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Infectious diarrhea
Cellular and molecular mechanisms

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Diarrhea caused by enteric infections is a major factor in morbidity and mortality worldwide. An estimated 2–4 billion episodes of infectious diarrhea occur each year and are especially prevalent in infants. This review highlights the cellular and molecular mechanisms underlying diarrhea associated with the three classes of infectious agents, i.e., bacteria, viruses and parasites. Several bacterial pathogens have been chosen as model organisms, including *Vibrio cholerae* as a classical example of secretory diarrhea, *Clostridium difficile* and Shigella species as agents of inflammatory diarrhea and selected strains of pathogenic *Escherichia coli* (*E. coli*) to discuss the recent advances in alteration of epithelial ion absorption. Many of the recent studies addressing epithelial ion transport and barrier function have been carried out using viruses and parasites. Here, we focus on the rapidly developing field of viral diarrhea including rotavirus, norovirus and astrovirus infections. Finally we discuss *Giardia lamblia* and *Entamoeba histolytica* as examples of parasitic diarrhea. Parasites have a greater complexity than the other pathogens and are capable of creating molecules similar to those produced by the host, such as serotonin and PGE_2. The underlying mechanisms of infectious diarrhea discussed include alterations in ion transport and tight junctions as well as the virulence factors, which alter these processes either through direct effects or indirectly through inflammation and neurotransmitters.

Introduction

At its core, diarrhea is simply an altered movement of ions and water that follows an osmotic gradient. Under normal conditions, the gastro-intestinal tract has tremendous capacity to absorb fluid and electrolytes, where 8–9 liters of fluid are presented to the intestine daily and only 100–200 ml are excreted in the stool. Enteric pathogens, however, can alter this balance towards net secretion, leading to diarrheal disease. The altered movement of ions can occur either through transporters or the lateral spaces between cells, which are regulated by tight junctions (**Fig. 1**). In this regard, some transporters seem to be tightly coupled with water movement, including sodium-dependent glucose transporter (SGLT1), Na^+/H^+ exchanger isoform 3 (NHE3) and the apical Cl^-/HCO_3^- exchanger, downregulated in adenoma (DRA). The classical secretory diarrhea caused by cholera toxin (CT) is due to cAMP-dependent activation of the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl^- channel (**Fig. 1**). Alternately, changes in Ca^{2+} levels increase the activity of the calcium activated chloride channel (CLCA). In some cases, as for CT, the increase in Cl^- secretion is paired with a decrease in Na^+ absorption. In addition the direct reduction of water transport proteins, such as aquaporins, results in less fluid absorption (**Fig. 1**).

Enteric pathogens can either directly modulate epithelial ion transport processes and barrier function or do so indirectly through inflammation, neuropeptides or loss of absorptive surface. For example, pathogens such as the intestinal parasite Giardia cause loss of brush border absorptive surface and diffuse shortening of villi. Similarly, enteropathogenic *E. coli* (EPEC) cause effacement of microvilli, which decreases the surface area for nutrient absorption and causes increased osmolarity of the intestinal contents and malabsorption. However, recent evidence suggests that the rapid onset of diarrhea induced by EPEC could result from direct effects on intestinal epithelial ion transport processes. Several invasive pathogens, including Shigella and Salmonella species, cause an inflammatory diarrhea characterized by fever and polymorphonucleocytes (PMNs) in the stool. PMNs regulate absorption through cytokine secretion but also have a more direct role through the secretion of a precursor to adenosine, a secretagogue that activates CFTR. *C. difficile* and rotavius infection also work indirectly through modulation of ion transport subsequent to cytokine secretion and activation of enteric nerves via neuropeptides.

This review highlights selected pathogens, which provide both a broad overview of the mechanisms pertaining to ion transport and barrier function that underlie pathophysiology of diarrhea, and presents recent advances regarding specific infectious agents. Major emphasis will be placed on the mechanisms underlying the pathogenesis of bacterial diarrhea, which has been extensively studied in recent years and has served as an important prototype for understanding regulation of intestinal epithelial processes at the cellular and molecular level. However, the mechanisms underlying recent advances in viral and parasitic diarrhea will also be discussed. An increased understanding of these regulatory mechanisms is important to completely define

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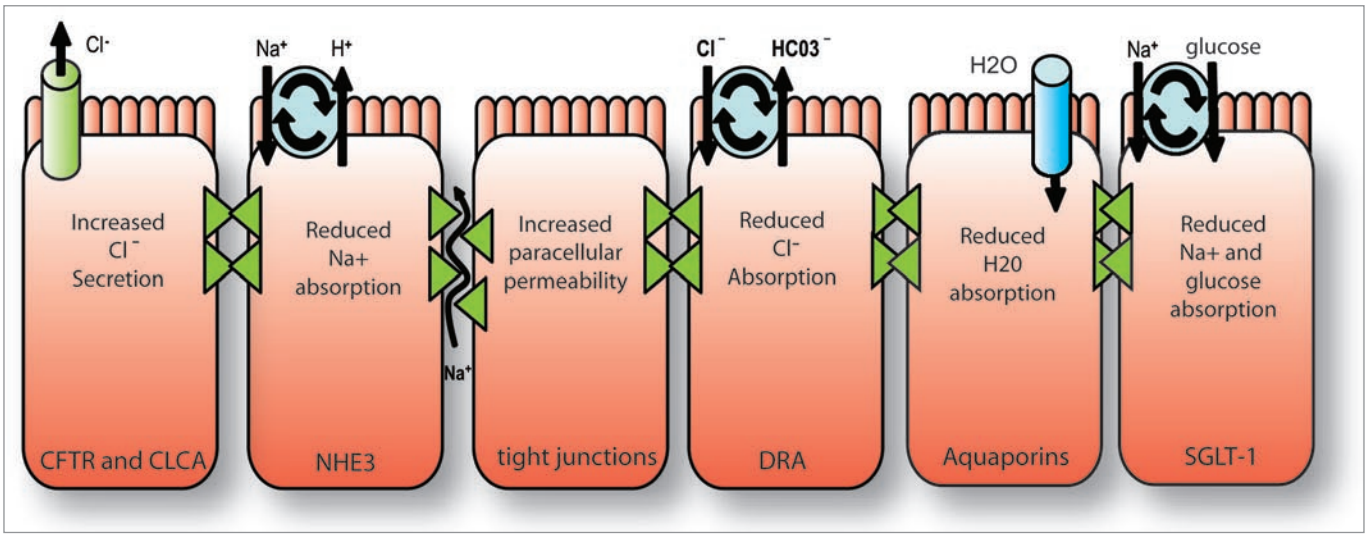

Figure 1. An overview of general mechanisms causing diarrhea. At the most basic level, diarrhea is caused by increased secretion or decreased absorption of fluids and electrolytes. Certain ion transport processes are particularly associated with diarrhea. These include CFTR and CLCA, which are chloride channels and the Na^+/H^+ exchange isoform, NHE3, which is involved in Na^+ absorption. Alterations in tight junctions create an important pathway for the movement of both ions and water. DRA is responsible for chloride absorption and is associated with congenital chloride diarrhea. Data on aquaporins is limited but they are expected to contribute to diarrhea when absorption is reduced. SGLT-1 transports sodium and glucose and is tightly coupled with water movement and is the basis for oral rehydration using glucose to enhance sodium absorption.

the pathophysiology of infectious diarrhea and explore the potential of novel anti-diarrheal drugs.

Pathophysiological Mechanisms of Infectious Diarrhea

Bacterial diarrhea. In developing countries, enteric bacterial pathogens and parasites are the leading cause of infectious diarrhea. Although even in the United States, the frequency of bacteria-induced illnesses is considerably high. Enterohemmorhagic *E. coli* (EHEC) infection causes disease in approximately 75,000 people per year,[1] whereas *C. difficile* remains the major cause of hospital acquired infections.[2] Bacterial diarrhea ranges in duration from a few hours for some released toxins to several weeks for active infections of enteroaggregative *E. coli*. Here we use *V. cholerae* as an example of secretory diarrhea and then discuss *C. difficile*-associated diarrhea, which relies on neuropeptides and inflammatory mediators for pathogenesis. In addition, Shigella species are used as an example of inflammatory and invasive diarrhea while *E. coli* have a variety of strategies, one of which is the reduction in absorption both through a loss of microvilli and through a direct effect on ion transporters. These four groups of pathogens have been selected to explain the major mechanisms involved in diarrhea.

Vibrio cholerae. Despite being the focus of scientific study since the 1800's, *V. cholerae* remains a threat today. While people with access to treated water are typically not exposed, areas without adequate chlorination or filtration still suffer from epidemic cholera. *V. cholerae* have several toxins, which are used to compromise the host, with the most important of these being cholera toxin (CT) itself. CT consists of an A subunit bound to a pentameric ring of B subunits, where the B subunits are responsible for delivery of the A subunit into the cell.[3] The A subunit ADP-ribosylates a GTPase, which regulates adenylate cyclase resulting in elevated cAMP production[4] (**Fig. 2**). The production of cAMP activates PKA, which then phosphorylates the regulatory domain of CFTR.[5] While CT is the most dangerous part of *V. cholorae's* arsenal, most of the modern work with CT actually provides insights into understanding key cellular mechanisms. For example, by studying CTs retrograde translocation and the eventual release of the A1 peptide into the host cytoplasm, the details of endogenous retrograde trafficking have been uncovered.[6] In addition, movement of CT through the Endoplasmic Reticulum-Associated protein Degradation (ERAD) pathway has shed light on the way cells deal with misfolded proteins in the ER.[7] Aside from the knowledge acquired in these studies, the CT B-subunit has shown great promise as an adjuvant in a number of recent vaccine studies. Although there have been additional insights into the transporters affected by CT. In addition to increased Cl^- secretion, the absorption of Na^+ is decreased through a cAMP-dependent mechanism where the activity of both apical sodium transporters, NHE2 and NHE3, is decreased[8] (**Fig. 2**). Together, this leads to an increase in NaCl levels in the intestinal lumen by enhancing secretion or decreasing absorption.

In addition to CT, *V. cholerae* encodes several other toxins, which modulate ion secretion and perturb barrier function to cause massive diarrhea. The toxins that affect ion secretion directly include accessory cholera toxin (ACE), which stimulates Ca^{2+} dependent Cl^- secretion; NAG-stable toxin, which activates guanylyl cyclase, thus stimulating cGMP production, which leads to PKG-mediated activation of CFTR; and, finally *V. cholerae* cytolysin (VCC), which creates anion permeable pores[9-11] (**Fig.2**). One of the phenotypes associated with VCC toxicity has been linked with the newly described phenomenon autophagy.[12]

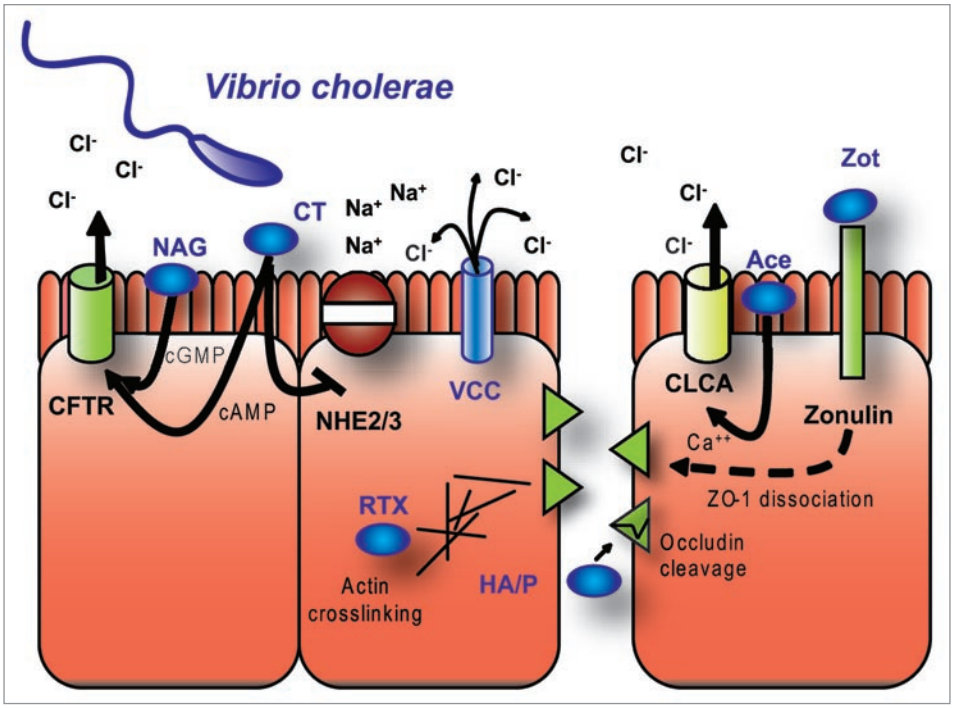

Figure 2. Mechanisms underlying *V. cholerae*-induced diarrhea. Cholera is characterized by severe watery diarrhea due to changes in ion secretion and absorption. Both CLCA and CFTR-dependent Cl^- secretion are activated, the first by Ace and the second by cholera toxin and NAG heat-stabile toxin. Increased cAMP levels also block sodium absorption through NHE2 and NHE3. *V. cholerae* also creates anion permeable pores through insertion of VCC. In concert with changes in ion transport, paracellular permeability is also decreased. Zot interaction with zonulin causes ZO-1 to dissociate from tight junctions while HA/P cleaves occludin and RTX interferes with the contractile actin ring.

The VCC toxin causes large vacuoles to form in host cells in addition to its cytolytic effect on red blood cells. While the mechanism behind this vacuole formation is only beginning to be understood, recent studies have shown that VCC is associated with double membrane vesicles and LC3-II accumulation which are characteristic of autophagy. In addition, mouse embryonic fibroblasts deficient for Atg-5 (which is needed for LC3 conversion) or inhibitors of autophagy including 3-methyladenine block the formation of vacuoles in response to VCC. While vacuole formation appears to be a severe phenotype, it is actually a protective cellular response and its inhibition results in a more than 50% decrease in cell survival after toxin exposure. In this case, vacuole formation appears to be a part of the natural removal of VCC from the cellular membrane, blocking further dysregulated Cl^- transport and protecting the cell.

The *V. cholerae* toxins which alter intestinal barrier function include hemagglutinin/protease or HA/P, RTX and Zot. HA/P is an extracellular protease, which cleaves a tight junction structural protein, occludin, that is known to regulate paracellular permeability (**Fig. 2**).[13] This results in the subsequent loss of the scaffolding protein ZO-1 from the tight junction. RTX cross links actin and induces cell rounding, loss of transepithelial resistance (TER) and an increase in permeability to FITC-dextran 3000.[14] Analysis of the crystal structure of RTX cross-linked actin and mass spectrometry showed that residues E270 and K50 form a covalent iso-peptide bond.[15] Interestingly, the fungal toxin phalloidin was able to partially restore the ability of actin to polymerize in the presence of the actin cross-linking domain of RTX. Actin and myosin light chain play a critical role in the regulation of tight junctions by forming a belt-like structure around the cell, which can be tightened to increase paracellular flow. In this case, the functionality of the junctional actinomyosin ring is lost due to the sequestration of actin in incorrectly polymerized aggregates. The third toxin, Zot, which also impacts barrier function, serves as both a phage assembly protein and an enterotoxin.[16] This is similar to what is seen with Ace, which is also a phage structural protein.[17] Many of the virulence factors associated with *V. cholerae* are part of an integrated prophage called φ, which is composed, in part, of Ace and Zot.[18] Zot is not functional in its phage associated form but is instead cleaved into an active 12 kDa peptide.[16] The Zot peptide binds to an apical receptor for a host protein called zonulin, which regulates permeability exclusively in the small intestine.[19] Loss of barrier function occurs only in rabbit ileum but not in colon, consistent with the localization of the zonulin receptor. Recently, a smaller fragment of Zot consisting of only 6 amino acids was shown to be capable of causing the same change in resistance as well as causing the dissociation of ZO-1 from tight junctions.[20] However, it should be noted that this work is considered controversial by some. Thus, *V. cholerae* has three different toxins that promote secretion and three toxins that promote the loss of barrier function and, in combination, this results in a particularly severe diarrhea (**Fig. 2**).

C. difficile. *C. difficile* is the leading cause of nosocomial diarrhea and accounts for almost all cases of pseudomembranous

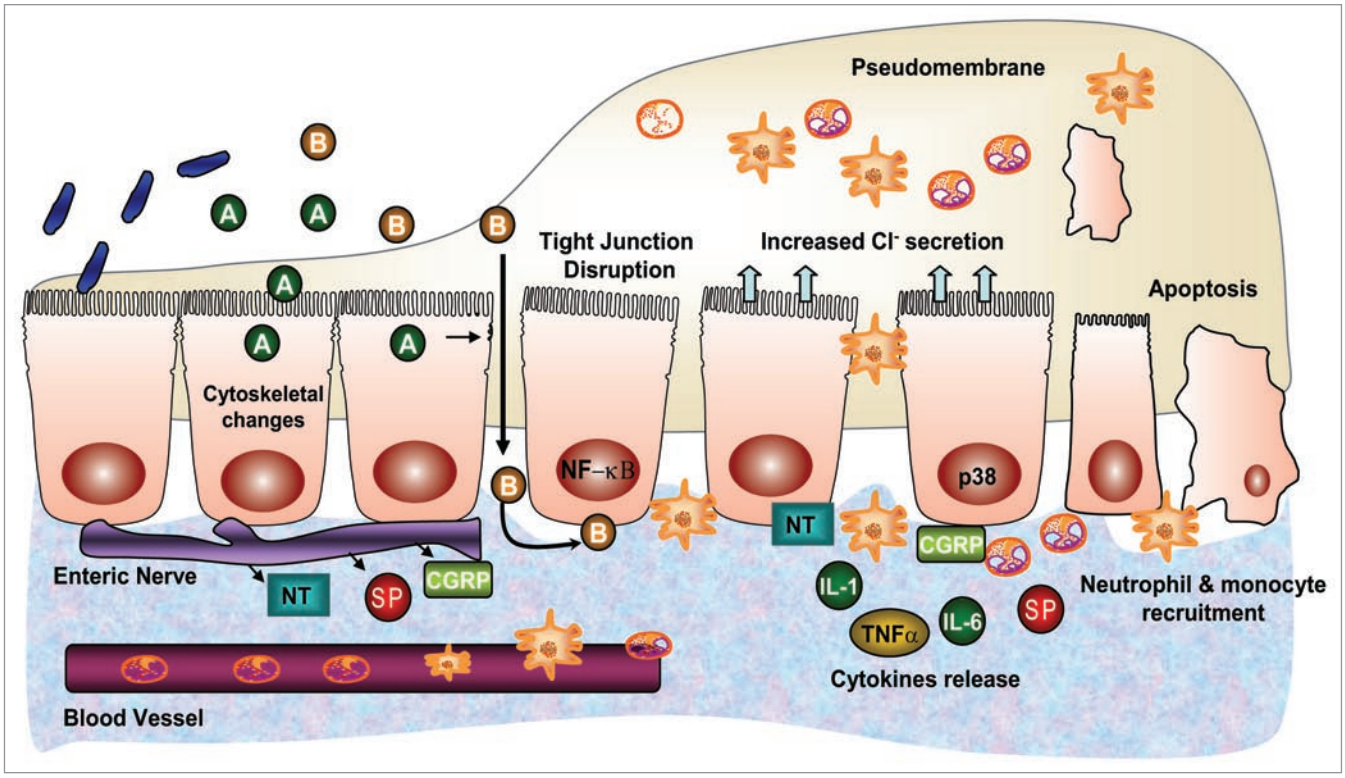

Figure 3. Pathogenesis of *C. diffcile*-associated diarrhea. *C. difficile* produces toxin A and toxin B (TcdA and TcdB). TcdA binds to the apical side of the cell and, after internalization, causes cytoskeletal modification and disruption of tight junctions. The resulting loss of epithelial barrier function facilitates TcdA and TcdB to cross the epithelium with preferential binding of TcdB to the basolateral cell membrane. Both toxins are cytotoxic and lead to production of proinflammatory cytokines, increase in vascular permeability, recruitment of neutrophils and monocytes, epithelial cell apoptosis and connective tissue degradation, resulting in pseudomembrane formation and diarrhea. Further, the activation and release of various neuropeptides by the toxins stimulates ENS to elicit fluid secretion, causing diarrhea.

colitis, which is an acute colitis characterized by formation of an adherent inflammatory membrane overlying the site of injury[21] (**Fig. 3**). The recent emergence of novel epidemic strains has caused increased concern in clinical settings because antimicrobial therapy predisposes patients to *C. difficile* associated diarrhea (CDAD).[22] The pathogenic process of *C. difficile* infection starts with initial colonization followed by the production of two distinct exotoxins, Toxin A and B (TcdA and TcdB), as well as an additional toxin called binary toxin (CDT) which is found in some hypervirulent strains of *C. difficile*.[23] TcdA binds effectively to the apical side of the host cell to glycoprotein gp96, which forms part of the receptor in humans.[24] In contrast, TcdB gains access to the basolateral side of the cell after tight junction disruption and binds preferentially to an unidentified receptor[25,26] (**Fig. 3**). TcdA and TcdB are potent cytotoxic enzymes that specifically glucosylate the small GTPase protein Rho, which leads to disruption of cytoskeletal integrity and cytotoxic effects.[27] CDT, an actin-specific ADP ribosyltransferase, potentiates the toxicity of TcdA and B and may increase the severity of CDAD.[28] While early studies in animals implicate TcdA as the primary factor in the pathogenesis of *C. difficile* infection, recent studies showed that disruption of the *tcdB* gene led to a significantly attenuated virulence phenotype in the hamster model suggesting that TcdB might play a key role in disease pathogenesis.[29]

Earlier studies showed that TcdA causes direct alterations in barrier function and ion transport. For example, purified toxin A has also been shown to cause net luminal accumulation of sodium, chloride and potassium in rabbit intestine in vivo.[30] Ussing chamber studies using isolated mucosal strips from guinea pig ileum demonstrated that TcdA increases transepithelial permeability and decreases electrogenic Na^+ absorption while eliciting a Cl^- secretory response.[31] Rounding up of cells and barrier function disruption corresponding with structural alterations of perijunctional actinomysin ring occurred in human intestinal epithelial T84 cells exposed to TcdA.[30,32]

Besides the direct effects of the toxins, other mechanisms underlying *C. difficile* associated diarrhea include inflammation and activation of neuropeptides. The *C. difficile* toxins initiate an extensive inflammatory cascade that causes increased damage to host tissues resulting in fluid exudation. TcdA causes release of several proinflammatory cytokines such as leukotriene, PGE_2, and tumor necrosis factor (TNFα in vivo).[33] It also directly activates monocytes to release IL-1 and IL-6,[34] and increase neutrophil migration in vitro.[35] Other toxin-mediated inflammatory effects include release of reactive oxygen species, activation of mitogen-activated protein kinases and NFκB activation.[33] A number of studies suggest that important cellular responses to *C. difficile* toxins such as p38 MAP kinase activation, mitochondrial

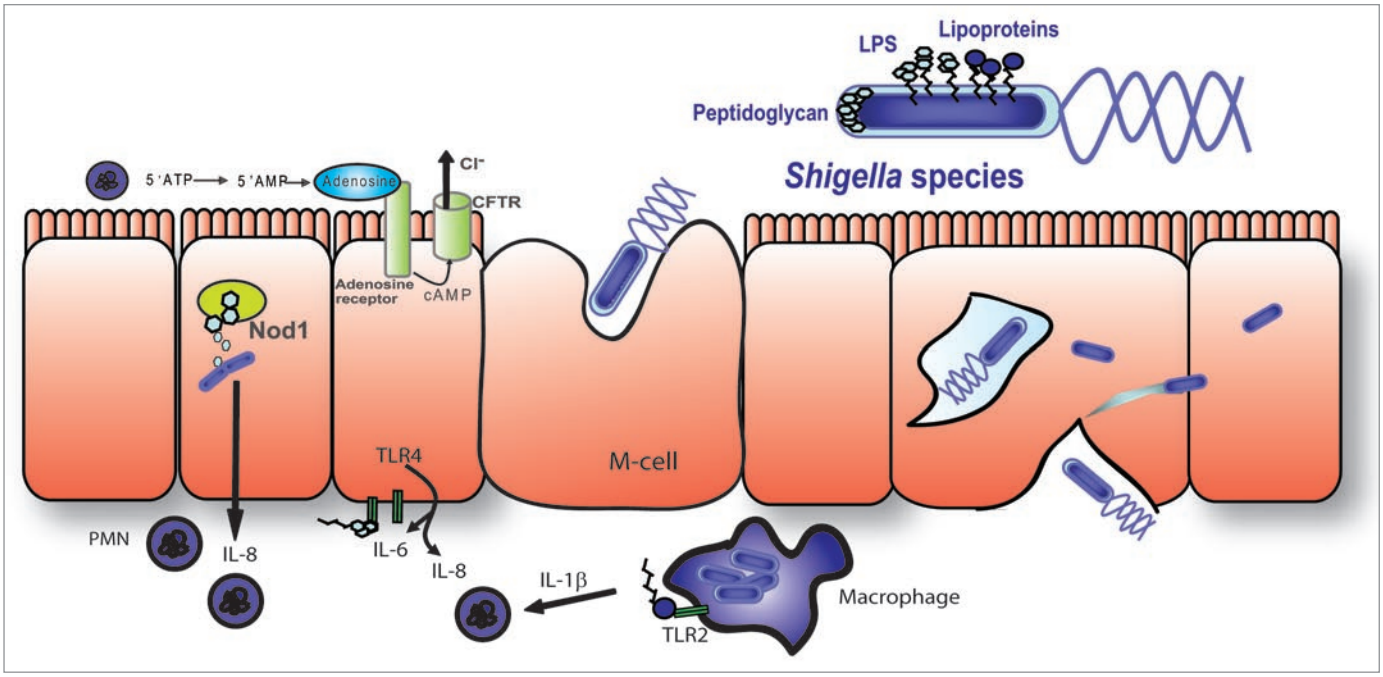

Figure 4. Invasion and inflammation caused by Shigella. Shigella species cross the epithelial barrier through M-cells where they encounter and eliminate macrophages. Binding of lipoprotein to TLR2 results in the production of the chemoattractant IL-1β. After translocation through M-cells LPS can bind to basolateral TLR4 which causes the production of IL-6 and IL-8. This effect is somewhat diminished due to the acetylation of LPS in Shigella. IL-8 is a potent chemoattractant for PMNs and is also produced due to activation of intracellular Nod1 by peptidoglycan. PMNs are the primary destructive force in Shigella infection. PMNs cause Cl^- secretion through generation of a precursor to the secretagogue adenosine and can also cause ulceration of the epithelium, which results in a decrease in the absorptive surface but also maximizes permeability and allows easy access of gut flora to the basolateral surface of cells further driving inflammation.

damage and IL-8 release occur prior to and independently of Rho glucosylation.[33]

Another striking feature of *C. difficile* toxin-associated intestinal responses is activation of enteric nerves and enhanced production or release of neuropeptides including Substance P (SP), calcitonin gene-related peptide (CGRP) and neurotensin,[36-38] which are known to elicit Cl^- secretion in intestinal epithelial cells. The antagonists of the Substance P receptor, Neurokinin-1 (NK1) and calcitonin gene-related peptide (CGRP), block fluid accumulation and mannitol flux in response to toxin A.[33] Also in a NK1-$R^{-/-}$ mouse ileal loop model, TcdA-mediated luminal fluid accumulation was considerably reduced.[39] Therefore, unlike cholera toxin and *E. coli* enterotoxins, which trigger intestinal secretion without intestinal inflammation, the pathophysiology of *C. difficile*-associated diarrhea involves a necroinflammatory reaction, which activates mast cells, nerves, vascular endothelium, and immune cells in addition to impairment of tight junctions (**Fig. 3**). Further studies pertaining to detailed analysis of the contribution of the electrogenic versus electroneutral components of ion absorption in the well-established animal models of *C. difficile* infection would benefit the modeling of disease pathogenesis and help elucidate the mechanisms of *C. difficile* associated diarrhea.

Shigella species. There are four major Shigella species that cause diarrheal disease. The most common species in the U.S. and other developed countries is *S. sonnei* followed by *S. flexneri*.[40] Two other Shigella species, *S. dysenteriae* and *S. boydii*, are found very rarely in developed countries and have a generally low infection rate over all, however, *S. dysenteriae* causes the most life-threatening of all of these infections due to the production of Shiga toxin, which can lead to hemolytic uremic syndrome (HUS). While all four species of Shigella are invasive due to a large virulence plasmid, there is some variation in the plasmid and *S. flexneri* is the best studied in this regard. The invasion process is complicated and occurs through a trigger mechanism on the basolateral side of epithelial cells after the bacteria have already passed through M-cells and potentially, macrophages.[41] In addition, Shigella have actin tails and are capable of cell to cell spread, increasing their ability to colonize the epithelium.[42] The type III secreted effector proteins involved in this process are numerous and have been recently and thoroughly reviewed by Schroeder and Hilbi.[43]

Invasion and inflammatory response. Shigella causes an inflammatory diarrhea and the cellular response to various steps of the invasion process are the primary cause of inflammation (**Fig. 4**). The destruction of macrophages after emergence from M-cells causes an initial release of IL-1β, which attracts PMNs.[44,45] PMNs release a precursor to the secretagogue adenosine, which activates Cl^- secretion. This early step in inflammation is exacerbated by the presence of free bacteria on the basolateral side of cells, which allows access to toll-like receptors. Shigella LPS is capable of activating TLR4, although recent studies suggest that it is about 50% less active than LPS from *E. coli* in activating NFκB due to a reduced level of acetylation.[46] Therefore,

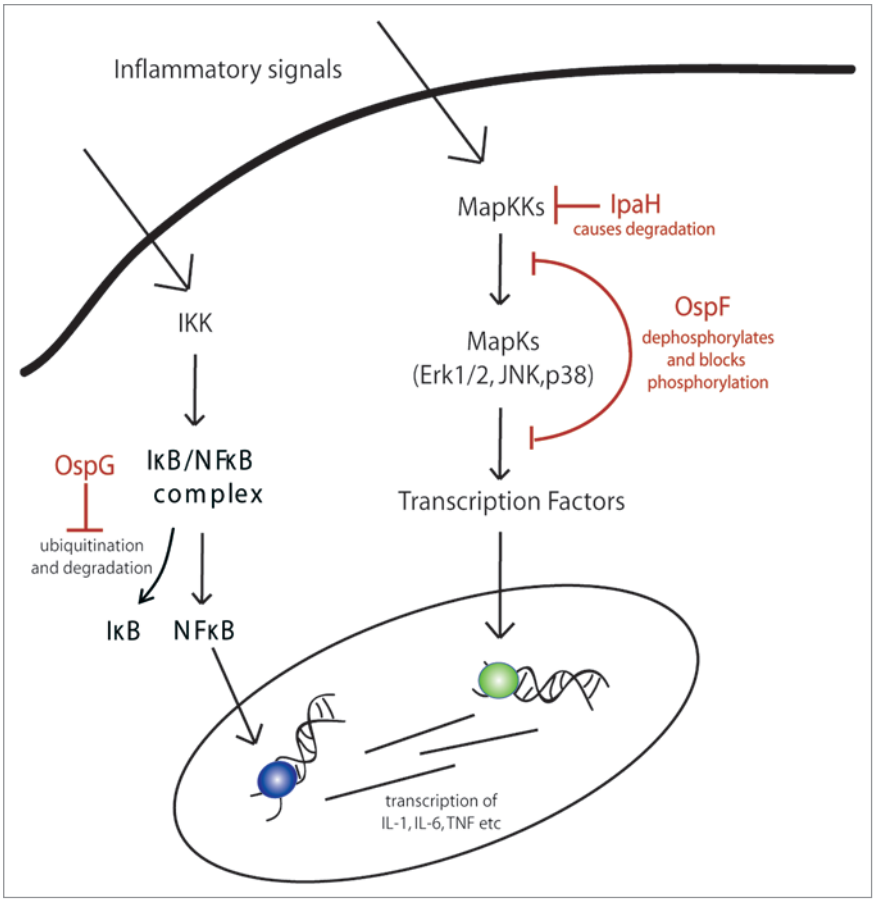

Figure 5. Modulation of pro-inflammatory signaling by Shigella T3SS effectors. Several type III secreted effector proteins have been shown to modulate the host immune response during Shigella infection. The first is OspG which interferes with the ubiquitination of phosphorylated IκB, preventing its degradation, thereby effectively blocking NFκB translocation into the nucleus. In contrast, IpaH actually induces ubiquitination and degradation of MapKKs, preventing further cascade activation. Finally, OspF has a unique way of cleaving the phosphate group and a few extra atoms from MapKs including Erk1/2, JNK and p38 which prevents further phosphorylation.

to some extent, Shigella actively evade TLR4 recognition, while TLR2 is activated by Shigella through lipoproteins.[47] In addition, the interaction of Nod1 with shed peptidoglycan from intracellular bacteria also leads to NFκB activation and IL-8 production.[48] IL-8 is another pro-inflammatory cytokine, which attracts PMNs. In this case, PMNs cause many of the symptoms of the disease but also lead to its eventual clearance.

Shigella are very closely related to *E. coli* and yet the host response is considerably different due to their ability to cross the epithelium and access basolateral toll-like receptors in addition to their ability to invade cells, where there are Nod proteins. Because of this, Shigella species have evolved a number of proteins, which counteract the host immune response. One method is preventative with mechanisms such as the alteration in LPS acetylation, which reduces TLR4 activation.[46] In addition, Shigella along with several other types of bacteria have been shown to have a recycling mechanism for their peptidoglycan, which is designed to prevent its release and interaction with Nod1. There are two permeases, AmpG and MppA, which transport free peptidoglycan from the periplasm to the bacterial cytoplasm.[48] Mice infected with an MppA mutant were able to clear an otherwise lethal dose of *S. flexneri*.[48] Masking the molecular patterns is not an entirely effective strategy, therefore, Shigella have additional strategies that block signal transduction after pathogen recognition.

Type III secreted effector proteins modulate inflammation. Shigella have a type III secretion system, which injects a number of effector proteins required for initial invasion, escape from the vacuole and movement within the cell. Because shigellosis is primarily due to inflammation, bacterial proteins that modulate the host immune response can differentially modulate the host diarrheal response as well. There are three effector proteins that meet this criteria: OspF, OspG and IpaH (**Fig. 5**). The first, OspF, has a unique way of inhibiting MAP kinase signaling. It is an entirely new type of enzyme which does not exist in mammalian cells called a phosphothreonine lyase.[49] OspF is capable of dephosphorylating threonine residues in a way that not only removes the phosphate group but also the oxygen atom, which is normally part of the amino acid, as well as a hydrogen atom from the adjacent carbon. In doing so it creates a carbon-carbon double bond within the backbone of the amino acid. In this form there is no hydroxyl group capable of being phosphorylated, thus the process is essentially irreversible. Several MAP kinases are affected by OspF, including Erk1/2, p38 and JNK.[49] These kinases activate transcription factors which control the production of proinflammatory cytokines including IL-1, IL-6 and TNFα. MAP kinases are also targeted by IpaH which is a ubiquitin ligase.[50] This was discovered using a yeast model where the yeast target was Ste7, a MAPKK. Ste7 is actively targeted for proteasomal degradation after being ubiquitinated by IpaH, thus impairing further MAP kinase activation and transcription of proinflammatory cytokines. In contrast, OspG actively interferes with the ubiquitination of phospho-IκBα, preventing its degradation.[51] IκBα normally binds to NFκB and prevents its translocation to the nucleus. Under normal conditions IκBα phosphorylation leads to its ubiquitination and subsequent degradation allowing NFκB translocation and transcriptional activation. OspG is autophosphorylated, leading to the association with ubiquitin conjugating enzymes such as UbcH5 and UbcH7.[51] While it is clear that OspG effectively blocks phospho-IκBα ubiquitination and degradation, it is unclear whether IκBα is a specific target. The ability of OspG to interact with multiple ubiquitin-conjugating enzymes suggests that there may be additional targets, but the presence of IpaH suggests that at least some ubiquitin-mediated degradation is left intact. OspF, IpaH and

OspG interfere with both the MAP kinase cascade and NFκB translocation, blocking many of the critical pathways required for transcriptional activation of host cytokines.

PMN activation can also lead to a more generalized loss of absorptive function through the destruction of the epithelial layer. In the case of Shigella, this actually enhances bacterial invasion by allowing greater access to the basolateral surface of cells.[52] However, it has been recently shown that Shigella actively opposes this loss of the epithelium through the interaction of OspE with the host protein integrin-linked kinase (ILK).[53] This interaction slows down turn over of focal adhesions, although the mechanism is not due to increased kinase activity but, instead, increased membrane association of ILK. While this mechanism prevents the initial loss of cells, it also prevents migration in wound healing assays so tissue which is already damaged will not heal. Studies of rectally infected guinea pigs suggest that OspE helps promote greater colonization, more severe diarrhea, inflammation and hemorrhaging. Maintaining epithelial integrity in this manner is somewhat unusual considering the disruption of cellular architecture caused by other pathogens; however, because Shigella colonize the epithelium intracellularly loss of cells which are already colonized could be detrimental to the bacteria.

Alterations in ion transport and barrier function. While diarrhea caused by Shigella is primarily due to host inflammatory processes and many Shigella effector proteins actively affect inflammation, there are additional factors that directly alter ion transport and tight junctions. Viable *S. flexneri*, in contrast to bacterial supernatant, heat killed bacteria or purified LPS is capable of decreasing transepithelial resistance.[54] A number of tight junction proteins including claudin-1, ZO-1, ZO-2 and occludin are altered by the presence of Shigella; however a specific effector protein responsible for these changes has yet to be identified. Shigella also possess three enterotoxins known as SigA, SepA and Pic, which are serine protease autotransporters or SPATES. As the name suggests, the primary function of SPATES is proteolytic. The autotransporter portion of the name refers to the exit mechanism from the bacterial outer membrane through a β-barrel pore formed by the C-terminus of the protein itself. While Pic has been associated with the cleavage of mucin and is believed to play a role in colonization, only SigA plays a clear role in fluid accumulation.[55] These proteases are also associated with various cytotoxic effects and induce cell rounding. The actual mechanism involved in SigA-mediated fluid increases in rabbit illeal loops has not been determined. A homologous protein in enteroaggregative *E. coli* called Pet has been shown to degrade spectrin, a cytoskeletal component, and cause changes in short circuit current in addition to cell lifting. So, while Shigella does produce toxins which alter fluid movement, and proteins which alter tight junction regulation, they are considered less important than inflammation in causing diarrhea.

Escherichia coli. *E. coli* is the most abundant facultative anaerobe of the human colonic flora and typically colonizes the gastrointestinal tract within few hours after birth.[56] Usually, commensal *E. coli* interact with the host in a mutually beneficial way; however, some strains of *E. coli* acquire virulence attributes that can cause a broad spectrum of disease. Several different classes of pathogenic *E. coli* strains responsible for diarrheal outbreaks have been recognized. These include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enetroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* and diffusely adherent *E. coli* (DAEC). This article focuses on diarrheagenic *E. coli* strains afflicting humans, in particular EPEC, which has been studied extensively in recent years, to gain understanding of the mechanisms underlying the pathophysiology of early diarrhea. A brief introduction regarding ETEC infection is provided primarily for comparison with these more modern studies.

Enterotoxigenic *E. coli*. ETEC causes toxigenic secretory diarrhea characterized by massive intestinal fluid secretion. The key virulence attributes of ETEC include adherence to epithelial cell surfaces by colonization factors and elaboration of heat labile (LT) and heat stable (ST_a) enterotoxins. Some strains of ETEC may also express Entero-aggregative heat stable toxin 1 (EAST1). Similar to cholera toxin, heat labile toxins elicit increases in Cl^- secretion via activation of cAMP. ST_a, however, is known to evoke secretion and diarrhea by elevation of intracellular cGMP. After binding to its receptor, guanylyl cyclase C (GC-C), ST_a through cGMP dependent pathways is known to stimulate CFTR translocation to the surface of villus enterocytes causing its activation.[57] There is net Cl^-, HCO_3^-, and water secretion as well as inhibition of Na^+/H^+ exchange in jejunal enterocytes by ST_a.[58] Other studies suggested that ST_a is only anti-absorptive and does not stimulate Cl^- secretion.[59] Although GC-C has been shown to be the primary receptor involved in ST_a mediated secretory response, recent studies have shown that a non-GC-C receptor exists in the mouse proximal intestine and functionally stimulates ST_a-induced duodenal bicarbonate secretion through a mechanism independent of CFTR.[60] The CFTR-independent mechanism is further supported by studies demonstrating stimulation in HCO_3^- secretion in the duodenum of CF patients[61] and CFTR knock-out mice.[62] Whether this mechanism is relevant in normal individuals or occurs as an adaptive response due to the loss of CFTR needs to be investigated.[60] Further elucidation of alternative bicarbonate secretory mechanisms modulated by ST_a utilizing DRA and PAT-1 knock-out mice would also be of interest.

LT has two serogroups, LTI and LTII, that do not cross react immunologically. LTI is expressed by strains of *E. coli* that are pathogenic for both animals and humans, whereas LTII is rarely found in human isolates and has not been associated with disease. Both LTI and LTII closely resemble CT structurally and functionally. LTI is composed of an enzymatic A subunit and a pentameric B subunit that binds strongly to ganglioside GM1. After binding to the membrane, LTI is endocytosed and undergoes trafficking through the trans-Golgi network (TGN).[63] LT targets adenylate cyclase leading to increased cAMP, activation of PKA leading to increased phosphorylation and activation of CFTR. The resulting stimulation in Cl^- secretion is a classical example of how LT and CT cause diarrhea. LT has been shown to decrease H^+/PEPT co-transporter through a cAMP dependent pathway in Caco-2 cells.[64] Thus, cAMP mediates the hypersecretory effects of LT resulting in decreased absorption and increased secretion of fluids and electrolytes.

Enteropathogenic *E. coli* (EPEC). EPEC is a major cause of persistent, watery diarrhea in infants, often accompanied by low-grade fever and vomiting. The pathogenic mechanisms of EPEC remained elusive for many years because, in contrast to prototypic enteric bacterial pathogens, EPEC does not produce classical enterotoxins such as LT in order to influence host cell pathways. In addition, EPEC is typically regarded as non-invasive in comparison to bacteria such as Shigella and Salmonella, although a limited number of the bacteria are internalized. However, EPEC does encode a T3SS and produces a characteristic attaching and effacing (A/E) lesion which is marked by effacement of microvilli on the epithelial surface at the site of bacterial attachment.[65] In addition, there is an accumulation of cytoskeletal proteins beneath adherent microcolonies leading to actin cup or pedestal formation, depending on cell type. This profound change in intestinal epithelial cells induced by A/E lesions contributes to the diarrheal phenotype due to loss of overall absorptive surface.[66] However, diarrhea occurs as quickly as 3–4 h after the ingestion of the pathogen, suggesting that mechanisms other than malabsorption are at work.[66,67] Progress is being made in unraveling the basis of EPEC pathogenesis at the molecular level and the mechanism(s) by which EPEC alters host epithelial responses are beginning to unfold. Studies over the past decade suggest that EPEC-induced diarrhea is a multi-factorial process and involves several factors including disrupted paracellular permeability and disturbances in ion transport. Recent advances suggest that EPEC has a direct effect on ion transport processes notably those related to solute, electrolyte, serotonergic and short chain fatty acid transporters. The following section will review potential mechanisms underlying EPEC-associated diarrhea.

Alterations in Na^+ and Cl^- transport. Investigation of the direct effects of EPEC on intestinal ion transport suggest that decreased intestinal NaCl absorption underlies the pathophysiology of the early onset of diarrhea rather than an increase in Cl^- secretion.[68-70] Earlier studies utilizing human intestinal epithelial Caco-2 cells showed that EPEC infection resulted in a small and transient increase in Cl^--dependent short circuit current (I_{sc}), a measure of charged ion movement.[71] In contrast, in T84 cells, a colonic crypt cell line widely used to study apical Cl^- secretion, there was no increase in Cl^- secretion but rather an inhibition of agonist-induced secretory responses.[70] Although secretion is minimally affected, EPEC alter Na^+ and Cl^- absorption in both Caco-2 and T-84 cells.[69] While net Na^+ uptake mediated by Na^+/H^+ exchangers (NHE) is actually increased by EPEC in Caco-2 cells, the activity of NHE isoforms is differentially modulated.[69] In the intestine, both NHE2 and NHE3 isoforms are apically localized, while expression of NHE1 is restricted to the basolateral membranes. Notably, EPEC infection of intestinal epithelial cells decreases the activity of NHE3, the major Na^+-absorbing isoform in the small intestine. In contrast, NHE1 and NHE2 isoforms are stimulated in response to infection with EPEC, which is thought to be a compensatory host response to alteration in NHE3 activity.[69] However, NHE2 knock-out mice do not have the same intestinal absorption defects or a diarrheal phenotype as NHE3 knock-out mouse mice.[72,73] Additionally, NHE2 is unable to prevent diarrhea in NHE3 knock-out mice.[74] This suggests that while absorption of Na^+ ions is important, it is not directly coupled with water movement in the case of NHE2. Studies utilizing EPEC mutants revealed that NHE3 inhibition is dependent on an intact T3SS III and occurs via the effector molecule EspF.[75] EPEC-induced inhibition of NHE3 activity also requires NHE regulatory factors (NHERF) that are important for regulation of NHE3 function and surface expression.[76] The inhibitory effect of EPEC on NHE3 is enhanced when PS120 cells are co-transfected with the scaffolding/regulatory proteins NHERF1 and NHERF2.[75] In addition to NHE3, EPEC has been shown to rapidly inactivate sodium-D-glucose transporter (SGLT1), the major contributor of fluid uptake in the small intestine.[77] Due to the critical role of SGLT1 in oral rehydration therapy, further research into the mechanism of this process would be of great benefit (**Fig. 6**).

Cl^--HCO_3^-(OH^-) exchangers function in concert with the Na^+/H^+ exchangers in mediating coupled electroneutral NaCl absorption in ileal and colonic epithelial cells. Parallel to EPEC mediated inhibition of NHE3, a decrease in apical Cl^-/OH^- exchange activity was demonstrated in Caco-2 and T84 cells.[68] Further studies to delineate the mechanisms underlying the decrease in Cl^-/OH^- exchange revealed an important role for the T3SS. Mutations in either the effector protein e*spG* or its paralog *espG2* partially attenuated the inhibitory effects of EPEC, whereas the double mutant completely abolished the inhibition of Cl^-/OH^- exchange activity.[68] Since EspG and EspG2 play an important role in disrupting the host microtubule network,[78] these studies suggested that EPEC-induced inhibition of Cl^-/HCO_3^-(OH^-) exchange activity is dependent on the disruption of microtubules. This was further confirmed by the observation that colchicine and nocodazole, microtubule-disrupting agents, decreased Cl^-/OH^- exchange activity in Caco-2 cells.[68] Among the candidate genes for luminal human intestinal Cl^-/HCO_3^- exchangers are two members of the SLC26 gene family: SLC26A3, or DRA, and SLC26A6, or PAT-1 (putative anion transporter-1). DRA is predominantly expressed in the colon and plays a major role in the apical Cl^-/HCO_3^- exchange process based upon its implication in congenital chloride diarrhea (CLD).[79] CLD is a rare genetic disorder characterized by voluminous diarrhea, massive loss of Cl^- in stool and metabolic alkalosis. In addition, in contrast to PAT-1 knock-out mice, DRA knock-out mice develop a robust diarrheal phenotype as well as serum electrolyte imbalances.[80,81] Both biochemical and immunofluorescence studies to assess surface expression of apical anion exchangers demonstrated reduced levels of DRA (but not PAT-1) on the apical plasma membrane in response to EPEC infection. This reduction in plasma membrane levels of DRA was EspG/EspG2 dependent.[68] An important role of DRA in the pathophysiology of diarrhea was further confirmed in vivo. A marked redistribution of DRA from apical to subapical compartments was demonstrated in EPEC-infected mouse colon.[68] Furthermore, mice challenged with *C. rodentium*, the mouse equivalent of EPEC, showed ~10 fold downregulation of DRA and fatal fluid loss.[82,83] A decrease in DRA and NHE3 activities impairs luminal NaCl absorption and underlies the pathophysiology of EPEC induced early diarrhea (**Fig. 6**).

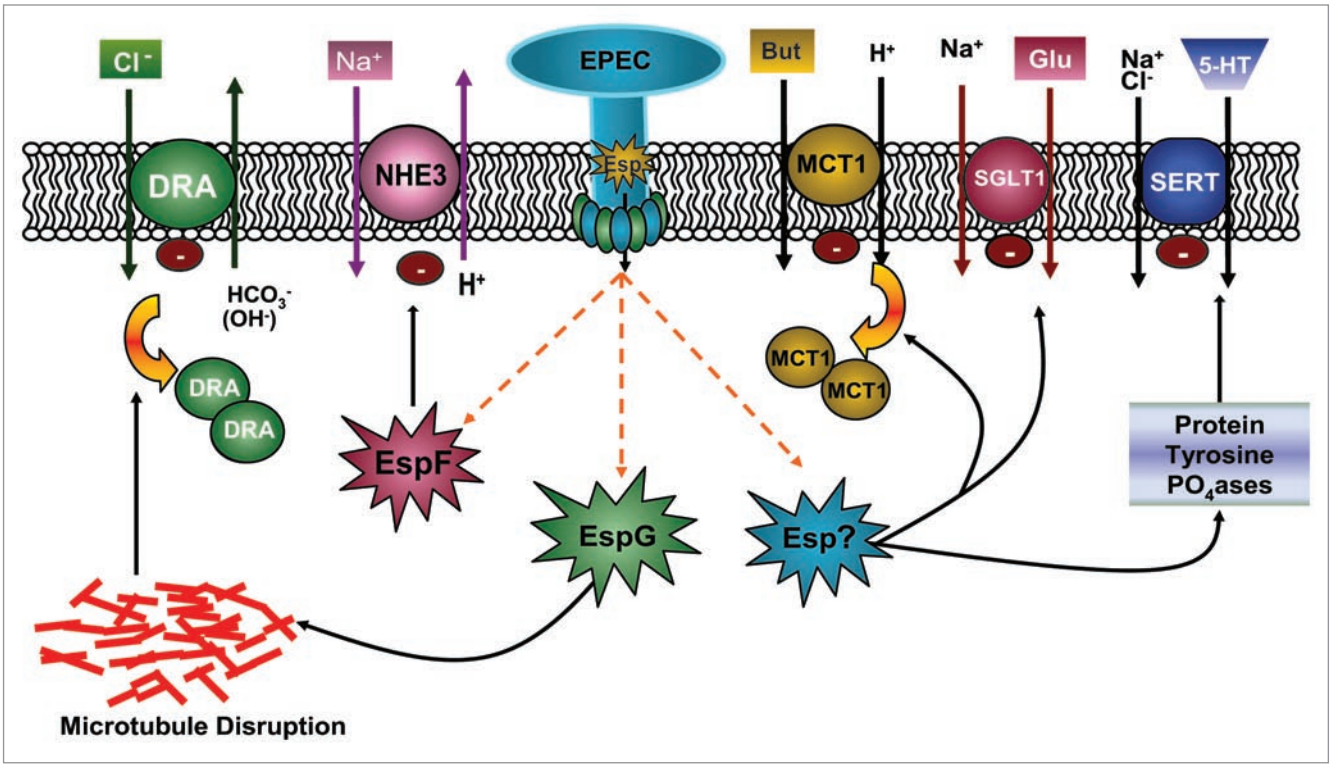

Figure 6. EPEC-induced early diarrhea is multifactorial. EPEC infection of human epithelial cells decreases epithelial ion absorption to cause diarrhea. The type III secretion system of EPEC releases *E. coli* secreted proteins (Esps) into the host cytosol. EspF inhibits the function of Na^+/H^+ exchange isoform 3, whereas EspG, via disruption of microtubules, decreases surface levels of DRA leading to a decrease in apical $Cl^-/OH^-(HCO_3^-)$ exchange activity. The resulting inhibition of coupled, electroneutral NaCl absorption in the infected intestine is thought to contribute to diarrhea. EPEC, via an unknown effector molecule, decreases butyrate absorption by decreasing the plasma membrane expression of monocarboxylate transporter 1. EPEC-induced activation of protein tyrosine phosphatases (PTPases) decreases the function of serotonin transporter (SERT) and increases 5-HT availability, which can further affect ion absorption and motility to cause diarrhea. Furthermore, SGLT-1 inhibition by EPEC is thought to promote fluid accumulation causing early diarrhea.

Water transport. Aside from modulating electrolyte transport, *C. rodentium* has been shown to directly impact water transport. During *C. rodentium* infection, water channels called aquaporins 2 and 3 (AQP2 and AQP3) redistribute from cell membranes to the cytoplasm partly due to EspF and EspG.[84] This altered AQP localization correlates with increased fluid levels in internal stool although the mice do not exhibit diarrhea per se. This loss of water absorption is thought to be a contributing factor to diarrhea during EPEC infection. In addition, genome-wide transcriptional array studies utilizing *C. rodentium* infected mouse colon showed a drastic downregulation of AQP8 mRNA[83] (**Fig. 6**).

Modulation of serotonin and butyrate transporters. EPEC is also known to alter other epithelial processes that indirectly influence electrolyte transport, epithelial integrity and immune responses in intestinal epithelial cells. 5-HT, a neurotransmitter and hormone of the GI tract, affects several physiological processes including absorption and secretion of fluids and electrolytes via serotonin receptors.[85] 5-HT is internalized through the highly selective Na^+ and Cl^--coupled serotonin transporter, SERT, which facilitates 5-HT degradation by intracellular 5-HT catabolizing enzymes. Therefore, SERT regulates 5-HT content and availability in the gut lumen. Enterotoxins such as CT are known to cause the release of serotonin in the small bowel which supports the secretory mechanism causing diarrhea.[86] Recent studies demonstrated that EPEC infection of Caco-2 cells decreases SERT function.[87] Protein tyrosine phosphatases (PTPases) were involved in inhibiting SERT in Caco-2 cells infected with EPEC. Decreased SERT expression and mucosal 5-HT content also occurred in EPEC-infected murine small intestine.[87] Future identification of the EPEC effector molecule(s) that activates PTPases and mediates inhibitory effects on SERT will be of importance in developing new targets to modulate the serotonergic system in treatment of infectious diarrheal diseases.

Another potential conributor to EPEC-induced diarrhea is the modulation of butyrate absorption mediated by monocarboxylate transporter (MCT1) in the intestine.[88] Butyrate, the major short chain fatty acid, provides energy for colonocytes and is known to stimulate electrolyte absorption.[88] Butyrate transport in epithelial cells is reduced by EPEC infection via a TTSS dependent mechanism and occurs via decreasing the levels of MCT1 on plasma membrane[88] (**Fig. 6**).

Disruption of tight junctions and immune responses to EPEC. Several lines of evidence demonstrate that EPEC infection of intestinal epithelial cells disrupts the tight junction barrier due to activation of myosin light chain kinase (MLCK) and dephosphorylation of occludin.[89,90] These effects are dependant on a

functional T3SS and several effector proteins including EspF, NleA and Map. The effect of NleA on tight junction integrity occurs via inhibition of host cell protein trafficking through COPII-dependent pathways, while the mechanism of EspF activity remains unknown.[90] The decrease in TER seen with EPEC infection also occurs in vivo in ileal and colonic mucosa.[91] This particular phenotype is EspF-dependent and correlates with the redistribution of occludin, replicating in vitro data.[91] Disruption of tight junctions increases paracellular permeability allowing electrochemical gradients to reach an equilibrium and has been shown to have a synergistic effect with altered ion transport in causing diarrhea.

Initiation of the inflammatory response is another effect of EPEC infection. EPEC is known to activate NFκB, which stimulates the transcription of IL-8, a prototypical cytokine that recruits PMNs to the site of infection.[92] EPEC-induced activation of NFκB also upregulates expression of the galanin-1 receptor, which results in increased Cl^- secretion and fluid accumulation within the lumen after activation by its ligand, galanin-1.[66] Although inflammation is certainly the net effect of EPEC infection, the disease is not as inflammatory as other bacterial pathogens such as Shigella and recent studies have demonstrated that EPEC effector proteins dampen the inflammatory response somewhat.[93] The ability of EPEC to suppress the degree or severity of the net inflammatory response was suggested to be essential for the survival of these noninvasive bacteria in the host.[93]

Collectively, these observations suggest that EPEC-induced diarrhea is a multifactorial process with perturbation of electrolyte, solute and water transport contributing to the development of early onset diarrhea induced by EPEC infection (**Fig. 6**). A prominent conclusion that emerged from these studies is the use of distinct effector proteins, suggesting selective effects of EPEC on ion transporters. For example, EPEC infection of host intestinal epithelial cells differentially modulates NHE2 and NHE3 activities as well causes a decrease in plasma membrane levels of DRA but not PAT-1.[68,69] Also, EspG which is involved in disruption of the host microtubule network, is involved in perturbation of Cl^- and water transport but does not modulate other apical membrane transporters such as Na^+ or butyrate transporters.[68,75,84,88]

Enterohemorrhagic *E. coli*. EHEC is another major intestinal pathogen that is a subset of Shiga toxin producing *E. coli* (STEC). EHEC adheres to epithelial cells, expresses a T3SS and causes A/E lesions much like EPEC.[63] Unlike EPEC, infection with EHEC can cause more severe symptoms including bloody diarrhea and life-threatening conditions such as hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic pupura. These symptoms are largely attributable to the production of Shiga toxins (Stx1 and/or Stx2), both of which are composed of one 32 kDa A subunit bound non-covalently to the pentameric ring of identical B subunits. The A subunit possesses N-glycosidase activity that catalyzes depurination of a single adenine residue of 60S ribosomes, rendering them inactive.[94] The B subunits mediate the binding of toxin to the globotriaosylceramide (Gb3) glycolipid receptor present on the plasma membrane of certain eukaryotic cells.[95] Although EHEC infections often originate from contaminated beef, EHEC colonization of ruminants is actually asymptomatic because of differences in Gb3 receptor distribution. CD77/Gb3 is present on kidney glomerular cells in humans but not in cattle, making HUS a serious complication only for humans. Interestingly, human intestinal epithelial cells do not even express GB3 receptors,[94,96] however, a novel binding site for Stx has been described at the base of small intestinal crypts of leiberkuhn in paneth cells.[97]

With respect to mechanisms of diarrhea, both Stx1 and Stx2 elicit luminal fluid accumulation in the intestine.[98] In this regard, purified Stx1 inoculated into adult rabbit ileum induces fluid accumulation and is associated with apoptosis in villus absorptive cells.[99] Similarly, Stx2 holotoxin evokes fluid accumulation in vivo and inhibits net absorptive water transport across human colon in vitro.[98] Recent studies utilizing T84 intestinal cells and a rabbit model of EHEC infection demonstrate a novel mechanism of Stx-mediated diarrhea. Stx1 infection decreases the secretion of galectin-3, a β-galactoside-specific lectin, resulting in mistargeting of several important brush border proteins.[100] Particularly, trafficking of villin, NHE2 and dipeptidyl peptidase is impaired, which is expected to alter epithelial absorption, leading to diarrhea.[100] Interestingly, decreased galectin-3 levels have also been described in Crohn's disease.[101] Whether EHEC virulence factors encoded by T3SS directly affect epithelial ion transport processes and contribute to diarrheal phenotype of this pathogen, as in the case of EPEC, requires further investigation.

Viral diarrhea. Viruses including norovirus, sapovirus, adenovirus, rotavirus and astroviruses are responsible for 30–40% of acute episodes of diarrhea in the US.[40,102] Compared to bacterial infections, many viral infections are mild and self-limited. Norovirus infection affects people of all ages and accounts for ~40% of non-bacterial diarrheal outbreaks in the US.[103] In contrast, rotavirus causes life-threatening gastroenteritis in children, accounting for 35% of the hospitalized cases of diarrhea in the US.[104] In the following section, we discuss three viral species that cause diarrhea, including rotavirus, which has the only known viral enterotoxin, as well as norovirus and astrovirus which are only beginning to be understood.

Rotavirus. Rotavirus is the leading cause of life-threatening diarrheal diseases among young children. Research over the past several years has provided important insights into mechanism of viral pathogenesis and led to successful development of live, attenuated vaccines for gastroenteritis.[105] Rotavirus primarily infects small intestinal villus cells and can cause watery diarrhea without any significant intestinal inflammation. The double stranded RNA genome of rotavirus encodes for 6 structural proteins that form virus particles (Vps) and 6 non-structural proteins (NSPs).[106] NSP4 is the first described virus-encoded enterotoxin and has been suggested to play a critical role in fluid and electrolyte secretion.[107] Interestingly, NSP4 is absent in the mature infective virion and is synthesized in infected villus enterocytes (**Fig. 7**). NSP4 and virus particles are released through the apical membrane of polarized epithelial cells by a non-classical secretory pathway.[105] However, NSP4 is also released from the basolateral side of infected enterocytes, although the role of basolaterally-released NSP4 in diarrhea is not clearly understood[108] (**Fig. 7**).

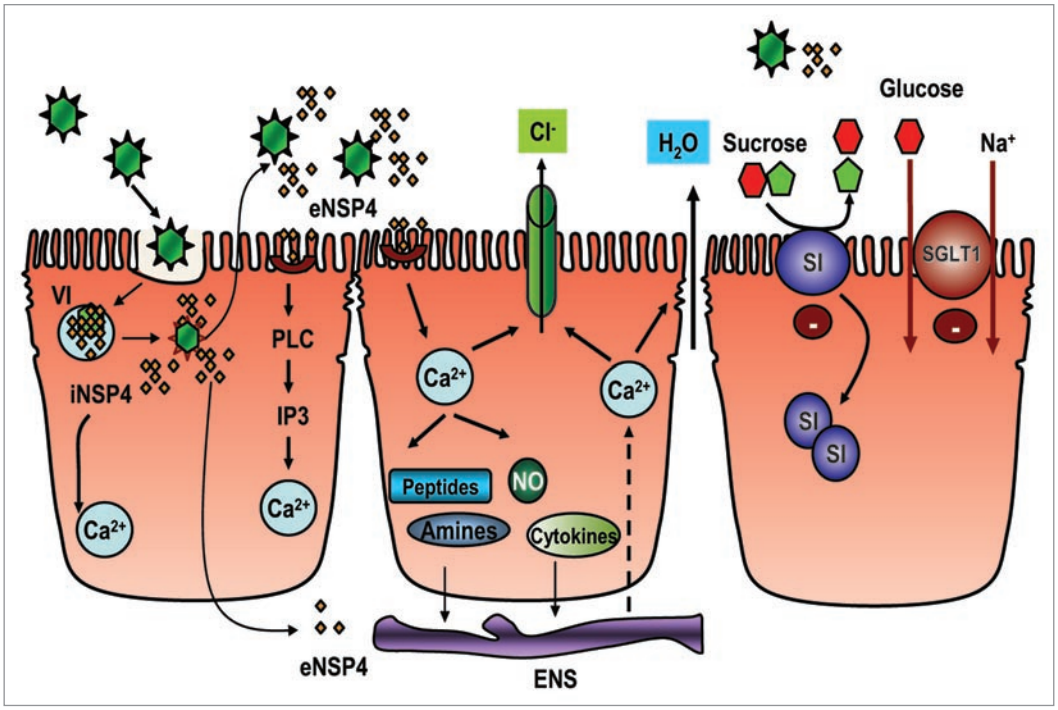

Figure 7. Mechanisms of rotavirus-mediated diarrhea. Rotavirus infection of enterocytes leads to virus entry, formation of viroplasms (VI) and release of the virus and its toxin, NSP4. Intracellular NSP4 (iNSP4) induces an increase in intracellular Ca^{2+} primarily through release from ER and a PLC-independent mechanism. NSP4 released from the apical side increases intracellular calcium levels through a receptor-mediated PLC-dependent mechanism. The increase in calcium by NSP4 disrupts microvillus cytoskeleton as well as barrier function, leading to an increase in the paracellular flow of water and electrolytes, causing diarrhea. The NSP4 induced increase in Ca^{2+} levels can also increase Cl^- secretion directly through a CFTR-independent mechanism and can cause release of amines, peptides, cytokines and reactive oxygen species, which can stimulate the enteric nervous system indirectly to increase Cl^- secretion. The basolateral release of NSP4 may also stimulate ENS. Maldigestion of carbohydrates due to a decrease in surface levels of sucrase-isomaltase and decreased function of SGLT-1 appears to be a major mechanism underlying diarrhea caused by rotavirus infection. eNSP4 is extracellular NSP4.

The virology and pathogenesis of rotavirus has been extensively reviewed recently.[105]

In contrast to classical secretory diarrhea, the viral enterotoxin, NSP4, induces diarrhea subsequent to maldigestion of carbohydrates concomitant with decreased water absorption, increased Ca^{2+} mobilization and a relatively mild Cl^- secretory component (**Fig.** 7). Maldigestion of carbohydrates has been suggested as a major mechanism underlying the pathophysiology of rotavirus-induced diarrhea. Rotavirus infection of Caco-2 cells decreases sucrose-isomaltase activity and apical expression in the absence of enterocyte destruction, suggesting the involvement of trafficking mechanisms.[109] Similarly, infection of young rabbits or mice with rotavirus decreases disaccharidase activity.[110,111] In addition, NSP4 applied exogenously is known to induce Ca^{2+} release from intracellular stores and plasmalemmal Ca^{2+} influx through a phospholipase C-dependent mechanism.[105] This NSP4-mediated Ca^{2+} mobilization can support diarrhea by influencing Ca^{2+}-dependent epithelial processes such as ion transport, barrier function or cytoskeletal regulation. Indeed, rotavirus has been demonstrated to increase paracellular permeability in Caco-2 cells.[112] In addition, NSP4-mediated Ca^{2+} mobilization may trigger the release of amines/peptides as well as the release of cytokines, prostaglandins and reactive oxygen species, which can alone or collectively activate the enteric nervous system (ENS).[113] This was further confirmed with studies demonstrating that net rotavirus-mediated fluid transport was inhibited by treatment of mice with drugs that affect ENS function.[114] Further, clinical studies in hospitalized, rotavirus-infected children show that an enkephalinase inhibitor reduces diarrhea duration.[115] Intracellular NSP4, however, is also known to increase intracellular calcium levels through a PLC-independent mechanism.[116] Utilizing NSP4-EGFP expression in HEK 293 cells, recent studies demonstrate that intracellular NSP4 causes actin reorganization in a calcium-dependent manner through decreased phosphorylation of the actin remodeling protein cofilin.[117] Modulation of subcortical actin dynamics and dysregulation of cofilin influences membrane trafficking events and ion transport processes.[118]

The Cl^- secretory component underlying the pathogenesis of rotavirus-associated diarrhea is complex, comprised of both pro- and anti-secretory components.[119] Unlike the purely secretory diarrhea caused by CT, rotavirus infection only moderately increases luminal Cl^- concentration.[119] An increase in luminal Cl^- concentrations could be a consequence of decreased absorption and/or increased secretion. Early studies demonstrated that NSP4 can cause diarrhea in young mice, which is associated with Ca^{2+} mobilization and potentiation of cAMP-dependent fluid secretion.[107] Interestingly, in CFTR-deficient mouse pups, NSP4 continues to result in diarrhea ruling out the involvement

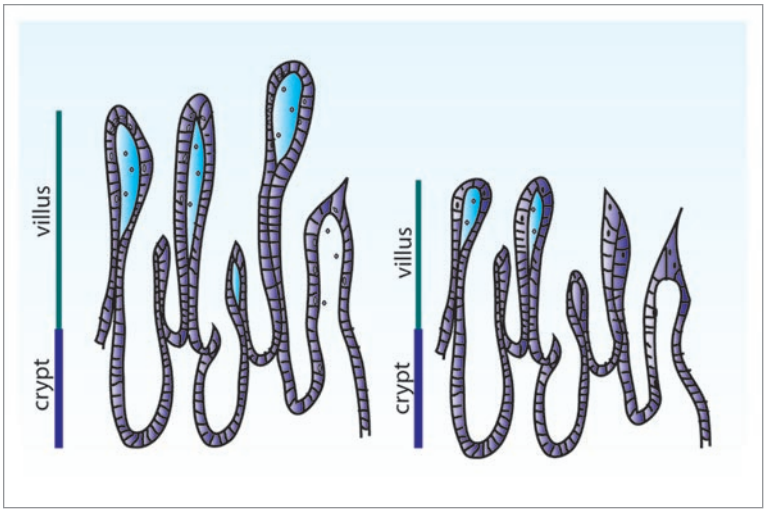

Figure 8. Shortening of villi associated with viral Infections. Viral infections including astrovirus and norovirus cause villus blunting or shortening of the villi. There is a decrease in the number of cells making up the villi, reducing the overall absorptive surface. Intestinal epithelial cells are generated from stem cells within the crypts and move toward the villus tip, where they are ultimately shed. Blunting can be caused by reduced cell proliferation, although in the case of norovirus it is associated with an apical villus infection, resulting in increased cell death. Villus blunting is also the cause of diarrhea associated with Celiac disease, which is autoimmune rather than pathogen caused.

of CFTR in fluid accumulation.[120] Unexpectedly, rotavirus infection of rabbits actually stimulates Cl^- absorption in intestinal brush border membrane isolated from villus cells and does not alter Cl^- secretory responses in crypt cells.[119] However, the net Cl^- secretory response is weak, suggesting that NSP4 exerts both secretory and anti-secretory actions to limit overall Cl^- secretion.[119] More in-depth studies are required to delineate the cellular mechanisms underlying rotavirus associated Cl^- secretory responses. The potential role of apical Cl^-/HCO_3^- exchangers, CLC chloride channels and key signaling events in the pathogenesis of rotavirus infection would be of utmost interest.

Norovirus. Noroviruses, previously known as Norwalk-like viruses, are a member of the Caliciviridae family.[40] They are one of a subset of viruses, which cause viral gastroenteritis, more commonly known as the stomach flu. This illness is typified by short term vomiting and diarrhea, both of which are infectious. The stomach flu is caused by a large number of viruses from several different families and includes sapoviruses, which are also members of the Caliciviridae family, a subtype of adenovirus, as well as astrovirus. In contrast to rotavirus infection, none of these viruses are particularly well-studied and infection rarely results in hospitalization, except in immune compromised individuals. A typical norovirus infection lasts anywhere from 1–3 days and not all infected individuals develop symptoms. Transmission is both foodborne and person-to-person via infectious GI fluids.

Research has been somewhat limited with regard to the molecular basis of norovirus induced diarrhea, since the virus has only recently been grown in tissue culture and earlier studies relied on transfection of viral genes into host cells. Several viral factors can interfere with basic cellular functions without causing cell death. One of these is the 3C-like proteinase ($3CL^{pro}$), which is similar to a previously described enzyme in rhinovirus called $3C^{pro}$. $3C^{pro}$ interferes with eukaryotic translation by cleaving poly(A)-binding protein (PABP).[121] Since both ion transporters and tight junction proteins have intermediate turnover times of 12–18 hours on average, inhibition of host translation could limit the abundance of these important regulators of intestinal homeostasis. Another mechanism which has the potential to interfere with host proteins involves the nonstructural protein p48 of Norwalk virus. P48 binds to a cellular protein known as VAMP associated protein or VAP-A, which associates with v-SNARES, regulators of vesicle trafficking.[122] As with the inhibition of translation, inhibition of protein trafficking is likely to interfere with cellular processes critical for maintaining absorptive function as well as barrier function.

While cell culture models have been impossible until recently and animal models are limited, a recent study was carried out using human intestinal biopsies from patients suffering from norovirus infection.[123] Initial observations showed that, in infected patients, villus length was decreased by 25%, while crypt length remained unchanged, reducing the overall absorptive surface of the epithelium (**Fig. 8**). Miniature Ussing chambers (0.049 cm^2) used to determine resistance, flux and short circuit current on biopsies samples, showed an increase in I_{sc} in patients infected with norovirus, which was dependent on active Cl^- secretion, consistent with CFTR activation. SGLT1 activity was also increased suggesting that electrogenic Na^+ absorption is not impaired. In addition to changes in Cl^- secretion, there was a marked decrease in transepithelial resistance that corresponded with a decrease in the levels of occludin as well as claudins 4 and 5. Because this work was carried out in human tissue, little is known about the mechanism which underlies these changes, however, recently developed norovirus cell culture models should prove useful.

Astrovirus. Astroviruses, like noroviruses, are another cause of the stomach flu. Astrovirus infection includes both diarrhea and vomiting and spreads in a similar manner to the previously described viruses. Little is known about the pathology of astrovirus infection as hospitalization is unlikely. One report is available describing the pathology of an immune-suppressed 4 year old male who showed villus shortening, which is consistent with what is seen in turkey models[124] (**Fig. 8**). In addition, in both these human biopsy specimens and in animal models, there is relatively little inflammation, suggesting that this is not an immune-mediated diarrhea.[125] However, there is some evidence that pro-inflammatory pathways are activated in the cell. In fact, activation of ERK1/2 is absolutely required for viral replication.[126] UV-inactivated astrovirus is also capable of activating ERK1/2 with a similar response at 15 minutes post infection followed by inactivation at 30 minutes.[126] The reduction in both viral protein and RNA levels compared to controls is most consistent with the case of HIV, which requires ERK activation for entry, however, the actual mechanism of ERK activation on adenovirus entry

and replication is not yet known. The activation of ERK in these cells is moderate and more is known about the inactivation of the innate immune response due complement inhibition. The astrovirus coat protein is capable of binding to the classical complement component C1q, blocking the subsequent cascade of complement cleavage and membrane attack complex formation.[127]

Astrovirus infection causes diarrhea with mild inflammation and has been shown to affect barrier function. Moser et al.[128] demonstrated a decrease in TER in Caco-2 cells infected with astrovirus. Interestingly, there was also a loss of TER in cells which were treated with UV-inactivated astrovirus, which is incapable of replication, or with virus-like particles, which lack RNA. This suggests that the entry step is responsible for a loss of TER and is consistent with the fact that other viruses are known to target tight junctions during entry. However, the cellular receptor for astrovirus remains unknown. The authors also suggest that there is a disruption of occludin in infected cells, however, cellular morphology is changed substantially after viral infection and is consistent with cells undergoing the early stages of apoptosis. The alteration of occludin could represent an early apoptotic event, although there is a loss in barrier function which is not influenced by early apoptotic events.

The association of astrovirus with cell death in humans is in question due to the lack of patient samples and the variability seen in animal models, specifically turkeys and pigs. In vitro, however, cell death has been seen in Caco-2 cells starting at 36 hours post-infection.[129] This apoptosis is blocked by the caspase inhibitor z-VAD-fmk. Interestingly, this viral activation of the caspase system is critical for the production of infectious virions. A precursor of the capsid protein called VP90 has a caspase cleavage site, which is clipped as a step in generating an intermediate capsid protein, VP70. As such, the caspase inhibitor z-VAD-fmk substantially inhibits viral processing to VP70. Virions can be formed by VP90, although they are not stable or infectious and in host cells the cleavage of VP90 to VP70 is critical for release from the cell. While cell death is not particularly associated with astrovirus infection, the characteristic blunting of villus tips could be explained through a loss of cells. This villus blunting along with changes in barrier function are the primary means through which astrovirus causes diarrhea, although changes in ion transport have not yet been studied.

Parasitic diarrhea. Diarrhea caused by parasites is unlike that of either bacterial or viral infections. For example, Giardia has a slow onset of diarrhea and can be present for months, while most bacterial and viral infections are limited to 1–2 weeks.[40] In addition, parasites are eukaryotic, which makes them larger and more complex than either viruses or bacteria and also more difficult to eradicate due to their similarity to the host. In fact, it is not unusual for parasites to make their own versions of host proteins: For example serotonin and PGE_2 are both made by *Entamoeba histolytica*. The advanced mechanisms used by the two important protozoan parasites, *Giardia lamblia* and *E. histolytica*, to cause diarrhea will be discussed below.

Giardia lamblia. *Giardia lamblia* (syn *G. duodenalis* and *G. intestinalis*) is the most common parasite of the human small bowel and causes waterborne diarrheal disease worldwide. Giardia is a non-invasive organism and trophozoites, the active form of the parasite, colonize the upper small intestine by adhering to the apical surface of the epithelium.[130] Symptomatic infection with Giardia causes acute or chronic diarrhea, dehydration, abdominal pain and malabsorption leading to malnutrition and weight loss. Also, this parasitic infection can trigger exacerbation of IBS[131] and may cause development of post-infectious functional gastrointestinal disorders.[132] Several studies utilizing a variety of cell systems, animal models and recent studies in humans with chronic infections have provided important insights into the pathophysiology of giardiasis. An important finding emanating from these studies is that malabsorption, secretion of electrolytes and impairment of tight junctions may underlie the luminal fluid accumulation during infection. Infections with Giardia species are known to cause a diffuse shortening of the microvillus brush border via activated T lymphocytes, accompanied by the reorganization of F-actin and ZO-1 in enterocytes through MLC phosphorylation.[133] Enterocyte apoptotic pathways are also induced by *G. lamblia* via caspase-3 activation, which may also adversely affect epithelial tight junctional integrity.[134] Previous studies from both in vitro and in vivo animal models demonstrated decreased absorption of glucose and Na^+ and reduced disaccharidase activity due to the loss of epithelial absorptive surface area.[135,136] Also, *G. lamblia*-infected mouse intestine demonstrated secretion of Na^+ and Cl^- that was protein kinase C dependent.[137] However, the role of Giardia virulence factors, such as proteinases and lectins, in modulating secretory responses needs to be fully determined.

The studies from in vitro and animal models were recently confirmed with observations from human subjects infected with Giardia.[138] It was established that *G. lamblia* infection is comprised of active electrogenic anion secretion, impaired barrier dysfunction and malabsorption. Analysis of tight junction proteins in membrane extracts from duodenal biopsy samples of infected patients showed a decrease in the epithelial tight junction protein pool primarily caused by reduction in mucosal surface area.[138] However, after mucosal surface area correction, only claudin 1 expression was differentially downregulated in chronic giardiasis.[138] Despite the decreased mucosal surface area, an increase in basal I_{sc} reflecting increased $Cl^-/HCO_3(OH)^-$ secretion was observed in giardiasis.[138] Importantly, the mucosal surface reduction in human biopsy samples is predominantly loss of villus surface while crypts, an important site of active anion secretion, are not affected. Additionally, apoptosis and a dramatic reduction in villus surface leading to impaired Na^+-dependent glucose absorption was observed in the duodenum of patients chronically infected with *G. lamblia*.[133,138] Detailed investigation of the signaling mechanisms and virulence factors underlying epithelial dysfunction following infection will be of importance to understand the pathophysiology of giardiasis as well as development of post-infectious IBS.

Entamoeba histolytica. *E. histolytica* is a single cell protozoan which causes amebiasis. Pathologic effects are due to both parasite virulence factors and the host response to the parasite. In fact, only about 10% of people infected with *E. histolytica* actually develop symptoms.[40] In those who present with invasive disease,

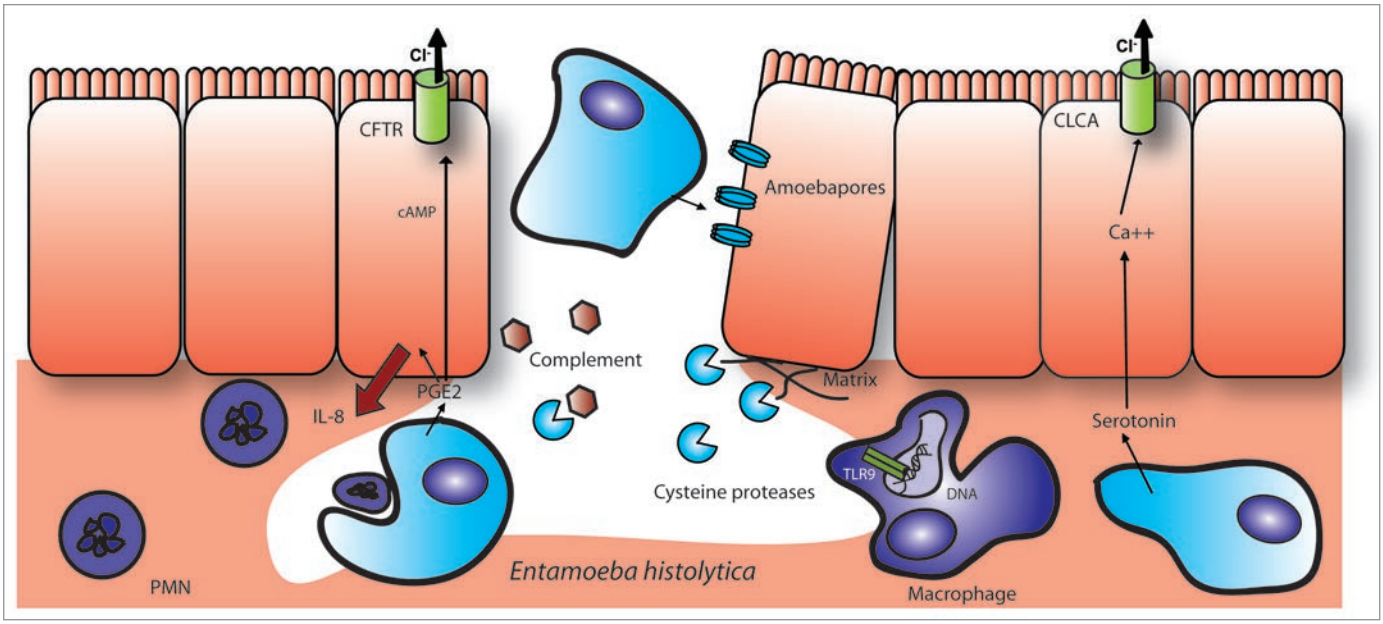

Figure 9. Epithelial destruction and inflammation caused by *E. histolytica*. *E. histolytica* induces a flask-shaped ulcer usually spanning several villi rather than several cells as shown here. The epithelial layer is destroyed both due to pathogen factors, such as the pore forming amoebapores and extracellular matrix cleavage by cysteine proteases, as well as host inflammatory factors. *E. histolytica* releases PGE_2 and causes a host PGE_2 response that results in colonic Cl^- secretion, IL-8 secretion and PMN recruitment. While PMNs can be neutralized and phagocytosed by the parasite and complement is degraded by cysteine proteases, the infection is still highly inflammatory. Macrophages have been shown to be activated by parasite DNA through TLR9 or alternately through lipophoshpopeptidoglycan activation of TLR 2 or 4. In addition to PGE_2, *E. histolytica* also secretes serotonin, which is capable of activating Ca^+ dependent Cl^- secretion in the small intestine.

there is an initial ulceration of the intestinal epithelium due to the expression of several cysteine proteases, which degrade the extracellular matrix, and due to amoebapores which cause cell lysis[139,140] (**Fig. 9**). This results in cell detachment, reducing the absorptive surface of the intestine. In addition, the cysteine proteases cause degradation of several complement factors including C3, C3a and C5a, as well as immunoglobulins, thus blunting the innate immune response. While *E. histolytica* infection is inflammatory, the parasite is resistant to neutrophils due both to complement degradation and a C59-like complement-inactivating adhesion on the surface of the parasite.[141] In addition, PMNs can be killed or alternately, neutralized through inhibition of cathepsin G, a serine protease, thus decreasing the activity of PMNs in areas of infection.[142,143] In addition, macrophages are activated due to stimulation of endosomal TLR-9 by parasite DNA as well as activation of TLR2 and 4 by lipopeptidophosphoglycan.[144,145] These inflammatory cells are thought to enlarge the initial flask-shaped ulcerations which are characteristic of *E. histolytica* infection. Therefore, a combination of parasite mediated destruction and subsequent inflammation causes the ulceration of the epithelium which reduces absorption and disrupts barrier function.

In addition to these morphological changes there are also direct effects on barrier function. Because of their ability to induce cell damage and make apparent holes in monolayers, work with *E. histolytica* and TER must be done early in infection. Leroy et al. found there was a drop in resistance as early as 1 hour post infection, while monlayers began losing cells between 3 and 6 hours post infection.[146] This decrease in TER was associated with an increase in mannitol flux and can not be replicated with soluble factors or sonicated trophozoites. Infection does not alter levels of occuldin or its localization but does cause a decrease in levels of ZO-1 without obvious dissociation from the tight junction.[146] While secreted cysteine proteases seem a likely cause of this drop in TER, typical protease inhibitors such as E-64 or aprotinin did not block the drop in resistance. However, inactivation of cysteine proteases has been shown to block the later drop in resistance associated with hole formation in monolayers.[147]

In addition to changes in tight junction activity, there are also changes in ion transport. Analysis of amebic lysates suggests that there are two distinct actions on Cl^- secretion: there is a Ca^{2+} dependent process, which is active in ileum and a cAMP-dependent process, which is active in colon (**Fig. 9**). The Ca^{2+}-dependent response is only active on the serosal surface, suggesting that these mechanisms are not activated until the epithelial layer is breached.[148] HPLC analysis and immunodetection found that amebic lysates actually contain low levels of serotonin and pathogenic strains contain twice the level of non-pathogenic strains. Serotonin is not the only host protein made by *E. histolytica*; recent studies show that prostaglandin E_2 (PGE_2) is also made when the amoebae are supplied with arachidonic acid (AA)[149] (**Fig. 9**). They have their own cyclooxygenase (COX)-like enzyme, which is capable of converting AA to PGE_2 but is not inhibited by indomethacin as are human COX enzymes.[149] However, this appears to be only a small part of the PGE_2 response because indomethacin is capable of blocking PGE_2 production in human

Table 1. An overview of the data presented in this review assessing the relative contribution of each phenotype to diarrhea for each pathogen presented

Pathogen	Type	Symptoms	Duration	Inflammation	Increased secretion	Tight junctions	Decreased absorption	Loss of cells*
Vibrio cholerae	gram (-)	profuse watery diarrhea	3–4 days	+	+++	+++	+++	-
Clostridium difficile	gram (+)	diarrhea + fever	3 days+	+++	++	+++	+	++
Shigella species	gram (-)	diarrhea + fever	5–7 days	+++	+	+++	?	+
Escherichia coli (EPEC)	gram (-)	diarrhea	1–3 weeks	+	-	+++	+++	-
Rotavirus	dsRNA	vomiting + diarrhea + fever	3–8 days	-	+	++	+++	+
Norovirus	(+) RNA	vomiting + diarrhea	1–2 days	+	++	+++	?	+++
Astrovirus	(+) RNA	vomiting + diarrhea + fever	1–4 days	+	?	++	?	++
Giardia	protozoan	diarrhea	2–6 weeks	-	+	+++	++	+++
Entamoeba histolytica	protozoan	diarrhea	weeks to months	++	++	++	?	+++

*Loss of cells includes shortening of villi.

intestinal xenografts in SCID mice.[150] The effects of PGE_2 are multifold with an increase in both production of IL-8, a potent chemoattractant, and an increase in Cl^- secretion.[150,151] Secretory effects in mouse colon are attributed to PGE_2 synthesis with only a minor Ca^{2+}-dependent component (25%).[151] Pretreatment of cells with PGE_2 causes desensitization to amebic lysates and the COX inhibitors indomethacin and piroxicam block the rise in I_{sc} almost entirely.[151] This mechanism is dependent on the production of cAMP and can be blocked with piroxicam, suggesting a CFTR-dependent mechanism in contrast to the Ca^{2+}-dependent mechanism found in small intestine[151] (**Fig. 9**). Both mechanisms are active on the basolateral side of cells, suggesting that this is not an early event but instead depends on initial infiltration of the epithelial layer by the parasite prior to activation. The overall picture of *E. histolytica* infection depends both on parasite components, including cysteine proteases, amoebapores, serotonin and PGE_2 as well as the host inflammatory response with no single component providing a complete answer.

Conclusion

The elucidation of the mechanisms underlying infectious diarrhea has progressed remarkably over the last decade and will continue to advance. Pathogen-specific virulence factors and signaling cascades affect wide-range of cellular functions such as ion secretion, absorption, barrier function and membrane trafficking events that elicit luminal fluid accumulation causing diarrhea (summarized in **Table 1**). This is further evident from the huge versatility in mechanisms utilized by pathogenic strains of *E. coli*, thus contributing to the complexity of pathogenesis. Remarkably, studies in recent years have progressed to define *E. coli*-secreted proteins (Esps) that specifically decrease ion and water absorption, in contrast to toxin producing strains, which stimulate secretion. Furthermore, activation of the mucosal immune and enteric nervous system as well as cytoskeletal arrangements and loss of absorptive surface seems to augment the outcome of infection (**Table 1**). Although, malabsorption seems to be a prominent factor in rotavirus and Giardia infections, much progress needs to be made on the molecular mechanisms and virulence factors underlying ion transport modulations associated with these infections. Further investigation into how pathogenic strains of *E. coli* modulate host physiological responses in vivo to cause diarrhea is critical to completely define the disease. Similarly, our understanding of the pathogenic mechanisms of noroviruses and astroviruses is inadequate and needs extensive research. Unraveling the pathophysiological mechanisms of these pathogens may help identify novel therapeutic targets not only for diarrhea associated with enteric infections but also for a variety of other gastrointestinal diarrheal disorders.

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