and potential difference in rabbit ileum studied in Ussing chambers in vitro (145). The secretory activity present in the EIEC culture filtrates was reported to be partially heat labile and iron regulated. The activity in the EIEC culture filtrates was named EIET (for enteroinvasive enterotoxin) but could not be ascribed to a single protein in this initial report. Most of the enterotoxic activity present in culture supernatants of EIEC has now been ascribed to a toxin called ShET2 (for *Shigella* enterotoxin 2 or EIEC enterotoxin), encoded by a plasmid gene (*sen*, formerly called *set2* [385, 386]). This toxin is also produced by *Shigella flexneri* and is discussed below in the section on that species. Notably, a *sen* EIEC deletion mutant had residual activity in Ussing chambers, suggesting that EIEC strains (and possibly *S. flexneri* as well as other *Shigella* spp. [see below]) possess another, as yet unidentified enterotoxic moiety. In addition, EIEC strains secrete a smaller cytotoxin (<30 kDa) with low-level Vero cell cytotoxicity (145). This cytotoxin is immunologically and genetically distinct from SLT-I and SLT-II, and its role in EIEC disease is unknown.

#### Enteropathogenic *Escherichia coli*

Epidemiologic studies indicate that EPEC is a common cause of diarrhea in children less than 1 year of age, particularly in developing countries (109). When a large inoculum ( $10^{10}$  organisms) of EPEC is administered to adult human volunteers with concomitant sodium bicarbonate, 60 to 100% will experience diarrheal disease with an onset as early as 3 h postinoculation (110) (Table 5). However, these strains are rarely associated with diarrheal illnesses in older children and adults. EPEC causes a distinctive histopathologic lesion in the human intestine, which involves destruction of microvilli and close adherence of the bacteria to the membrane of the enterocyte with cup-like pedestals upon which each bacterium rests (549). F actin, myosin, and other cytoskeletal elements are clustered just beneath the attached bacteria (151, 288, 334). This classic histopathologic lesion is termed the AE lesion (373).

Expression of the AE phenotype requires several virulence genes located in a 35-kb region of the chromosome called the LEE (356). This 35-kb region is also present in EHEC, the rabbit pathogen RDEC-1, and strains of *Hafnia alvei* associated with diarrheal disease (356). Genes contained in this region include the *eaeA* and *eaeB* (for EPEC attaching-and-effacing A and B loci, although *eaeB* has recently been renamed *espB* for EPEC secreted protein B [273]) genes, as well as *sep* genes, which are necessary for secretion of extracellular proteins (including the EaeB protein) by EPEC (110, 111, 244, 356). Additional virulence genes that enhance the AE lesion are located on the EAF (for EPEC adherence factor) plasmid and include the *bfpA* (for bundle-forming pilus A) and *per* (for plasmid-encoded regulator) genes (108, 186, 241). Recent studies indicate that formation of the AE lesion requires both intimin, the 94-kDa outer membrane protein product of the *eaeA* gene, and a secreted protein encoded by the *eaeB* (*espB*) gene (163, 240, 242, 244). In volunteer studies, infection with strains lacking the EAF plasmid or the *eaeA* gene causes diarrhea in only 20 to 36% of volunteers, indicating the importance of intimate colonization and AE lesion formation in the stimulation of intestinal secretion (110, 312).

Formation of the AE lesion has also been associated with the triggering of several host cell signal transduction events in nonintestinal epithelial cell lines. These host cell signal transduction events include activation of a tyrosine kinase with phosphorylation of a 90-kDa eukaryotic cell protein (464), release of inositol phosphates (127, 164, 289), elevation of intracellular calcium levels, and activation of PKC/calcium calmodulin-dependent kinases with phosphorylation of myosin light chain and other cytoskeletal proteins in eukaryotic cells including, in some cases, intestinal cells (e.g., Caco-2 cells) (11, 14, 127, 289, 334). As discussed above, each of these host cell signal transduction pathways regulates ion transport in intestinal epithelial cells. Although the exact sequence of these host cell events is not yet clear, it appears that activation of a host cell tyrosine kinase precedes the release of inositol phosphates. Release of inositol phosphates presumably would lead to release of intracellular calcium and activation of PKC. In studies with a nonintestinal epithelial cell line, the protein encoded by the *eaeB* (*espB*) gene was shown to be necessary to trigger these eukaryotic cell signal transduction events associated with EPEC infection (163). Thus, the EaeB (EspB) protein (which remains to be purified and characterized) is an attractive bacterial protein candidate potentially central to the pathogenesis of the intestinal secretion associated with EPEC infection.

Recent studies have begun to identify specific changes in the physiology of intestinal epithelial cell monolayers infected with EPEC. Both T84 and Caco-2 monolayers manifest delayed decreases in resistance after infection with EPEC as a result of a change in tight junctional function (43, 81, 491, 514). The diminished resistance in T84 monolayers is not due to damage to the monolayers as assessed by electron microscopy or release of the intracellular enzyme lactic dehydrogenase but is associated with phosphorylation of myosin light chain and is abrogated by pretreatment with dantrolene, which sequesters intracellular calcium (514, 588). Although experiments to correlate direct measurements of intracellular calcium with changes in the physiology of intestinal epithelial cell monolayers after EPEC infection have not yet been reported, preliminary data (233) suggest that EPEC infection of T84 cells increases intracellular calcium levels at a time point associated with AE lesion formation and diminished monolayer resistance in these other studies (491, 514). Together, these data suggest that release of intracellular calcium and/or phosphorylation of myosin light chain regulate tight junctional permeability after EPEC infection (514, 588). Although no increase in  $I_{sc}$  has been observed in T84 monolayers infected with EPEC to date, more detailed Ussing chamber studies of EPEC-infected Caco-2 monolayers have revealed a rapid but largely transient increase in  $I_{sc}$  (81). Furthermore, the  $I_{sc}$  response was observed in monolayers infected with an *eaeA* deletion mutant but not an *eaeB* (*espB*) deletion mutant. Although EPEC strains have not previously been recognized to produce any cell-associated or secreted toxins that alter the physiology of the intestinal epithelium (305, 323), these data suggest that the secreted EaeB (EspB) protein is a newly recognized EPEC enterotoxin and may account for the rapid onset of diarrhea reported in human volunteers infected with EPEC (110). Similar to T84 monolayers, Caco-2 monolayers infected with wild-type EPEC but not an *eaeA* deletion mutant later develop diminished resistance, suggesting a role for intimin in both AE lesion formation and development of tight junctional permeability. Phosphorylation of myosin light chain has also been identified in polarized Caco-2 cells as well as human intestinal epithelium experimentally infected with EPEC (289, 334), but these findings have not yet been linked to physiologic changes as discussed for T84 monolayers above.

Presently, a multistage hypothetical model of the pathogenesis of EPEC disease is proposed (109). The first stage is localized adherence of the bacteria to the epithelial cells in a nonintimate fashion which involves the bundle-forming pilus of these organisms. The second stage involves eukaryotic cell signal transduction events triggered by secreted products of the organism including the *eaeB* (*espB*) gene product. The third stage is intimate attachment of EPEC to the intestinal epithelial cell, which is dependent on both bacterial intimin and the bacterial product of the *eaeB* (*espB*) locus and is possibly promoted by intracellular calcium release (13, 127). After the stage of intimate attachment, a subset of bacteria can invade intestinal epithelial cells but are unable to multiply. Net intestinal secretion is postulated to occur as a result of stimulation of one or more signal transduction pathways in the intestinal epithelial cell and as a result of interference with absorption by the destruction of the microvillus structure by adherent bacteria. Although diarrhea due to EPEC is not a classic inflammatory diarrhea, a local inflammatory response in intestinal biopsies has been reported (549) and recent data have identified the neutrophil product lactoferrin in stools of patients infected with EPEC (367), suggesting that an inflammatory response may also contribute to the pathogenesis of EPEC diarrheal disease.

Although the first and third stages of this model are supported by studies with cultured intestinal epithelial cells, animal models, and human volunteer studies, the crucial signal transduction events of the second stage have been studied primarily in nonintestinal cell lines with more fragmentary results available for intestinal epithelial cell models of EPEC disease. For example, the presence of the 90-kDa host cell tyrosine-phosphorylated protein has been confirmed in unpolarized human intestinal epithelial cells but not consistently in polarized monolayers of these cells (233, 243, 464). Similarly, inositol phosphate release is detectable in unpolarized human intestinal epithelial cells, but similar studies of polarized cells are not yet available (164). Furthermore, although release of total inositol phosphates was measurable, specific release of inositol triphosphate (presumably the mediator releasing intracellular calcium) was not detectable. Time course studies linking EPEC-stimulated signal transduction events to specific changes in intestinal epithelial transport are not yet available. The interpretation of the available data in intestinal epithelial cells is further complicated by recent data suggesting that activation of tyrosine kinases or PKC has both stimulating and inhibitory actions on intestinal epithelial cell transport processes (18, 116, 341, 489, 552). Additional studies are necessary to determine, in either in vitro or in vivo intestinal models of disease, which of the eukaryotic cell signal transduction events associated with EPEC infection are linked to the rapid onset of intestinal secretion observed in the human volunteer experiments or to the more protracted diarrhea observed in some infants with EPEC disease.

#### Enterotoxigenic *Escherichia coli*

ETEC strains are important causes of traveler's diarrhea and dehydrating diarrheal illnesses in children in the developing world. These organisms produce heat-stable and heat-labile enterotoxins (ST and LT, respectively), which are among the best characterized of all enterotoxins. STa is associated with disease in both humans and animals, and STb is associated primarily with diarrhea in piglets, although one recent report indicates that it might also be associated with human disease (409). LT-I is associated with disease in both humans and animals, while LT-II has been associated only with animal



disease. Strains of ETEC isolated from human disease produce STa only, LT-I only, or STa and LT-I together.

*E. coli* heat-stable enterotoxin a (STa, STI, ST<sub>p</sub>, ST<sub>h</sub>). STa is a cysteine-rich, 18- or 19-amino-acid peptide with a molecular mass of ca. 2 kDa (120, 531). The toxin is encoded by the transposon-associated *estA* gene, which is located on a plasmid (509). STa is produced as a 72-amino-acid precursor that is cleaved by signal peptidase 1 to a 53-amino-acid peptide (455). This peptide is translocated to the periplasm, where three intramolecular disulfide bonds crucial to toxin activity are formed by a protein termed DsbA prior to secretion by the bacteria (585). A second proteolytic event occurs extracellularly to produce biologically active STa of 18 or 19 amino acids (455). Through nuclear magnetic resonance spectroscopy and X-ray crystallography, the structure of STa has been shown to consist of a folded peptide backbone with three  $\beta$  turns stabilized by three disulfide bonds (176, 177, 410, 412). The second  $\beta$  turn at residues 11 to 14 (and especially Ala-13) is proposed to be most important to the toxicity of STa and its interaction with its receptor (412, 478). These four amino acids (residues 11 to 14, Asn-Pro-Ala-Cys) are conserved in all ST family members and are partially conserved in guanylin and the EAEC EAST 1 toxin (Ala-Cys). These results are consistent with these proteins all acting via binding to the same or a similar receptor on intestinal epithelial cells. In most but not all studies (563), STa residues 5 to 17 or 6 to 18 confer full binding and enterotoxic activities, and it is this region that shares a striking identity with heat-stable enterotoxins secreted by other enteric pathogens including *Y. enterocolitica* and *V. cholerae* non-O1 strains (523, 524, 586).

STa acts by binding to a protein intestinal epithelial receptor localized in the brush border membrane. STa receptors are found throughout the human small intestine and colon, with decreasing receptors along the longitudinal axis of the gut (295). In addition, a large number of receptors are present in infants and the number rapidly decreases with increasing age (76). This latter observation may help to explain the increased severity of diarrhea in young children infected with STa-producing ETEC strains. The size and nature of the STa receptor(s) have been an area of intensive investigation, resulting in the identification of at least one definitive STa receptor, guanylate cyclase type C (GC-C), located in the apical membrane of intestinal epithelial cells (77, 485, 532). GC-C (a 120-kDa protein unglycosylated and a 140- to 160-kDa protein after N-linked glycosylation) is one of a family of receptor cyclases that include the atrial natriuretic peptide receptors, GC-A and GC-B (55, 213, 551). The endogenous agonist for GC-C is a 15-amino-acid hormone called guanylin, which contains four cysteines and is less potent than STa in activating GC-C and in stimulating chloride secretion (45, 86, 161, 296). Guanylin presumably plays a role in basal gut homeostasis, and STa opportunistically utilizes GC-C to alter ion transport in the gut. Although GC-C is clearly a STa receptor, solubilization and STa receptor cross-linking experiments with native intestinal tissue (including human) have repeatedly identified both small and large STa-binding proteins, generally of 45 to 80 kDa and 120 to 160 kDa, respectively (77, 98, 199, 213, 234, 532, 554). Recently, these smaller STa-binding peptides were shown to cross-react with antibodies to GC-C, suggesting that proteolysis of the extracellular domain of GC-C generates these peptic fragments (77, 551). In addition, similar lower-molecular-weight STa-binding peptides have been identified in naive cells transfected to express GC-C, which is consistent with the hypothesis that the appearance of low-molecular-weight STa receptors is related to the expression of GC-C and not to the expression of multiple genes encoding STa receptors (98, 551).

An unresolved issue to date has been how these data account for receptors of high and low affinity identified in native intestinal tissue and, more recently, human intestinal epithelial cells in vitro (T84) (161, 225). Other investigators, however, have identified only low-affinity receptors in T84 cells (85, 193, 560). A recent study has identified both high- and low-affinity STa-binding sites in COS-7 cells expressing GC-C, consistent with expression of different oligomers of the protein by these cells (99). In addition, STa binding to GC-C shifts the affinity of the protein for STa. Together, these data suggest that ST-binding sites with different affinities on intestinal cells reflect expression of a single protein, GC-C, not multiple STa-binding proteins (99). Nonetheless, non-GC-C-linked STa receptors may exist (3, 335).

After STa binding, GC-C is activated, although the molecular details of cyclase activation and inactivation are not fully understood. However, available data suggest that adenine nucleotides regulate GC-C as well as the interaction and/or response of GC-C to STa (85, 178, 418, 553). In addition, removal of the kinase domain of GC-C by deletional mutagenesis results in constitutive activation of GC-C without further responsiveness to *E. coli* STa (467). These data suggest that, similar to atrial natriuretic peptide stimulation of GC-A or GC-B (64, 290), the kinase domain of GC-C is inhibitory to cyclase activity and that activation of GC-C may require a conformational change in the kinase domain potentially induced by the binding of *E. coli* STa to GC-C. Lastly, GC-C appears to be an oligomer in the presence or absence of STa, and STa-mediated activation of GC-C may require the interaction of at least two cyclase domains (467, 553). It is unknown whether the other bacterial enteric toxins that elevate intracellular cyclic GMP levels, namely, EAST 1 of EAEC and Yst of *Y. enterocolitica*, activate GC-C similarly to *E. coli* STa.

Activation of GC-C results in increased levels of intracellular cyclic GMP that stimulate chloride secretion and/or inhibit NaCl absorption, resulting in net intestinal fluid secretion (150, 192, 224, 227, 453). In addition, examination of the ion transport changes stimulated by STa in rabbit ileum most consistently reveals inhibition of NaCl absorption, a finding potentially explained by enhanced binding of STa to villus enterocytes which are responsible for NaCl absorption (4, 78). In vivo chloride secretion may occur through activation of a cyclic GMP-dependent protein kinase, type II, present in the apical membrane of the enterocyte (92, 553). Conflicting data in vitro with human intestinal epithelial cell lines suggest involvement of either cyclic GMP-dependent kinase or cyclic AMP-dependent kinase, which ultimately activates a linear chloride channel known as CFTR (162, 317, 534). The observation that a PD response, but not a cGMP response, to STa is absent in the rectum of cystic fibrosis patients further supports the importance of CFTR in the secretory response to STa (185). More recently, treatment of Caco-2 cells with STa inhibited uptake of the nutrient amino acid taurine (which is also coupled to sodium absorption) by activation of PKA but not PKG or PKC (39). This mechanism also potentially contributes to diminished absorption in STa-mediated disease. Although STa is a classic enterotoxin without in vivo or in vitro evidence of histologic damage, the secretory response to STa in vitro in T84 cells was recently reported to involve microfilament (F-actin) rearrangement only at the basal pole of these polarized cells (343).

An interesting dissociation between identification of STa receptors, the cGMP response, and the short-circuit response to STa in the human intestine has been reported (295, 296). Although STa stimulation of GC-C is greater in the human small intestine than in the colon (194, 296) and, in general,



fewer STa receptors are found in the human colon (76, 295), the short-circuit current response to STa is greater in the colon (296). The mechanism accounting for this observation is unknown. However, one possibility is that STa is inactivated at different rates in different regions of the gut, as shown previously in the jejunum and ileum by Cohen et al. (79). Alternatively, non-GC-C STa receptors exist in the colon. If so, these non-GC-C STa receptors may be linked to an alternative signalling pathway, accounting for the greater colonic  $I_{sc}$  response reported for STa (296). Consistent with these data, the presence of a novel STa receptor not coupled to guanylate cyclase activity has been suggested by studies with the intestinal cell line, IEC-6, and expression of non-GC-C-linked STa receptors has been induced in cells by human cDNA containing a specific Alu repeat sequence (3, 335).

Whether cyclic GMP alone accounts for the full secretory response to STa is controversial. In rat jejunum, the secretory response to STa has been completely abrogated by 5-HT (serotonin) receptor antagonists without altering the cyclic GMP response to STa (30). These results suggest that serotonin mediates STa secretion, possibly through an effect on prostaglandin synthesis (HT-2 receptors) and/or activation of neurons (HT-3 receptors). However, the potential role of prostaglandins or serotonin in mediating STa-stimulated secretion is controversial (30, 122, 189, 190, 296, 426, 461, 530). Most recently, the  $I_{sc}$  response of human intestine to STa was not inhibited by the cyclooxygenase inhibitor indomethacin, suggesting that prostaglandins do not mediate the secretory response to STa (296). In addition, on the basis of inhibitor studies and studies with isolated rat intestinal epithelial cells, a role for phosphatidylinositol and diacylglycerol release with elevation of intracellular calcium levels and activation of PKC has been proposed to be important in STa-stimulated secretion (16, 17, 60, 61, 174, 191, 286, 287, 530). Although in vitro studies have shown release of these potential secretory mediators, correlation with a secretory response is not yet available. In contrast, in T84 cells, STa has been demonstrated only to increase cyclic GMP levels (yielding chloride secretion) without elevating intracellular calcium levels or hydrolysis of phospholipids (227, 555). Lastly, a role for the enteric nervous system in STa-stimulated secretion has been suggested by studies of myoelectric activity in STa-treated rabbit small intestine and by studies with neuronal inhibitors that diminished the STa secretory response in vivo and in vitro (130, 339, 461).

***E. coli* heat-stable enterotoxin b (STb, STII).** The plasmid-associated *E. coli* STb gene (*estB*) encodes a 71-amino-acid precursor protein containing four cysteines that is proteolytically processed in the periplasmic space to a mature 48-amino-acid protein with two disulfide bonds (123, 168, 297, 301, 429). Unlike *E. coli* STa, this trypsin-sensitive protein is secreted extracellularly without further processing. Neither the toxic domain nor the intestinal receptor for STb has been characterized. However, the charged amino acid residues Lys-22, Lys-23, Arg-29, and Asp-30 have been identified as necessary for toxic activity (123, 169). Derivatives of STb with the Arg-29 or Asp-30 residues individually mutated have reduced secretory activity and, furthermore, do not inhibit the secretory activity of wild-type STb, suggesting that these residues are important in the binding of STb to its receptor. Similarly, substitution of the Lys-22 or Lys-23 residues caused a marked reduction in secretory activity but the potential mechanism(s) involved is unknown (169).

Initially, the biological activity of STb was thought to be limited to secretion in ligated piglet intestinal segments. However, Whipp demonstrated that the apparent species specificity of STb was due to proteolytic cleavage of STb by trypsin in the

small intestines of rats and mice (574, 575). In the presence of trypsin inhibitor, STb is secretory in rats and mice, although rat intestine is more sensitive to STb. STb does not appear to have an effect on human small intestine mounted in Ussing chambers (573), which is consistent with the infrequent occurrence in human disease of *E. coli* strains expressing this toxin (409, 573). Unlike STa, STb induces histologic changes in intestinal epithelium, consisting of loss of villus epithelial cells and partial villus atrophy (577, 578). Consistent with these observations, sucrose activity is diminished in STb-treated swine intestine and the  $I_{sc}$  response to the amino acid alanine is reduced, suggesting impaired absorption in STb-treated tissues (576). In addition, the major anion secreted in vitro and in vivo in response to STb appears to be bicarbonate, not chloride as is observed in STa-stimulated secretion (570).

The mechanism by which STb stimulates intestinal secretion is not known but does not involve cyclic nucleotides (272, 426). In renal and intestinal epithelial cell lines (MDCK and HT29/C1, respectively), STb stimulates a dose-dependent increase in intracellular calcium levels which requires the presence of extracellular calcium and is blocked by somatostatin and pertussis toxin, thereby suggesting that STb activates a GTP-binding protein-regulated calcium channel in the plasma membrane (121). However, the relevance of this increased intracellular calcium level to the intestinal pathophysiology of STb has not been demonstrated. Additional data also indicate that STb treatment of rat intestinal loops stimulates the release of the intestinal secretagogues serotonin and PGE<sub>2</sub> and that antagonists of these secretagogues inhibit the secretory response to STb (205, 426). Whether release of these mediators is stimulated directly or indirectly by STb from the epithelium or submucosal tissue has not yet been determined.

***E. coli* heat-labile enterotoxin I (LT-I).** The LT-I enterotoxin is closely related to CT, sharing approximately 80% protein sequence identity in the A and B subunits (107). LT-I and CT share many characteristics including holotoxin structure, primary receptor identity, enzymatic activity, and activity in animal assays (reviewed in reference 510). LT-I shows no major differences in these aspects, and so a discussion of the mode of action will be deferred to the section on CT below. However, there is a clear difference in disease caused by *E. coli* producing LT-I compared with that caused by *V. cholerae* producing CT. Although some infections due to LT-I-producing *E. coli* strains resemble cholera in severity, the majority of LT-I-producing *E. coli* infections are generally milder and of shorter duration than infections due to *V. cholerae* expressing CT. The reason for this difference is not known, but many factors could play a role, including the more efficient secretion of toxin by *V. cholerae*, the production of neuraminidase by *V. cholerae*, or differences in intestinal colonization. Thus, the difference is in all probability not due to sequence differences in toxins but to other bacterial factors which affect toxin delivery or colonization.

***E. coli* heat-labile enterotoxin II (LT-II).** The LT-II toxins share many characteristics with LT-I but have been isolated primarily from animals and rarely from humans (494). The LT-II A subunits show 55 to 57% identity with the A subunits of LT-I and CT, but the LT-II B subunits show essentially no homology with the B subunits of LT-I or CT (431, 432), a result consistent with the differing ganglioside-binding specificities of these toxins (see below). Two distinct members of the LT-II family have been described, LT-IIa and LT-IIb, which share 71 and 66% identity in the predicted A and B subunit sequences, respectively (432).

Like LT-I, LT-II increases intracellular cyclic AMP levels by activating adenylate cyclase through the GTP-dependent

ADP-ribosylation of G $\alpha$  (56), although some differences in substrate specificity have been noted (302). Unlike LT-I, which binds primarily to GM $_1$  ganglioside, LT-II $_a$  binds best to GD1 $_b$  ganglioside and LT-II $_b$  binds best to GD1 $_a$  ganglioside (170). Purified LT-II increases vascular permeability in rabbit skin and is 25 to 50 times more potent than CT or LT-I in causing rounding of cultured Y-1 adrenal cells (216). However, purified LT-II (unlike CT or LT-I) does not cause secretion in ligated rabbit ileal loops (216), although activity in the sealed adult mouse model has been noted (unpublished results cited in reference 118). These species differences in secretory activity are most probably due to the ganglioside specificities of LT-II. Cyclic AMP levels have not been measured in the tissues of either of these mouse or rabbit models after treatment with LT-II. Thus, correlation of a secretory response with an increase in tissue cyclic AMP levels is not yet possible.

#### Other *Escherichia coli* Toxins

**Cytolethal distending toxin.** In 1987, Johnson and Lior (252) described a new toxin produced by *E. coli* isolated from a small number of children with diarrhea, which produced a novel effect on CHO cells. This activity produced elongation of CHO cells at 24 h followed by progressive cellular distension and cytotoxicity at 96 to 120 h. The initial cellular distension of CHO cells seen at 24 h is indistinguishable from that caused by *E. coli* LT and may account for some false-positive results for LT when CHO cells are used. However, unlike *E. coli* LT, CLDT is not active on Y-1 adrenal cells. This CHO cell activity has been found with *E. coli* from sporadic cases of gastroenteritis as well as with 6.4% of *E. coli* strains belonging to classic EPEC serotypes (38). The CLDT activity is heat labile, but the toxin has not yet been purified. Crude *E. coli* CLDT stimulates an erythematous response in rabbit skin but is not secretory in rabbit ligated ileal loops or the suckling-mouse assay (252). The genes encoding CLDT activity, *cdt*, have been cloned from *E. coli* E6468/62 and contain three open reading frames encoding proteins with predicted molecular masses of 25.5, 29.8, and 20.3 kDa (488). Heterogeneity of CLDT is indicated by the cloning of *cdt* genes from another strain of *E. coli*, 9142-88, that have the same genetic structure but whose predicted protein products share only 38, 56, and 37% identity with the three open reading frames from strain E6468/62 (430). CLDT activity has also been found in *Campylobacter* species and *Shigella* species (251, 253, 411). No studies on intestinal cells have been reported for this toxin.

**Cytotoxic necrotizing factors.** Necrotizing strains of *E. coli* (NTEC strains) have been associated anecdotally with extraintestinal infections (e.g., septicemia and urinary tract infections) and enteritis in humans and animals (34, 44, 97). NTEC strains of largely differing serogroups produce two bacterial cell-associated protein toxins, CNF1 and CNF2 (34, 97). Limited data suggest that CNF1 strains are more common than CNF2 strains in human infections and that both CNF1 and CNF2 strains are isolated from normal and diarrheal stools of animals and humans (34). Although the structural genes for these toxins are highly related, encoding ca. 115-kDa monomeric proteins with 86% identical and 99% conserved amino acid residues, these toxins differ in their gene location and biological activities and are only partially related by immunologic assays (91, 139, 413). The gene encoding CNF1 (*cnf1*) is chromosomal and closely linked to a distinct hemolysin gene (139); the gene for CNF2 (*cnf2*) is located on a Vir or F plasmid (413). On the basis of genetic analysis, both toxins show a significant homology with the *Pasteurella multocida* toxin that is important in the pathogenesis of progressive rhinitis in pigs.

Characteristic features of CNF1 and CNF2 are the ability to cause dermonecrosis in rabbit skin and multinucleation in several cell lines (e.g., HeLa, HEP-2, Vero). However, CNF2 is 100 times more potent than CNF1 in the rabbit skin necrosis assay and the patterns of morphologic changes in response to CNF1 and CNF2 differ in HeLa cells (91). Overall, CNF2 is more necrotic and lethal than CNF1 in selected biological assays and, unlike CNF1, causes secretion in ligated intestinal segments after 24 h in most rabbits tested.

The molecular mechanism of action of these toxins is unknown. However, CNF1 treatment alters F-actin and tubulin arrangement in cells, decreases the number of microvilli in nonintestinal epithelial cells (152), and induces epithelial cells to become phagocytic, thereby enabling uptake of noninvasive bacteria (140). In addition, treatment of nonintestinal epithelial cells with CNF1 for 24 h leads to increased expression of F-actin (but not G-actin or monomeric actin) and increased cell volume (153). Treatment of cells with CNF2 also yields striking changes in the distribution of F actin with development of thick stress fibers observable by phalloidin staining (413). These dramatic changes in the cell cytoskeletal architecture precede the development of multinucleation and are thought to be responsible for impairing subsequent cell division. Although it is unclear whether the mechanisms of action of CNF1 and CNF2 are identical, preliminary data suggest that both toxins covalently modify Rho proteins, small GTP-binding proteins related to Ras which modulate actin assembly in eukaryotic cells (153, 155, 413). Available data suggest that CNF1 induces actin polymerization by constitutive activation of the Rho GTPase and that CNF2 covalently modifies Rho by a mechanism not involving ADP-ribosylation or phosphorylation. No data linking the cytoskeletal effects of CNF1 or CNF2 to intestinal pathophysiology are available.

#### *Plesiomonas shigelloides*

*Plesiomonas shigelloides* has been associated with sporadic cases of diarrhea in a variety of countries. Both heat-labile and heat-stable enterotoxins capable of inducing fluid accumulation in rabbit ligated ileal loops without any alteration in intestinal histology have been reported for this species (340, 476). However, the toxins have not been purified and genes encoding these toxins have not been cloned. In addition, a heat-labile toxin that causes elongation of CHO cells similar to that seen with CT is expressed under low-iron culture conditions; the effect on CHO cells is also neutralized by incubation with antiserum against CT (175). It is not known whether the heat-labile toxins described by Gardner et al. (175) and Saraswathi et al. (476) are the same. Studies with DNA probes for CT, LT, ST $_a$ , and ST $_b$  have detected no homologous sequences in *P. shigelloides* (340).

#### *Salmonella typhimurium*

Nontyphoidal *Salmonella* serotypes such as *S. typhimurium* most often cause gastroenteritis with watery diarrhea. However, the pathogenesis of the secretory response to *Salmonella* infection is incompletely understood. In a monkey model of disease, regional differences in the intestinal response to *Salmonella* infection have suggested that both a toxin and invasion of the epithelium by the organism with stimulation of an inflammatory response contribute to secretion (466). In this model, jejunal secretion was observed with only minimal histologic changes and without organism invasion (i.e., consistent with a toxin-mediated mechanism). In contrast, secretion in the ileum and colon paralleled the presence of organism invasion, morphologic damage, and inflammation. Similarly, stud-

ies with ligated rabbit ileal segments in vivo and with Ussing chambers in vitro indicated that invasion is necessary but not sufficient for *Salmonella* strains to elicit secretion (167, 180), suggesting that another factor in addition to invasion is necessary for fluid secretion. These two potential mechanisms for *Salmonella*-stimulated secretion, toxins and invasion, will be discussed separately.

**Toxins.** Lysates of *Salmonella* organisms have been reported to stimulate secretion in 18-h ligated rabbit ileal segments, to elongate CHO cells, and to increase permeability in rabbit skin (181, 421, 424). The secretory response in rabbit ligated ileal segments is associated with increases in cyclic AMP and PGE<sub>2</sub> levels and is neutralized with antisera to CT, suggesting immunologic relatedness of the *Salmonella* enterotoxin to CT or *E. coli* LT-1 (124, 422, 424, 446). This secretory activity is reportedly due to a heat-labile protein toxin with a molecular mass of ca. 25 kDa, which tends to aggregate into complexes of greater than 100 kDa, making purification to homogeneity elusive to date (422, 446). Interestingly, *S. typhi* has also been reported to produce an LT-like enterotoxin with identical biologic activity. However, the structural gene for this toxin has not yet been identified (148).

A chromosomal structural gene for the 25-kDa *Salmonella* toxin (*stn*) has been cloned and encodes a predicted 29-kDa protein (69). The *stn* gene has an atypical initiation codon and lacks an optimal upstream ribosomal binding sequence, resulting in inefficient production of the toxin by the organism (69). Expression of the cloned *stn* gene in *E. coli* has reproduced all of the biological properties ascribed to crude lysates of *Salmonella* organisms (59, 68, 422, 446, 447). Only minor histologic changes have been reported in intestinal epithelium treated with lysates of clones expressing the *Salmonella* enterotoxin (Stn) (422). Curiously, *stn* was cloned from a strain of *S. typhimurium* that does not give a secretory response in rabbit ligated ileal segments, suggesting that other virulence factors may be necessary to elicit an enterotoxic response to this pathogen.

Despite the immunologic relatedness of Stn to CT (59, 68, 422, 446, 447), Stn lacks subunit structure and the biologically active protein is a single polypeptide (59, 69, 422). In addition, the predicted amino acid sequence is quite dissimilar from CT or *E. coli* LT-I, with only limited conservation in two regions including the potential active site of the toxin, which is predicted to be an ADP-ribosylating toxin (69, 422). Similarly, although Stn binds to GM<sub>1</sub> and GM<sub>1</sub> neutralizes biologic activity, the affinity of Stn for GM<sub>1</sub> is ca. 3,000-fold lower than the affinity of CT for GM<sub>1</sub> (446).

Because toxin is produced at only low levels by *S. typhimurium* strains, its role in disease pathogenesis has been questioned. One hypothesis addressing this concern is that invasion facilitates toxin delivery to its presumed intracellular target, adenylate cyclase (424, 446). Consistent with this hypothesis, the cyclic AMP level is elevated in rabbit crypt epithelial cells after *Salmonella* infection (424). However, the cyclic AMP level is also elevated in the lamina propria of *Salmonella*-infected intestine, consistent with either release of Stn by the bacteria or stimulation of adenylate cyclase by prostaglandins formed during the inflammatory response to the bacteria (see below) (424).

Although increases in cyclic AMP and/or prostaglandin levels may account for the secretory response to the *Salmonella* toxin, other mechanisms may play a role in secretion stimulated by the crude *Salmonella* toxin. For example, of several agents tested, the secretory response to a concentrated lysate of *S. typhimurium* was most completely inhibited by a combination of the potential PKC antagonist, H-7, and indomethacin,

an inhibitor of prostaglandin synthesis (278). In addition, the intracellular calcium level was reportedly elevated on Stn-treated tissue and no additional secretory response to the PKC activator, phorbol myristate acetate, was noted in rat small intestine treated with this crude *Salmonella* toxin (278). These data suggest that *Salmonella* toxin or another protein in the lysate may act, in part, through the activation of PKC and/or calcium in the intestinal epithelium. However, in this study, cyclic AMP levels were not measured in the tissue, making correlation with other studies unclear.

In addition to Stn, other investigators have reported one or more heat-labile proteins produced by *Salmonella* strains that stimulate secretion in rabbit ligated ileal segments, elongate CHO cells, and increase permeability in rabbit skin but are not neutralized by antisera to CT or *E. coli* LT or by gangliosides (15, 201, 449). A cytotoxin that inhibits protein synthesis in both Vero cells and rabbit intestinal epithelial cells and causes detachment of Vero and HeLa cell monolayers has also been described (291). Lastly, a heat-labile PLA (ca. 43 to 67 kDa) has been purified from *S. newport* and stimulates secretion in 18-h rabbit ligated ileal segments (388). The importance of any of these additional potential toxins to the secretion observed in *Salmonella* infections is unknown.

**Invasion and/or inflammation.** Invasion of the intestinal epithelium is a well-known virulence trait of *Salmonella* species and has been linked to stimulation of potentially secretory epithelial cell signal transduction pathways including activation of mitogen-activated protein kinase by tyrosine phosphorylation, activation of PLA<sub>2</sub>, and stimulation of an increase in intracellular calcium levels by activation of a host cell PLC or via leukotriene D<sub>4</sub> (414, 465, 469). Invasion by *Salmonella* species has also been linked with tyrosine phosphorylation of the epidermal growth factor receptor (172), although this finding has been disputed by others (165, 465) and a linkage of epidermal growth factor receptor phosphorylation and secretion due to *Salmonella* species has not been proposed. However, invasion alone does not always correlate with secretion (167, 180), suggesting that local activation of these signal transduction mechanisms may not be sufficient to stimulate chloride secretion.

In contrast, a more pronounced inflammatory response to a *Salmonella* strain appears to correlate with stimulation of both cyclic AMP production in intestinal mucosa and the secretory response (181, 466). In addition, secretion is inhibited by indomethacin or depletion of PMNs (179, 181). These data suggest that inflammation may trigger the production of prostaglandins with subsequent adenylate cyclase activation and secretion. Most recently, the ability of a *Salmonella* strain to elicit the transepithelial migration of PMNs in vitro (following stimulation of new protein synthesis in the epithelial cell and the bacterium) was found to correlate with the ability of the strain to cause human enteritis, indicating that this as-yet-unidentified transcellular chemotactic factor is a key virulence mechanism of *Salmonella* species (354, 355). Transepithelial recruitment of PMNs with production and apical delivery of 5'-AMP may then stimulate epithelial cell chloride secretion (330) (see the section on potential mechanisms of intestinal secretion, above). Lastly, the pathogenesis of secretion stimulated by *Salmonella* strains is probably amplified by the ability of *Salmonella* strains to stimulate the production of proinflammatory and/or secretory cytokines (e.g., IL-1, IL-6, IL-8, tumor necrosis factor alpha, and gamma interferon) in the intestinal epithelium (128, 280, 354).



### *Shigella* Species

Although *Shigella* species are known primarily for causing dysentery, a sizable percentage of cases exhibit only watery diarrhea more characteristic of small bowel involvement rather than dysentery. The molecular mechanisms by which *Shigella* organisms invade epithelial cells have been extensively studied (184), but there are no data relating epithelial cell entry and the accompanying cytoskeletal changes to ion transport changes in intestinal epithelial cells.

***Shigella dysenteriae*.** *S. dysenteriae* I produces Shiga toxin, which has secretory activity in the rabbit small intestine as described above (see the section on EHEC). An isogenic strain of *S. dysenteriae* specifically mutated in genes encoding Shiga toxin was studied in monkeys by Fontaine et al. (159). The toxin-positive and toxin-negative strains gave equivalent diarrheal stool volumes, but the stools in animals receiving the toxin-positive strain were consistently more bloody than those in animals receiving the toxin-negative strain. Histopathologic examination revealed that the toxin-positive strain produced profound changes, including destruction of capillary vessels within the connective tissue of the colonic mucosa, severe inflammatory vasculitis of the peritoneal mesothelium, and an influx of inflammatory cells into the colonic mucosa. This study did not support a major role for Shiga toxin in stimulating the diarrhea due to *S. dysenteriae* 1, but it did indicate that the Shiga toxin exacerbates the dysentery seen with this organism. The striking colonic vascular and inflammatory changes observed with the toxin-positive organism (reminiscent of similar results with SLT-I in RDEC-1 [502]), which are not observed when the small bowel intestinal epithelium is treated with purified Shiga toxin (261, 269), could reflect regional intestinal differences in the response to Shiga toxin. Alternatively, the invasion of colonocytes with possible submucosal multiplication of the organisms may deliver Shiga toxin to sensitive target cells (such as the vascular endothelium) in the intestinal wall. Another potential contributor to the inflammatory response to and pathogenesis of infection with *S. dysenteriae* (as well as possibly other *Shigella* spp.) is local production of cytokines, as recently shown in human rectal biopsies (454). The potential impact of cytokines on intestinal secretion has been discussed above in the section on potential mechanisms of intestinal secretion.

The other *Shigella* species do not produce Shiga toxin. There are reports that *Shigella* species other than *S. dysenteriae*, as well as *E. coli* from the normal flora and *E. coli* K-12, produce low levels of Shiga toxin-like activity defined as cytotoxicity in HeLa cells that is neutralizable by antibodies against Shiga toxin (reviewed in reference 400). However, this activity has not been linked to diarrheal disease, and the gene encoding this activity has not yet been cloned.

***Shigella flexneri* 2a.** Two distinct enterotoxins have recently been described in *S. flexneri* 2a that may account for the watery diarrhea that often precedes the onset of scanty dysenteric stools in infections with this species. The genes for both of these toxins have been cloned, and the predicted protein sequences show no apparent similarity to previously described proteins, thus indicating that these are novel toxins.

The first toxin, called ShET1 for *Shigella* enterotoxin 1, is encoded on the chromosome by the *set1* gene (146). Fasano et al. (146) report that the purified toxin increases PD in rabbit ileal tissue mounted in Ussing chambers and induces fluid accumulation in rabbit ileal loops with no apparent histologic damage. Expression of the toxin was detected only under iron-limiting growth conditions. The cloned *set1* genes form two open reading frames; the *set1A* open reading frame encodes a

predicted protein of 20 kDa, while the *set1B* gene encodes a predicted 7-kDa protein. The active toxin is 55 to 60 kDa in size, suggesting a configuration of 1 A subunit to 5 B subunits. Convalescent-phase sera from volunteers experimentally infected with *S. flexneri* 2a neutralize the effect of the toxin, thereby indicating that ShET1 is produced during infection.

The second enterotoxin produced by *S. flexneri* 2a is called ShET2 or the EIEC enterotoxin, and, like ShET1, iron-limiting growth conditions favor expression of this toxin. Nataro et al. (385) have cloned the gene encoding this toxin (*sen*, for *Shigella* enterotoxin 2, previously called *set2* [399]) by employing Ussing chambers to screen for clones capable of increasing the PD in rabbit ileal tissue. Subcloning and DNA-sequencing studies revealed that the active gene contained a single open reading frame encoding a predicted protein of 62.5 kDa. Expression of this gene in a pTAC expression system yielded an active purified toxin migrating as a single band of 63 kDa. The *sen* genes from *S. flexneri* 2a and EIEC share 99% identity and are located on the ca. 140-MDa plasmid associated with invasion in these pathogens. DNA hybridization studies revealed homologous genes in 60 of 80 EIEC strains and 27 of 34 *Shigella* strains examined, including members of all four *Shigella* species. In contrast, the *set1* gene encoding the ShET1 enterotoxin has rarely been found outside of *S. flexneri* 2a (399). An isogenic EIEC strain with a mutated *sen* gene showed significantly lower rises in  $I_{sc}$  in Ussing chambers than did the wild-type parent. However, some enterotoxigenic activity still remained with the mutant, suggesting that EIEC (and possibly *Shigella* spp.) contains another enterotoxigenic moiety distinct from both ShET1 and ShET2. Similar isogenic mutants of *S. flexneri* must be constructed to evaluate the role of ShET1 and ShET2 in *Shigella* pathogenesis.

### *Staphylococcus aureus*

*S. aureus* is a leading cause of food-borne illnesses characterized by the onset of emesis within 6 h of ingestion of one or more preformed heat-stable enterotoxins. Some cases are also associated with diarrhea (451). Clinical illness is attributed to the ability of the organism to produce one or more serologically distinct protein exotoxins named A, B, C1 to C3, D, or E (537). All of these toxins are small proteins (24 to 30 kDa) with similar biological activities but with sequence homology ranging from 30 to 86% (248). The genes encoding the staphylococcal enterotoxins may be chromosomal or plasmid associated or may be carried by members of a polymorphic family of temperate bacteriophages (5, 29). The mechanisms by which these toxins stimulate emesis and diarrhea are not clearly understood. In human disease (417) and after oral challenge of monkeys with purified enterotoxin B (274), a gastritis of rapid onset (within hours) and resolution (within 2 to 4 days) is observed with prominent infiltration of PMNs. All seven toxins induce emesis in monkeys when given intragastrically (537). The emetic reflex is abolished by combined abdominal vagotomy and sympathectomy, suggesting that the toxins stimulate local neural receptors in the upper intestinal tract (133). Additional studies with monkeys suggest a correlation between levels of arachidonic acid metabolites (PGE<sub>2</sub>, leukotriene B<sub>4</sub>, and 5'-HETE/HPETE) in plasma and gastrointestinal illness (245). Consistent with this observation, human colonic epithelial cells (HT29 cells) produce these metabolites after stimulation with enterotoxin B (unpublished results cited in reference 245). Alternatively, the source of these metabolites may be the influx of PMNs noted histologically in human and experimental disease. Studies of the effect of staphylococcal enterotoxins A, B, and/or C on flounder, canine, or rat small



intestine suggest that these toxins can inhibit absorption and/or stimulate secretion, but the mechanism(s) for these responses has not been studied (131, 223, 519). Enterotoxin B has also been shown to activate mast cells in skin via stimulation of primary sensory neurons containing substance P (2). Although similar data in intestinal systems are lacking for this toxin, this observation may be important for the pathophysiology of the disease, because substance P-containing neurons are important in the secretory response to *C. difficile* toxin A in rats (436).

The most exciting recent observation on this class of toxins is the recognition that they can be classified as "superantigens" (238, 248) along with toxins produced by several other bacterial species (e.g., *Streptococcus* spp., *Clostridium perfringens* [CPE]). Notably, the staphylococcal enterotoxins are the most potent T-cell activators known and are able to stimulate lymphocyte proliferation and cytokine production at concentrations of  $10^{-13}$  to  $10^{-16}$  M (248). Superantigens can be presented directly (i.e., they do not require processing by an antigen-presenting cell) to the T-cell receptor by cells containing major histocompatibility complex class II molecules. The major histocompatibility complex class II molecule is the receptor for these toxins. Intestinal epithelial cells express major histocompatibility complex class II molecules and thus can present the staphylococcal enterotoxins to submucosal T cells stimulating cytokine production and proliferation of these lymphocytes. The role of cytokine production in the pathogenesis of the emesis, diarrhea, or histologic changes observed in response to the staphylococcal enterotoxins is unknown but is likely to be significant (see the section on potential mechanisms of intestinal secretion). However, the ability of these toxins to act as superantigens is crucial to the biological responses such as fever, hypotension, and enhancement of endotoxin lethality noted when these proteins are administered systemically. Some, but not all, preliminary data suggest that different domains of these toxin molecules may account for their emetic and mitogenic activities (36, 248, 511).

Almost all human clinical isolates of *S. aureus* also produce delta toxin, a 26-amino-acid (approximately 3-kDa) amphipathic protein exotoxin classified with the staphylococcal cytolysins or hemolysins (i.e., alpha, beta, gamma, and delta toxins). The contribution of this toxin to the pathogenesis of human intestinal or extraintestinal disease is unknown. This toxin is of interest because in vivo and in vitro data obtained with guinea pig ileum indicate that the toxin rapidly alters intestinal transport by increasing potential difference and decreasing resistance (264, 265, 403). Higher concentrations of toxin also alter the histology of the intestinal epithelium (402). The changes in potential difference and resistance precede any evidence of histologic damage. Although delta toxin stimulates delayed increases in cyclic AMP levels in guinea pig intestinal epithelium, the rapid onset of secretion suggests that mechanisms other than cyclic AMP account for the secretory response (402, 403). The amphipathic nature of the peptide with separate hydrophilic and hydrophobic faces has led to the hypothesis that association of six molecules of the toxin may create a transmembrane "pore" lined by the hydrophilic portions of the monomers (27, 264). Consistent with this hypothesis, subsequent experiments revealed that treatment of planar lipid bilayers with  $<1$   $\mu\text{g}$  of delta toxin per ml resulted in the appearance of weakly cation-selective ion channels with voltage-dependent and -independent characteristics (275, 363). Data indicating that delta toxin forms channels in intestinal epithelial cells are lacking. Alternatively, delta toxin has been reported to activate  $\text{PLA}_2$  in mouse fibroblasts, releasing arachidonic acid and stimulating the synthesis of prostaglandins (27). If this mechanism is operative in intestinal epithelial

cells, prostaglandins could contribute to the secretory response to delta toxin.

### *Vibrio cholerae*

***V. cholerae* O1 or O139.** The severe diarrheal scourge, cholera, is caused by *V. cholerae* of the O1 and O139 serogroups. The prototypic rapidly dehydrating noninflammatory diarrhea caused by this bacterium is largely due to its ability to produce CT. Volunteer studies showed that ingestion of 25  $\mu\text{g}$  of pure CT produced severe diarrhea exceeding 20 liters of rice water stools (308). However, CT-negative mutants of *V. cholerae* still cause mild to moderate diarrhea in some individuals (310). This observation has led to the discovery of two additional toxins produced by *V. cholerae*, Zot (for zonula occludens toxin) and Ace (for accessory cholera enterotoxin). Data on these *V. cholerae* toxins and how they may contribute to the pathogenesis of diarrhea in cholera are presented below. For detailed discussions of and primary references for the *V. cholerae* toxins and the genetics of toxin expression, the reader is referred to recent reviews (262, 263, 510).

**(i) Cholera toxin.** CT is the prototypic A-B subunit toxin (A/B ratio, 1:5), where B is the subunit (11.6 kDa) responsible for binding of the holotoxin to its  $\text{GM}_1$  receptor and A is the subunit responsible for the intracellular changes in cyclic AMP levels. The A subunit consists of two components generated by proteolysis, A1 (21.8 kDa), containing ADP-ribosyltransferase activity, and A2 (5.4 kDa), which links the A1 and B subunits. X-ray crystallography reveals the structure of CT to consist of five B subunits forming a pentamer with a central "barrel" or pore, where the carboxy terminus of A2 sits; A2 is connected to the triangular A1 subunit by a long alpha helix. The cellular receptor for the B subunit of CT is the  $\text{GM}_1$  ganglioside, which is present on all eukaryotic cells and accounts for the ubiquitous effects of CT. CT acts by ADP-ribosylation of the alpha subunit of the GTP-binding protein, Gs, which stimulates adenylate cyclase activity. Upon ADP-ribosylation, the intrinsic GTPase activity of the alpha subunit of Gs is inhibited resulting in constitutive activation of the adenylate cyclase. The onset of action of CT occurs with a 15- to 60-min lag time, accounted for by the time it takes for A1 to translocate the cell membrane and be delivered to its substrate. Because the majority of Gs in the intestinal epithelial cell is located in the brush border membrane and the adenylate cyclase is located in the basolateral membrane, the precise sequence of events in CT activation of adenylate cyclase remains unclear. Potential models of activation include (i) A1 translocation through the membrane with diffusion through the cytoplasm to the adenylate cyclase; (ii) A1 modification of brush border membrane Gs, which then diffuses to the basolateral membrane to activate adenylate cyclase; and (iii) endocytosis of CT with delivery of active A1 in the endosomal membrane to the basolateral adenylate cyclase (reviewed in references 262 and 263; specific references on polarized intestinal epithelial cells include references 303 and 304). Although further investigation is necessary to distinguish among these possibilities, most recent data support the third model. Notably, in intestinal epithelial cell monolayers (T84), brefeldin A, a reversible inhibitor of trans-Golgi vesicular transport, appears to inhibit CT activity by blocking the transport of CT-containing membranes into a compartment where reduction and presumably translocation of the A1 subunit to act on  $\text{Gs}\alpha$  occurs (303).

Figure 3 depicts potential mechanisms by which CT may contribute to intestinal secretion. The first-recognized and most ubiquitous activity of CT is stimulation of intracellular

cyclic AMP production via activation of adenylate cyclase. In rabbit ileum *in vivo*, this results in a slow onset of inhibition of NaCl absorption and stimulation of Cl<sup>-</sup> secretion (149), similar except in time course to the effect of *E. coli* STa on ion transport in this tissue (see the section on ETEC, above). In a human intestinal epithelial cell line (T84) studied in Ussing chambers *in vitro*, membrane-permeant analogs of cyclic AMP stimulate chloride secretion, most probably mediated by up-regulation of CFTR and/or activation of A kinase with phosphorylation of CFTR, a major chloride channel present in intestinal epithelia (63, 96, 171, 428, 517, 536). In addition, cyclic AMP may directly or indirectly activate other ion-transporting proteins such as an outwardly rectifying chloride channel or the Na/K/2Cl cotransporter which contribute to the chloride secretory response (200, 342, 344). Together, these data strongly imply that elevation of intracellular cyclic AMP levels by CT stimulates secretion of chloride by intestinal epithelial cells. However, a combination of *in vivo* and *in vitro* observations also suggests that the pathogenesis of secretion in response to CT may be considerably more complex. Most probably, the additional mechanisms described below by which CT may stimulate intestinal secretion serve to augment and/or supplement (but do not supplant) cyclic AMP-mediated intestinal secretion.

First, *in vitro* and *in vivo* data strongly implicate prostaglandins of the E series (PGE<sub>1</sub> and PGE<sub>2</sub>) (423, 425, 426, 540) and, recently, platelet-activating factor (PAF) (196) in the pathogenesis of intestinal secretion stimulated by CT. In ligated rabbit ileal segments, CT stimulates the release of both cyclic AMP and PGE<sub>2</sub> (425, 540); in the same system, the secretory response to cyclic AMP was less pronounced than the secretory response to PGE<sub>2</sub>. Notably, in one study, the increase in PGE<sub>2</sub> was noted in the absence of an inflammatory response in the intestinal epithelium, suggesting epithelial cell production of PGE<sub>2</sub> (540). Similarly, in *in vitro* studies with the human colonic epithelial cell line HT29/C1.19A, de Jonge (91) found that mepacrine, an inhibitor of PLA<sub>2</sub>, inhibited the secretory response to CT. Together, these data suggest that CT activates directly (e.g., via ADP-ribosylation of Gs in the apical membrane of the intestinal epithelial cell) or indirectly (e.g., via cyclic AMP activation of PKA) an epithelial cell PL. Recent studies with rabbit intestinal mucosa and CHO cells have attempted to delineate the order in which these intracellular signals occur. It is unclear whether the elevation in epithelial cell cyclic AMP level stimulated by CT in rabbit intestinal mucosa is responsible for the production of eicosanoids (PGE<sub>2</sub> and PGF<sub>2α</sub>, and leukotriene C<sub>4</sub>) detected in the secreted fluid (423). Notably, in this study, dibutyl cyclic AMP, but not CT, diminished eicosanoid production in adult rabbit ligated ileal loops, suggesting that cyclic AMP is not the intermediary leading to the synthesis of eicosanoids *in vivo* following CT treatment. However, cyclic AMP did appear to elicit 5-HT release, presumably from enterochromaffin cells, with subsequent activation of the enteric nervous system (423, 426; also see below). In contrast, preliminary data with CHO cells suggest that CT stimulates an increase in cyclic AMP levels which increases PAF levels. PAF then stimulates an increase in PGE<sub>2</sub> levels, which precedes the detection of CHO cell elongation (528). To date, although CT stimulates PAF production by intestinal epithelial cells and PAF antagonists inhibit CT-stimulated intestinal secretion (196), the relationships between cyclic AMP, PAF, and prostaglandins have not been clearly established in an intestinal model. Another potential site of production of prostaglandin may be the submucosal compartment of the intestinal epithelium. The mechanism(s) by which CT might stimulate arachidonic acid metabolism in the submucosa is

unknown. Potentially, diffusion of PAF from the epithelial cells may activate PLA<sub>2</sub> in submucosal cells (196) or CT may stimulate 5-HT release from enterochromaffin cells, which, in turn, stimulates submucosal prostaglandin synthesis (31).

Second, CT alters the activity of the enteric nervous system, possibly by stimulating peptide hormone release by intestinal enterochromaffin cells and/or by increasing the smooth muscle activity of the small bowel. Serotonin (5-HT) and VIP are released into the human small bowel *in vivo* after treatment with CT (389). Both of these hormones can directly stimulate intestinal epithelial cell secretion, suggesting a possible role for these hormones in CT-induced secretion. Conversely, a recent study disputes the role of serotonin in mediating CT-stimulated secretion in human jejunum (129). Serotonin can also stimulate neuronal release of VIP, and the increased release of VIP can be blocked by the ganglionic blocker tetrodotoxin, indicating a neuronal source for the VIP. Furthermore, recent evidence reveals that CT specifically binds to and activates VIP-containing neurons in the intestinal submucosa of guinea pigs (246). Nocerino et al. (398) have also implicated the enteric nervous system in the mediation of CT-stimulated intestinal secretion by separately perfusing the jejunum and colon in the same rat. Addition of CT to the jejunum not only induced a local secretory response in this part of the intestine but also resulted in secretion in the colon which was not exposed to CT. This distal secretory effect was eliminated by transecting the bowel between the jejunum and colon, thus interrupting the enteric nervous system. In addition, rabbit ileum treated *in vivo* with CT develops a highly organized myoelectric response, resulting in smooth muscle contractions and aboral propulsion of intraluminal contents (338). These data and additional *in vivo* data showing that several neurotransmitter and ganglionic blockers inhibit CT-induced secretion strongly support a role for the enteric nervous system in the pathogenesis of cholera (47–50).

Third, CT may alter the barrier function of the intestinal epithelium. Although reports on the effect of CT on intestinal permeability in the small bowel (62, 540) vary, CT treatment increased intestinal permeability in the rat (332). Thus, similar to Zot (see below), a change in the paracellular pathway may yield a more “leaky” epithelium enhancing the fluid secretion stimulated by CT. However, the classic study by Field et al. (149) of CT action on rabbit ileal tissue in Ussing chambers, as well as subsequent work (443), revealed a significant increase in tissue resistance after treatment with crude or purified CT. Similarly, in contrast to untreated or volume-expanded controls, morphologic evaluation of rabbit intestinal tissue after CT treatment revealed close opposition of the lateral membranes of the intestinal epithelial cells by light and electron microscopy, consistent with a high tissue resistance (103). However, recent ultrastructural studies of duodenal biopsy specimens from patients infected with *V. cholerae* O1 revealed widening of the villus intercellular spaces and alteration of apical junctional complexes (336). Whether these changes are secondary to CT or other virulence factors of *V. cholerae* O1 in humans is unknown.

Fourth, recent data suggest that CT as well as certain *V. cholerae* O1 strains may stimulate a modest intestinal inflammatory response. CT treatment of rat intestinal epithelial cells *in vitro* or mouse ligated intestinal loops stimulates production of the proinflammatory cytokine IL-6 (280, 359), thereby activating the enteric immune system and potentially generating arachidonic acid metabolites such as prostaglandins or leukotrienes that stimulate chloride secretion. Duodenal biopsies of patients infected with *V. cholerae* O1 also revealed modest inflammation (336). In addition, stools of human volunteers

infected experimentally with an El Tor  $\Delta ctxA \Delta zot \Delta ace$  vaccine strain contained high levels of lactoferrin, which is normally found in the secondary granules of PMNs (500). The mechanism by which this strain stimulates an inflammatory response is unknown. However, it seems likely that this inflammatory response may account, at least in part, for the residual reactivity and diarrhea observed in these volunteers (521) (see the section on potential mechanisms of intestinal secretion, above).

**(ii) Other *V. cholerae* O1/O139 toxins.** The second toxin identified in *V. cholerae* O1 strains is Zot (for zonula occludens toxin). The *zot* gene encodes a predicted 44.8-kDa peptide (21), whose native form has not yet been purified. The *zot* gene is present in most O1 and O139 strains, and if a strain is CT positive, it is almost always *zot* positive (249). Crude Zot diminishes the resistance of rabbit ileal tissue in Ussing chambers without causing detectable changes in potential difference (143). The onset of action of crude Zot is immediate, and the activity is reversible. Electron microscopy has revealed penetration of an electron-dense marker into the zonula occludens of rabbit ileum after Zot treatment, and freeze-fracture analysis of the zonula occludens reveals a marked decrease in the number of junctional strands. Furthermore, Zot has been reported to cause F-actin rearrangement in rat intestinal epithelial cells (IEC-6 cells) in vitro and rabbit ileum in vivo (144). In an endothelial cell line, Zot treatment increased the proportion of F to G actin (144). Together, these observations indicate an effect of Zot on the zonula occludens or epithelial tight junctions, possibly through a rearrangement of F actin, and suggest that Zot may contribute to diarrhea in cholera by altering the permeability of intestinal tissue. Several signal transduction mechanisms (e.g., calcium, PKC, tyrosine kinase, cyclic AMP) have been shown to regulate tight junctions in intestinal epithelial cells (483, 561), and current data implicate PKC in the response of intestinal cells to Zot (144). Evidence for in vivo expression of Zot is found in serum from volunteers experimentally infected with wild-type or attenuated *V. cholerae* strains. Convalescent-phase but not prechallenge sera from these volunteers are capable of neutralizing the effect of Zot in Ussing chambers (142). Despite these data, a role for Zot in the diarrhea due to  $\Delta ctxA$  strains of *V. cholerae* is not supported by volunteer data (521). In this study, a  $\Delta ctxA \Delta zot \Delta ace$  derivative of El Tor Ogawa *V. cholerae* remained reactogenic in human volunteers (similar to a  $\Delta ctxA$ -alone strain), suggesting, at most, a limited role for Zot and Ace (see below) in the secretory response to *V. cholerae*.

The third toxin identified in *V. cholerae* O1 is Ace (for accessory cholera enterotoxin), a 11.3-kDa protein encoded by the *ace* gene (541). Although this toxin has not yet been purified, the predicted protein sequence shares homology with eukaryotic ion-transporting proteins including CFTR and calcium pumps. Ace is predicted to be an amphipathic molecule, leading to the hypothesis that it forms multimers which insert into the eukaryotic cell membrane, creating an ion-permeable pore. Direct proof of this hypothesis is not yet available. In rabbit ileal tissue mounted in Ussing chambers, crude Ace stimulates a delayed increase in  $I_{sc}$  and PD. Inoculation of ligated rabbit ileal segments with a CT- and Zot-negative *V. cholerae* strain containing the cloned *ace* gene results in fluid accumulation. Both of these observations are consistent with Ace stimulating electrogenic chloride secretion, which may contribute, in a limited fashion (see above), to the pathogenicity of *V. cholerae* O1.

The genes encoding CT (*ctxAB*), Zot (*zot*), and Ace (*ace*) are located on a 4.5-kb chromosomal "core region" or "virulence cassette" (541). These genes are flanked by repeated se-

quences (RS1 elements) that could potentially lead to amplification or deletion of all three toxin genes as a unit (183). Thus, given this close alignment of one known (*ctxAB*) and two potential (*zot* and *ace*) virulence genes, important studies which remain to be done include examining the pathophysiologic interactions of CT, Zot, and Ace. Although it is unclear whether these potential interactions contribute to disease, these studies could yield new insights into the regulation of tight junctions and ion transport in the intestinal epithelium.

*V. cholerae* O1 and O139 strains also produce a cytotoxic protein, which produces fluid accumulation in animal models. This toxin, known as the El Tor hemolysin or hemolysin-cytolysin, is cytotoxic for a variety of erythrocytes and mammalian cells in culture and rapidly lethal for mice (217). The cytotoxin is initially made as an 82-kDa protein and processed in two steps to a 65-kDa active protein (584). When added to planar lipid bilayers, this toxin forms anion-selective channels (294). Injection of the purified toxin into rabbit ileal loops produces fluid accumulation, and the accumulated fluid is usually bloody with mucus (230). The purified cytotoxin also produced fluid accumulation in suckling mice within 1 h and reached a maximum level at 3 h (230). Histologic examination of the mouse intestinal tissue after toxin addition showed considerable villus degeneration, accumulation of cellular exudate and mucin, and a collection of inflammatory cells in the lamina propria (346). A *V. cholerae* strain specifically deleted for the *ctx* genes was further mutated to inactivate the *hlyA* gene encoding this cytotoxin. Ingestion of this strain by volunteers still produced the mild diarrhea and nondiarrheal symptoms seen in infections with other CT-negative strains, thus casting doubt on the importance of this cytotoxin in human disease (310).

***V. cholerae* non-O1 or non-O139 strains.** Strains of *V. cholerae* belonging to serogroups other than O1 and O139 occasionally cause diarrhea or extraintestinal infections. The diarrhea associated with these serogroups can range from mild illness to profuse, watery diarrhea comparable to that seen in patients infected with *V. cholerae* O1 (377). No toxins unique to *V. cholerae* non-O1/non-O139 serogroups have been reported, but some strains of these serogroups may produce one or more toxins that have been previously characterized in *V. cholerae* O1 or other species (218). Most *V. cholerae* non-O1/non-O139 strains produce a cytotoxic protein apparently identical to the El Tor hemolysin (hemolysin-cytolysin) of *V. cholerae* O1 (583). The majority of non-O1/non-O139 strains do not contain genes encoding CT, Zot, or Ace, but a few strains have been shown to possess genes for these toxins (249). Some strains of *V. cholerae* non-O1/non-O139 produce a 17-amino-acid heat-stable enterotoxin (designated NAG-ST for nonagglutinable *Vibrio* ST) that shares 50% sequence homology with the STa of ETEC (8). In a volunteer study, one subject who ingested a CT-negative *V. cholerae* non-O1 strain producing NAG-ST purged over 5 liters of diarrheal stool (379). This toxin is found only in a minority of non-O1/non-O139 *V. cholerae* strains. In one study, 6.8% of *V. cholerae* non-O1 strains from Thailand and none of the strains from the United States or Mexico produced this toxin (215). In another study, 2.3% of all non-O1 *V. cholerae* strains isolated from Calcutta, India, contained genes for this toxin (416). The *tdh* gene encoding the thermostable direct hemolysin of *V. parahaemolyticus* (see below) has also been found in *V. cholerae* non-O1/non-O139 strains (220). In one strain, the *tdh* gene was found on a plasmid, suggesting that this toxin gene could be readily transferred to other species.



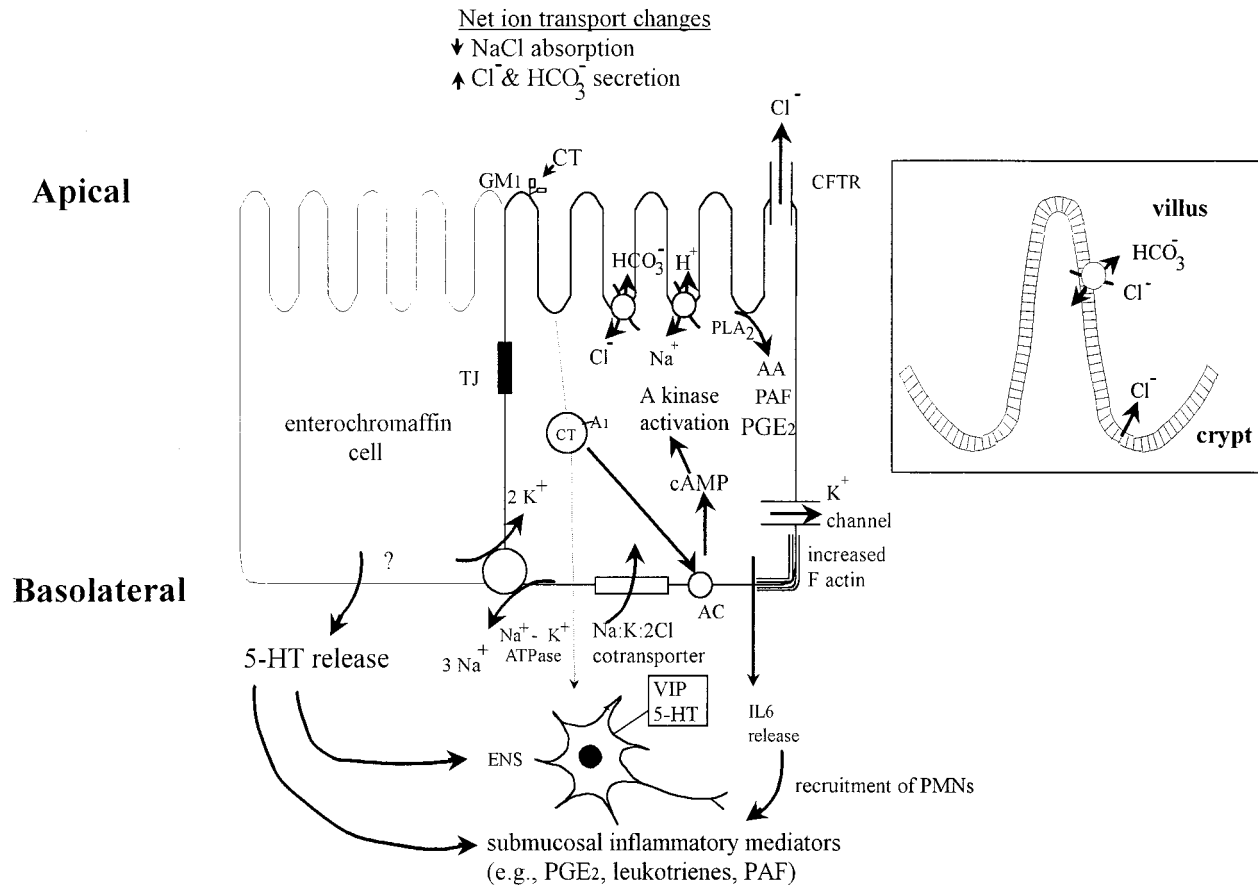


FIG. 3. Model of the pathogenesis of noninflammatory diarrhea for CT. This figure presents potential mechanisms by which CT may act to stimulate intestinal secretion. Although NaCl absorption and chloride secretion are primarily functions of intestinal villus and crypt cells, respectively, a single ion-transporting intestinal epithelial cell is shown for ease of presentation. Some of the depicted data are derived from specific studies of the action of CT, and others are derived from studies of the effect of cyclic AMP analogs or other cyclic AMP-dependent agonists on intestinal secretion. Binding of CT to an ion-transporting intestinal epithelial cell most probably leads to endocytosis of CT with delivery of the active A1 subunit to the basolateral adenylate cyclase (AC). A small amount of CT may also be delivered intact to the submucosal compartment. Activation of adenylate cyclase increases intracellular cyclic AMP levels, leading to A kinase activation, which regulates intestinal epithelial cell transporters, as well as possibly other host cell enzymes (e.g., PLA<sub>2</sub>). Increases in intestinal epithelial cell cyclic AMP levels result in inhibition of NaCl absorption and stimulation of chloride secretion. Stimulation of chloride secretion is dependent on coordinate interaction of several intestinal epithelial cell transporters including the basolaterally located Na/K/2Cl cotransporter, Na<sup>+</sup>/K<sup>+</sup> ATPase, and K<sup>+</sup> channels and the apically located CFTR. As chloride ions diffuse out of the crypts, the apical membrane Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger (present in the more highly differentiated epithelial cells higher on the crypt-villus axis) is stimulated, resulting in enhanced HCO<sub>3</sub><sup>-</sup> secretion (see the inset). This sequence of ion transport changes may account for the striking HCO<sub>3</sub><sup>-</sup> losses in the stool and the systemic acidosis reported in clinical cholera. Increases in cyclic AMP levels also appear to upregulate expression of both CFTR and Na/K/2Cl cotransporters in intestinal epithelial cell membranes and affect cytoskeletal proteins including microtubules (involved in upregulation of CFTR) and F actin. After cyclic AMP stimulation, F-actin staining is denser at the basolateral membrane of the cell and F actin regulates the activity of the Na/K/2Cl cotransporter. Cyclic AMP may also regulate tight junctional permeability, which potentially contributes to intestinal secretion. Additional mechanisms also are likely to contribute to the intestinal secretion observed in response to CT. First, CT stimulates the release of arachidonic acid metabolites (AA) such as prostaglandins (e.g., PGE<sub>2</sub>) and PAF, although the precise site of production of these mediators is unclear. Second, CT secretion involves the enteric nervous system (ENS) and/or increased smooth muscle activity of the bowel wall (not depicted). The release, for example, of 5-HT or VIP from either enterochromaffin cells and/or the enteric nervous system can directly stimulate intestinal epithelial cell secretion. Delivery of CT to the submucosal compartment after endocytosis may also enable CT to bind directly to and stimulate neurons of the enteric nervous system. Recent data also reveal that CT stimulates secretion in bowel segments not exposed to the toxin via stimulation of the enteric nervous system. Third, a modest intestinal inflammatory response has been observed in patients infected with *V. cholerae* O1, and CT stimulates intestinal epithelial cells to produce IL-6, a proinflammatory cytokine, suggesting that submucosal inflammatory mediators may contribute to the secretory response to CT. See the sections of text on potential mechanisms of intestinal secretion and on *V. cholerae* for detailed discussion and references.

### *Vibrio fluvialis*

*V. fluvialis* was named and its association with human disease was heightened after being isolated from over 500 patients with apparently inflammatory diarrhea in Bangladesh (228). This organism is also occasionally isolated from cases of diarrhea linked to the consumption of raw oysters in the United States. Both whole cultures and culture supernatants produce rapid (ca. 4 h after inoculation) fluid accumulation in suckling mice (321, 394, 395). At least three potential toxins have been described for this species: a CHO cell cytotoxin, a CHO cell-rounding toxin, and a CHO cell elongation factor, which are all

heat-labile factors (320, 321). Crude concentrates of each factor cause fluid accumulation in infant mice (321). Only the CHO cell-rounding toxin appears to stimulate secretion (sometimes hemorrhagic) in ligated rabbit ileal loops (321). In contrast, *V. fluvialis* infection of mice leads, in some cases, to hemorrhagic intestinal secretion, suggesting that other virulence factors are produced by these organisms (321). None of these CHO cell-active proteins have been purified, nor have structural genes for these proteins been identified. However, the CHO cell elongation factor and CHO cell cytotoxin have been partially purified and found to be 45- and 12.2-kDa pro-



teins, respectively (495, 565). No histopathologic or cultured intestinal cell studies have been reported for any of these toxins.

#### *Vibrio hollisae*

*V. hollisae* causes diarrhea and septicemia in humans, with most cases linked to the consumption of raw seafood. In addition to producing a toxin similar to the thermostable direct hemolysin of *V. parahaemolyticus* (see below), *V. hollisae* produces a toxin which causes elongation of CHO cells (293). The purified *V. hollisae* toxin (HT) has a molecular weight of 80,000, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and no apparent subunit structure. The toxin is heat labile (30 min at 56°C) and causes fluid accumulation in suckling mice. HT does not react with antisera against CT, and no DNA sequences homologous to *ctx* have been found (293). Although HT can cause elongation of CHO cells, there is no accompanying increase in intracellular cyclic AMP levels in this cell line and the mechanisms by which HT leads to CHO elongation and intestinal secretion are unknown. As discussed above, it is important to note that this toxin and the *Aeromonas* toxin described by McCardell et al. (347) represent the first reported dissociations between CHO cell elongation and detection of an increase in intracellular cyclic AMP levels.

#### *Vibrio parahaemolyticus*

*V. parahaemolyticus* is an important cause of diarrheal illness resulting from ingestion of contaminated seafood, particularly in Japan. The major diarrheagenic factor produced by *V. parahaemolyticus* is a 23-kDa protein called TDH or the Kanagawa phenomenon hemolysin. This toxin was first discovered and studied on the basis of its ability to lyse several species of erythrocytes. For many years, the only linkage of this toxin to diarrheal disease was the near-exclusive isolation of TDH-positive strains from diarrheal stools whereas nonclinical isolates of this species were usually TDH negative (reviewed in reference 392). Volunteer studies showed that TDH-positive strains caused diarrhea whereas TDH-negative strains did not cause diarrhea, even at inocula of  $10^{10}$  (475). Using recombinant DNA techniques, Nishibuchi et al. (390) constructed an isogenic mutant strain in which the chromosomal *tdh* genes were specifically mutated and compared the activity of this strain with that of the TDH-positive parent strain from which it was derived. The TDH-positive strain caused fluid accumulation in ligated rabbit ileal loops, whereas the TDH-negative strain caused no fluid accumulation. Culture supernatants from the TDH-positive strain increased  $I_{sc}$  in rabbit ileal tissue mounted in Ussing chambers, whereas supernatants from the TDH-negative strain did not increase  $I_{sc}$ . The  $I_{sc}$  response was blocked by preincubation with the trisialoganglioside GT1<sub>b</sub> but not GM<sub>1</sub>, suggesting that GT1<sub>b</sub> is the intestinal receptor for TDH in the rabbit ileum. Further work with purified TDH in Ussing chambers suggests that TDH stimulates chloride secretion, based on the abrogation of the  $I_{sc}$  increase when sulfate ion replaces chloride ion in the Ringer's solution bathing the tissue (450). Inhibition of the  $I_{sc}$  response by preincubation with the calcium-buffering compound BAPTA/AM implicates calcium as the intracellular mediator of intestinal secretion due to TDH. In the rat intestinal cell line IEC-6, TDH caused a rapid, greater than 10-fold increase in intracellular calcium levels that gradually returned to baseline values over ca. 15 min. This time course correlates well with the  $I_{sc}$  increase in rabbit ileal tissue, further supporting a role for intracellular calcium in mediating the observed chloride secretion.

Strains of *V. parahaemolyticus* also produce a second hemolysin called the TDH-related hemolysin (TRH); the gene for TRH shares ca. 69% nucleotide homology with the *tdh* gene (396). Although TRH has been linked epidemiologically to diarrheal disease (499), there are no reported studies on the secretory activity of this toxin. Other toxins produced by *V. parahaemolyticus* include a PLA<sub>2</sub> (thermolabile hemolysin) (498) and a factor causing morphological changes in CHO cells similar to those produced by CT (221). However, there are no data supporting a role for these toxins in disease.

#### Other *Vibrio* spp.

*V. mimicus* has also been implicated in diarrheal disease and may produce TDH and possess the *tdh* gene (393). Some *V. mimicus* strains may produce an ST-like toxin (452). Other marine vibrios produce cytolytic toxins unrelated to TDH, but only a cytotoxin purified from *V. metschnikovii* has been shown to cause fluid accumulation in an animal model. *V. metschnikovii* is rarely isolated from clinical specimens, but one strain isolated from a stool sample from a patient with diarrhea was studied and a 50-kDa cytotoxin immunologically unrelated to other *Vibrio* toxins was purified (370). This toxin was capable of lysing several species of erythrocytes as well as Vero and CHO cells. More significantly, the purified toxin was found to cause fluid accumulation in infant mice and increased vascular permeability in rabbit skin (370). No further information about potential secretogenic mechanisms is available.

#### *Yersinia enterocolitica*

*Y. enterocolitica* produces several disease syndromes including watery diarrhea, ulcerative enterocolitis, and mesenteric adenitis unassociated with diarrhea. Strains of *Y. enterocolitica* secrete a heat-stable enterotoxin (Yst for *Yersinia* heat-stable toxin) postulated to be important in the pathogenesis of the watery diarrhea syndrome. Yst is the product of a chromosomal gene (*yst*) encoding a 71-amino-acid preprotoxin that is processed, similar to *E. coli* STa, in two proteolytic steps to a biologically active 30-amino-acid protein (94). Most, if not all, pathogenic *Y. enterocolitica* strains contain DNA sequences homologous to *yst*. The carboxy terminus of this peptide is highly homologous with *E. coli* STa in residues that form the active site of the toxin for stimulation of intestinal particulate GC-C (524), and purified Yst increases cyclic GMP levels in mouse intestine and cultured cell lines (232). Thus, the mechanism by which Yst stimulates secretion is thought to be similar or identical to that used by *E. coli* STa. Because Yst is produced in vitro only at temperatures below 30°C, its relevance to disease pathogenesis has been questioned (94). However, recent examination of *yst* deletion mutants in a rabbit model of intestinal disease indicates that the *yst* gene is expressed in vivo and is associated with virulence (93). In addition, *yst* transcription can be induced at 37°C by increasing the osmolarity and pH of the culture medium to values normally present in the ileum lumen (366).

It has also been proposed that *Y. enterocolitica* can produce a second heat-stable, small (ca. 10- to 30-kDa) protein toxin that is active in suckling mice (similar to *E. coli* STa); this toxin has tentatively been called Yst-II (459). Although it is immunologically cross-reactive with Yst, Yst-II is genetically distinct, with no detectable hybridization to a *yst* gene probe. Crude Yst-II increases  $I_{sc}$  in rabbit ileum without altering the response to cyclic GMP or cyclic AMP agonists and, similarly, does not elevate cyclic nucleotide levels in the T84 human intestinal epithelial cell line, suggesting a cyclic nucleotide-

independent mechanism of action. How commonly *Y. enterocolitica* strains produce Yst-II is unknown.

### ENTERIC BACTERIAL TOXINS: MECHANISTIC CLASSIFICATION

The extent to which the mechanism of action of each enteric bacterial toxin is understood varies considerably. In a few instances, precise molecular details on the mechanism of action of a toxin are available; in most instances, the enteric bacterial toxins can be grouped by a type of activity (e.g., cytotoxin and enterotoxin) but even preliminary specific mechanistic information is lacking. Table 6 categorizes the enteric bacterial toxins into three groups: (i) toxins with established mechanisms of action; (ii) toxins with possible or postulated mechanisms of action; and (iii) toxins with identified activities but unknown mechanisms of action. A mechanism of action is considered to be established only if the molecular mechanism is known and linkage to intestinal secretion has been demonstrated. Notably, certain toxins appear in more than one list. For example, while the mechanism by which CT elevates intracellular cyclic AMP concentrations is established at the molecular level, the mechanisms by which neural activity contribute to the secretory response to CT are not clearly delineated. The details of each toxin's mechanism of action as currently understood have already been summarized, and detailed references are available above in the section on the functional classification of enteric bacterial toxins by genus and species. In this section, the discussion will focus on the extent of information available on particular mechanisms by which these toxins act and the limitations of these data. In the section on possible or postulated mechanisms of action (below), only limited examples are provided to illustrate the approach used in this review to categorize toxins by potential mechanisms of action.

#### Toxins with Established Mechanisms of Action

Established mechanisms of action for enteric or nonenteric toxins are typically categorized into three general groups: (i) toxins with intrinsic enzymatic activity (most often, these toxins have an A-B structure in which the B subunit mediates cell binding and the enzymatically active A subunit is translocated across the eukaryotic cell membrane to its cytosolic substrate); (ii) toxins that bind to a receptor stimulating the release or production of a second messenger (e.g., calcium and cyclic GMP), most often with subsequent stimulation of a protein kinase; and (iii) toxins that insert directly into the cell membrane, creating an ion-permeable pore (582). Enteric toxins have been definitively demonstrated only to have either intrinsic enzymatic activity (e.g., CT) or to stimulate the release of a second messenger, namely, cyclic GMP, in response to *E. coli* STa. Conspicuously absent from the list of established mechanisms of enteric bacterial toxin action are toxins known to stimulate secretion by calcium/PKC-dependent, calcium/calmodulin-dependent, or tyrosine kinase-dependent mechanisms and toxins that stimulate secretion by formation of an ion pore. Initial data on several toxins (particularly *E. coli* STb and *V. parahemolyticus* TDH) are very suggestive of a calcium-dependent mechanism, but the mechanism is not definitively established because either direct measurements of intracellular calcium levels are missing or the detection of the potential intracellular mediator has not been correlated with a secretory response (see below). Similarly, membrane insertion with creation of an ion-permeable pore is not an established mechanism of action linked to intestinal secretion for any enteric

bacterial toxin and thus is discussed below under possible or postulated mechanisms of action.

Among the established mechanisms of action, the best-understood mechanisms at the molecular level are the ADP-ribosyltransferase activity of CT and *E. coli* LT (262, 263, 510), the *N*-glycosidase activity of Shiga and Shiga-like toxins (526), the ADP-ribosyltransferase activity of the *C. botulinum* C2 toxin (556), and, only recently discovered, the glucotransferase activity of *C. difficile* toxins A and B (256, 258). In each of these examples, the specific residue modified by the enzymatic activity of the toxin has been identified. CT, *E. coli* LT, and the Shiga and Shiga-like toxins are all examples of A-B toxins; *C. botulinum* C2 toxin is a two-component toxin (not covalently or noncovalently linked) in which component I contains the ADP-ribosyltransferase activity; and *C. difficile* toxins A and B are single polypeptides. For CT and *E. coli* LT, the ADP-ribosyltransferase of the toxin transfers the ADP-ribose moiety of NAD to arginine 201 of the alpha subunit of the GTP-binding protein, Gs, leading to the constitutive activation of adenylate cyclase and thereby raising intracellular levels of cyclic AMP. The *N*-glycosidase activity of the Shiga and the Shiga-like toxins cleaves adenine 4324 from the 3' end of the 28S rRNA component of the eukaryotic ribosomal complex, inhibiting peptide elongation and terminating protein synthesis. The C2 toxin of *C. botulinum* ADP-ribosylates arginine 177 of G actin, preventing polymerization of G to F actin. Each of these toxins is secretory in one or more experimental intestinal or intestinal epithelial cell models. *C. difficile* toxins A and B modify threonine 37 of Rho subtype proteins by addition of a single molecule of glucose, resulting in inactivation of Rho and dissociation of F-actin microfilaments (256, 258). Although this modification has not been linked to secretion or identified in intestinal epithelial cells, these toxins have been tentatively included in this group because the primary action of *C. difficile* toxins A and B on intestinal epithelial cells is not to induce secretion but to alter the cellular cytoskeleton (see the section on *C. difficile* [above] for a detailed discussion). Thus, this recently identified modification of Rho subtype proteins by *C. difficile* toxins A and B appears likely to account for its toxicity to intestinal epithelial cells.

Another class of enteric bacterial toxins acts by activating GC-C. The best studied of this class is *E. coli* STa, but a precise characterization of the activation of GC-C by *E. coli* STa is not yet available (see the section on enterotoxigenic *E. coli* [above]).

#### Possible or Postulated Mechanisms of Action

Toxins have been included in the category of possible or postulated mechanisms of action on the basis of one of several criteria. The first criterion for inclusion in this category is when the mechanism of action of the toxin has been inferred by identification of a biologic activity specific for another toxin with a known mechanism of action. For example, both the *C. jejuni* heat-labile enterotoxin and the *S. typhimurium* heat-labile enterotoxin have been reported to activate adenylate cyclase, thereby elevating intracellular cyclic AMP levels. However, neither toxin has been purified, and DNA hybridization experiments or sequence analysis has revealed only limited, if any, homology to CT. Thus, although the mechanism of action of these toxins may be related to their ability to increase cyclic AMP levels in cells, the molecular mechanisms of action of these toxins are unknown and may be dissimilar to the mechanism of action of CT or *E. coli* LT. In addition, *C. jejuni* produces a cytotoxin active on HeLa cells which is neutralizable by rabbit anti-Shiga toxin or by a monoclonal antibody to

the B subunit of Shiga-like toxin I, suggesting a potentially similar mechanism of action among these toxins (374). However, the *C. jejuni* Shiga-like toxin is genetically distinct from Shiga-like toxin I, and there are no direct data on its mechanism of action.

A second criterion for including a toxin in this category is when the possible mechanism of action has been demonstrated only in nonintestinal cells. For example, recent data have suggested that in addition to *C. difficile* toxins A and B (see above), two other enteric bacterial toxins, *E. coli* CNF1 and CNF2, alter the GTP-binding protein Rho which modulates microfilament structure in eukaryotic cells (105, 153, 155, 254, 258, 413). The molecular basis for this activity of *E. coli* CNF1 and CNF2 has not been identified, and the activity of these toxins has not been studied in intestinal epithelial cells or linked to secretion. In addition, the effects of *C. difficile* toxins A and B and *E. coli* CNF1 and CNF2 on F actin structure appear to be polar opposites, suggesting unique molecular effects on Rho by these two groups of toxins. Similarly, the EAEC 120-kDa heat-labile toxin has been shown to elevate intracellular calcium levels and to stimulate the phosphorylation of eukaryotic cell proteins in a calcium-dependent manner in the nonintestinal epithelial cell line HEP-2 (12). However, similar data obtained with intestinal epithelial cells are not yet available.

A third criterion for inclusion in this category is when the toxin has been shown to elevate the level of a second messenger in intestinal epithelial cells, but the relevance to secretion has not yet been demonstrated. This criterion applies to the increases in intracellular calcium level reported for *E. coli* STa and STb. *E. coli* STa has been reported to elevate intracellular calcium levels and to stimulate the release of diacylglycerol in isolated rat intestinal epithelial cells. However, the relevance of these observations to secretion has not been demonstrated. Notably, the secretory response to *E. coli* STa in vitro in T84 monolayers has a rapid onset and terminates after approximately 90 min (313). This time course shows an excellent correlation with the time of onset of an increase in intracellular cyclic GMP levels, but no elevation in calcium or phospholipid release is demonstrable in this in vitro system in response to *E. coli* STa. Because secretory responses to calcium and/or PKC (activated by diacylglycerol) agonists are often brief, it is unclear whether the increases in calcium levels and/or the diacylglycerol release noted in isolated rat intestinal cells relate to the more prolonged in vitro chloride secretory responses stimulated by *E. coli* STa. *E. coli* STb has also been shown to elevate intracellular calcium levels in several cell types including the human intestinal epithelial cell line HT29/C1 (121). This appears to occur through activation of a calcium channel regulated by a GTP-binding protein. Similar to the data on *E. coli* STa, experiments to demonstrate a secretory response correlating with the increase in intracellular calcium levels stimulated by *E. coli* STb have not yet been done.

A fourth criterion for inclusion in this category is when the mechanism of action of the toxin has been suggested by experiments with inhibitors or chelators of, for example, protein kinases or calcium, respectively, but direct data demonstrating kinase activation or increases in intracellular calcium are lacking. For example, the *S. typhimurium* heat-labile enterotoxin has been reported to activate adenylate cyclase. In addition, it has recently been shown that the potential PKC inhibitor H-7 inhibits the secretory response to this toxin in Ussing chambers in vitro (278). These data, combined with the observation that phorbol esters (direct activators of PKC) did not stimulate secretion in toxin-treated tissue, suggest that PKC may be involved in the mechanism of action of the *S. typhimurium* heat-labile enterotoxin. However, the inhibitory constant ( $K_i$ )

of H-7 is 6  $\mu$ M for PKC, 5.8  $\mu$ M for cyclic GMP-dependent kinase, and 3  $\mu$ M for cyclic AMP-dependent kinase, indicating that this compound is not necessarily selective or specific for PKC (212).

A fifth criterion for inclusion in this category is when the mechanism of action of the toxin is based on computer modeling of the structure of the toxin deduced from the DNA sequence for the toxin or a possible mechanism is inferred from a nonphysiologic system. This criterion applies to potential pore-forming enteric bacterial toxins. For example, the potential pore-forming toxin, Ace, of *V. cholerae* O1 is predicted by sequence analysis to be amphipathic, suggesting that multimers of these toxin molecules could insert in the intestinal epithelial cell membrane to create an ion-permeable channel (541). In addition, by using planar lipid bilayer experiments, both *S. aureus* delta toxin and *C. perfringens* CPE have been shown to cause the formation of ion-permeable channels (275, 363, 518). However, patch clamp experiments to detect new ion channels in the apical membranes of intestinal epithelial cells after treatment with these toxins have not been done. Similarly, evidence that membrane pore formation alone is sufficient to yield a secretory response in native intestinal tissue is not available, but the available data do indicate that *S. aureus* delta toxin and CPE are secretory in intestinal models (265, 350, 403).

The last criterion for inclusion in this category is when a toxin has been shown to stimulate the release of potential secretory mediators in the intestine but the mechanism(s) leading to the production and release of the mediators is unknown. Notably, several toxins, including CT, *C. difficile* toxin A, *E. coli* STb, and the *S. typhimurium* heat-labile enterotoxin, stimulate the release of arachidonic acid metabolites in the intestine. Furthermore, each of these toxins has been linked to secretion in one or more experimental intestinal models. However, the mechanistic steps between binding of the toxin to its intestinal epithelial cell receptor and the ultimate release of arachidonic acid metabolites are likely to be multiple and have not been delineated for any of these toxins. Thus, although arachidonic acid metabolites are likely to contribute to the pathophysiologic response to each of these toxins, the absence of detail regarding the mechanisms involved led to the classification of the arachidonic acid cascade as a possible or postulated mechanism of action.

#### Toxins with Unknown Mechanisms of Action

Most enteric bacterial toxins can be classified by demonstrating various biological activities in one or more experimental systems. However, even for the best-studied enteric bacterial toxins, biological activities have been identified for which precise mechanistic explanations do not exist at present. For example, the mechanism of action of toxins which increase intracellular levels of cyclic nucleotides may also involve remodeling of F actin. Recently, secretion stimulated by 8-bromo-cyclic AMP has been shown to be inhibited when F actin is immobilized in vitro in intestinal epithelial cells (T84) by phalloidin (496). Furthermore, changes in F-actin distribution have been identified after treatment of these intestinal epithelial cells with 8-bromo-cyclic AMP (496) or *E. coli* STa (343). Thus, the secretory response to cyclic nucleotide-dependent agonists appears to involve remodeling of actin in the basolateral aspect of the epithelial cell, which is linked to activation of the Na/K/2Cl cotransporter located in the basolateral cell membrane (342, 344). The mechanism by which the F-actin distribution in the intestinal epithelial cell is altered has not been investigated. One hypothesis requiring experimental test-



TABLE 6. Mechanistic classification of enteric bacterial toxins<sup>a</sup>

Type of mechanism	Toxin
Toxins with established mechanisms of action <sup>b</sup>	
Cyclic nucleotides	
Cyclic AMP	CT <i>E. coli</i> LT I and II
Cyclic GMP	<i>E. coli</i> STa EAEC heat-stable enterotoxin (EAST 1) <sup>c</sup> <i>Y. enterocolitica</i> heat-stable enterotoxin I (Yst) <sup>c</sup>
ADP-ribosylation	CT <i>E. coli</i> LT I and II <i>C. botulinum</i> C2 toxin
Inhibition of protein synthesis	Shiga toxin Shiga-like toxins I and II
Glucosylation of Rho	<i>C. difficile</i> toxin A <sup>d</sup> <i>C. difficile</i> toxin B <sup>d</sup>
Toxins with possible or postulated mechanisms of action	
Cyclic AMP	<i>A. hydrophila</i> 15- and 44-kDa toxins <i>C. jejuni</i> heat-labile enterotoxin <i>S. typhimurium</i> heat-labile enterotoxin (Stn)
Inhibition of protein synthesis	<i>C. jejuni</i> Shiga-like toxin <sup>e</sup> <i>Shigella</i> Shiga-like toxin <sup>e</sup> <i>E. coli</i> Shiga-like toxin <sup>e</sup>
Arachidonic acid cascade	<i>A. hydrophila</i> 44-kDa toxin CT <i>C. difficile</i> toxin A <i>C. perfringens</i> type C PLC <sup>f</sup> <i>E. coli</i> STb <i>S. typhimurium</i> heat-labile enterotoxin <i>S. aureus</i> delta toxin <i>S. aureus</i> enterotoxins <sup>g</sup>
Calcium/PKC	<i>C. perfringens</i> enterotoxin (CPE) <sup>h</sup> <i>C. perfringens</i> type C PLC <sup>f</sup> <i>E. coli</i> STb <i>V. parahaemolyticus</i> TDH <i>Vibrio cholerae</i> O1 Zot <i>E. coli</i> STa EAEC 120-kDa heat-labile toxin <i>S. typhimurium</i> heat-labile enterotoxin
Pore formation	<i>S. aureus</i> delta toxin <i>C. perfringens</i> CPE <sup>h</sup> <i>C. perfringens</i> beta toxin <i>V. cholerae</i> O1 Ace EAEC 120-kDa heat-labile toxin <i>V. cholerae</i> O1 and non-O1 hemolysin <i>Aeromonas</i> aerolysin
Alteration of the GTP-binding protein, Rho	<i>E. coli</i> CNF1 and CNF2
Toxins with unknown mechanism of action <sup>i</sup>	
Enterotoxins	<i>Aeromonas</i> aerolysin <i>Aeromonas</i> 70-kDa and CT-cross-reactive toxins

Continued

TABLE 6—Continued

Type of mechanism	Toxin
	EAEC 108-kDa heat-labile toxin EIEC enterotoxin (ShET2) <i>P. shigelloides</i> heat-stable and heat-labile enterotoxins <i>S. flexneri</i> 2a (ShET1 and ShET2) Toxin from other <i>Shigella</i> spp. (ShET2) <i>S. aureus</i> enterotoxins A–E <i>V. fluvialis</i> CHO cell cytotoxin, CHO cell-rounding factor, CHO cell elongation factor <i>Y. enterocolitica</i> heat-stable enterotoxin II (Yst-II)
Cytotoxins	<i>B. cereus</i> diarrheal toxin (HBL) <i>C. jejuni</i> cytotoxin <i>C. jejuni</i> CLDT <i>C. perfringens</i> beta toxin EAEC 108-kDa heat-labile toxin EIEC cytotoxin Enterotoxigenic <i>B. fragilis</i> toxin <i>E. coli</i> CLDT <i>V. fluvialis</i> CHO cell cytotoxin, CHO cell-rounding factor <i>V. metschnikovii</i> cytolyisin
Cytoskeleton-altering toxins	<i>Aeromonas</i> 15-, 44-, and 70-kDa and CT-cross reactive toxins <i>C. jejuni</i> CLDT CT <sup>j</sup> Enterotoxigenic <i>B. fragilis</i> toxin <i>E. coli</i> CLDT <i>E. coli</i> STa <sup>k</sup> <i>E. coli</i> LT-I and LT-IV <sup>l</sup> <i>S. typhimurium</i> enterotoxin (Stn) <sup>j</sup> <i>V. fluvialis</i> CHO cell-rounding factor, CHO cell elongation factor
Neural activity	<i>B. cereus</i> emetic toxin CT <i>C. difficile</i> toxin A <i>E. coli</i> STa <i>S. aureus</i> enterotoxins A–E

<sup>a</sup> Adapted in part from reference 490.<sup>b</sup> A toxin is considered to have an established mechanism of action if the molecular mechanism of action has been identified and linkage to intestinal secretion has been demonstrated.<sup>c</sup> EAST 1 of EAEC and Yst of *Y. enterocolitica* are assumed to act in a similar manner to *E. coli* STa, although this has not been conclusively demonstrated.<sup>d</sup> *C. difficile* toxins A and B have only tentatively been included as toxins with an established mechanism of action. Glucosylation of Rho has not been linked to intestinal secretion and has not been directly studied in intestinal epithelial cells (256, 258). However, Rho appears to be glucosylated by both toxins in all cell lines studied to date, and the primary action of *C. difficile* toxins A and B on intestinal epithelial cells is not to induce secretion but to alter the cellular cytoskeleton (see the section of text on *C. difficile* for a detailed discussion). Thus, this recently identified modification of Rho by *C. difficile* toxins A and B appears likely to account for its toxicity to intestinal epithelial cells.<sup>e</sup> *C. jejuni*, *Shigella* spp. other than *S. dysenteriae*, and *E. coli* K-12 may produce low levels of Shiga toxin-like activity defined as cytotoxicity in HeLa cells that is neutralizable by antibodies against Shiga toxin (401). However, neither the protein nor the gene accounting for this activity has been identified. This activity is not due to SLT-I or SLT-II.<sup>f</sup> Serosal application of *C. perfringens* alpha toxin or PLC stimulates an increase in  $I_{sc}$  in rat colon that, from inhibitor studies, appears to be dependent on prostaglandins and/or calcium (104). However, direct measurements of intracellular calcium or prostaglandin levels are either negative or not yet available.<sup>g</sup> Studies to date identifying release of arachidonic acid metabolites have been done only with *S. aureus* enterotoxin B.

Continued on following page



<sup>h</sup> The mechanism of action of CPE involves calcium-independent and -dependent steps (see the text). Although CPE creates a high-conductance, linear, ion-permeable channel in lipid bilayers consistent with pore formation (518), other data indicate that CPE is not amphipathic in the absence of eukaryotic membrane proteins and, furthermore, forms a complex with eukaryotic membrane proteins, suggesting that the pore formation detected in artificial lipid bilayers is not physiologically relevant (349, 352, 579).

<sup>i</sup> For this section, toxins are classified by currently described activities, using the definitions for enteric toxins in Table 2. However, inadequate data exist, for example, to state that no intestinal damage occurs for those listed as enterotoxins.

<sup>j</sup> Chloride secretion in intestinal epithelial cell monolayers stimulated by cyclic AMP analogs or cyclic AMP-dependent agonists is regulated by microtubules and F actin through effects on CFTR and the Na/K/2Cl cotransporter, which play a key role in transepithelial Cl<sup>-</sup> secretion (171, 342, 344, 496, 536). By analogy, a similar response to toxins increasing intracellular cyclic AMP levels (e.g., CT, *E. coli* LT-I and LT-II, or the *S. typhimurium* enterotoxin) is hypothesized.

<sup>k</sup> *E. coli* STa has been shown to alter the appearance of F actin in the basal portion of T84 cells, a human intestinal epithelial cell line, but the mechanism of this effect is unknown.

ing is that actin is a substrate for the cyclic nucleotide-dependent kinases activated by the increases in intracellular cyclic GMP or cyclic AMP levels stimulated by GC-C- or adenylate cyclase-activating toxins produced by ETEC (STa, LT), EAEC, *Y. enterocolitica*, *V. cholerae*, or *Salmonella* spp.

### THE COMPLEXITY OF INTESTINAL PATHOPHYSIOLOGY: ACCESSORY BACTERIAL OR HOST FACTORS INFLUENCING TOXIN ACTION ON THE INTESTINE

#### Bacterial Factors

The pathogenicity of an enteric bacterial organism is most often dependent on multiple virulence mechanisms that include intestinal colonization, delivery of one or more secretory or cytotoxic toxins, and/or invasion of the epithelium. The expression of these virulence proteins is regulated by a variety of conditions including, for example, the anaerobic environment of the intestine (362). Furthermore, the expression of one virulence protein may alter the effect of another virulence factor. For example, production of CT by *V. cholerae* O1 enhances colonization by *V. cholerae* O1 in rabbits (433). Similarly, the production of neuraminidase by *V. cholerae* O1 represents another potential interaction between bacterial virulence factors. By cleaving sialic acid, neuraminidase catalyzes the conversion of higher-order gangliosides to GM<sub>1</sub>, the receptor for CT. Consistent with this enzymatic activity, in vitro and in vivo studies have suggested that production of neuraminidase by *V. cholerae* O1 may enhance the binding and secretory response to CT under defined conditions (173). Future studies investigating the potential expression of new bacterial or intestinal epithelial cell proteins in the host-bacterium interaction or the interactions of different bacterial proteins may yield novel mechanisms by which bacterial pathogens alter the physiology of the intestinal epithelium.

#### Host Factors

Several lines of evidence suggest that the secretory response to certain enteric bacterial toxins is a complicated event involving not only the intestinal epithelial cell but also the submucosal compartment containing the enteric immune and nervous systems and, in some instances, possibly systemic immune responses. The precise contribution of each potential mode of action to intestinal secretion or the impact of one activity on the other physiologic sequelae of the toxin (i.e., signal transduction crosstalk) in vivo in animals or humans is not known.

TABLE 7. Enteric bacterial toxins with more than one potential mechanism of action

Enteric bacterial toxin	Potential mechanisms
<i>V. cholerae</i> CT	Cyclic AMP F actin Prostaglandins of the E series PAF Serotonin (5-HT) <sup>a</sup> Stimulation of proinflammatory cytokines (e.g., IL-6) Neural activity
<i>E. coli</i> STa	Cyclic GMP F actin Serotonin (5-HT) Calcium/PKC (?) Neural activity
<i>C. difficile</i> toxin A	O-glucosylation of Rho proteins Inflammatory mediators <sup>b</sup> Neural activity

<sup>a</sup> Notably, recent data examining the role of 5-HT in CT-induced secretion in human jejunum found that 5-HT antagonists enhanced, rather than inhibited, CT-induced secretion (129).

<sup>b</sup> These inflammatory mediators include leukotrienes, PGE<sub>2</sub>, PAF, and histamine.

As summarized in Table 7 and in the functional discussion of toxins grouped by genus and species (see above), CT, *E. coli* STa, and *C. difficile* toxin A provide the best-studied examples of toxins with several potential mechanisms for causing diarrhea. Recent data provide additional support for the hypothesis that the secretory response to an enteric pathogen and/or its toxins is modulated by host factors. For example, the secretory responses to both *E. coli* STa and cyclic AMP-dependent agonists such as VIP and PGE<sub>2</sub> in T84 monolayers are synergistically augmented by the acetylcholine analog carbachol and/or the mast cell mediator histamine (102, 227, 313, 325, 568). Both carbachol and histamine appear to stimulate secretion by a calcium/PKC-dependent mechanism. Although the mechanism of synergy appears to involve the cooperative interaction of potassium channels and CFTR (46), activators of PKC or purified PKC also increase *E. coli* STa-stimulated GC-C activity in intestinal epithelial cell membranes and the phosphorylation of GC-C is promoted by PKC activators (85, 553). Similarly, CT may stimulate the epithelial cell to secrete IL-6, a proinflammatory cytokine (359). Delivery of cytokines to the submucosa from the intestinal epithelial cell could lead to the recruitment of inflammatory cells with local production of mediators (e.g., prostaglandins and leukotrienes) that can stimulate intestinal epithelial cell secretion. These experiments suggest that host factors such as neurohormones released by enterochromaffin cells and/or cytokines released from intestinal epithelial cells or submucosal immune cells, as well as enteric nervous system neurotransmitters, may enhance secretion in response to certain bacterial enteric toxins.

Together, these data indicate that the mechanisms by which an enteric bacterial toxin stimulates intestinal secretion are dependent on numerous factors including other bacterial virulence traits as well as the response of the epithelial and nonepithelial intestinal components. Precise determination of the contribution of each potential secretory mechanism to these enteric infections is not likely to be experimentally feasible. However, constructing a comprehensive scheme for toxin action encompassing these potential contributions to secretion constitutes the challenge and excitement of research on the gastrointestinal pathophysiology of enteric bacterial toxin action today.

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