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Enteric infection meets intestinal function: how bacterial pathogens cause diarrhoea

V. K. Viswanathan^{*,§}, Kim Hodges^{‡,§}, and Gail Hecht[‡]

^{*}Department of Veterinary Science & Microbiology, University of Arizona, Tucson, Arizona 85721, USA

[‡]Department of Medicine, Section of Digestive Diseases and Nutrition, University of Illinois and Jesse Brown Veterans Affairs Medical Center, Chicago, Illinois 60612, USA

Abstract

Infectious diarrhoea is a significant contributor to morbidity and mortality worldwide. In bacterium-induced diarrhoea, rapid loss of fluids and electrolytes results from inhibition of the normal absorptive function of the intestine as well as the activation of secretory processes. Advances in the past 10 years in the fields of gastrointestinal physiology, innate immunity and enteric bacterial virulence mechanisms highlight the multifactorial nature of infectious diarrhoea. This Review explores the various mechanisms that contribute to loss of fluids and electrolytes following bacterial infections, and attempts to link these events to specific virulence factors and toxins.

Infectious diarrhoea, particularly that due to pathogenic bacteria, is a major health problem worldwide. In developed countries, infectious diarrhoea contributes primarily to morbidity but, in the developing world, it is responsible for a high level of mortality, particularly in children below 5 years of age¹. Although the overall frequency of diarrhoea in children below 5 years of age has not changed significantly since the mid 1980s (median of 3.2 episodes of diarrhoea per child–year), measures such as use of oral rehydration therapy have reduced global mortality from nearly 4.6 million per year in 1982 to 2.5 million per year by 2000 (REF. 2). As significant brain and synapse development in humans takes place within the first 2 years of life, impaired absorption of nutrients during this period can result in long-term developmental disabilities. Repeated bouts of early childhood diarrhoea contribute significantly to growth shortfalls (up to 8.2 cm by age 7) and impairment in fitness, cognition and schooling³. By one estimate, recurrent diarrhoea in the first 2 years of life can contribute to a loss of 10 IQ points and a year of schooling by 9 years of age³.

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Correspondence to G.H. gahecht@uic.edu.

[§]These authors contributed equally to this work.

DATABASES

Entrez Genome Project: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj>

Citrobacter rodentium | *Salmonella enterica* serovar

Typhimurium | *Shigella flexneri* | *Vibrio parahaemolyticus*

OMIM: <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>

congenital chloride diarrhoea

UniProtKB: <http://www.uniprot.org>

CFTR | DRA | MyD88 | NHE2 | NHE3

FURTHER INFORMATION

Gail Hecht's homepage: <http://www.uic.edu/com/dom/gastro/hechtlab.html>

ALL LINKS ARE ACTIVE IN THE ONLINE PDF

At its most basic level, diarrhoea results from an imbalance of absorption and secretion of ions and solute across the gut epithelium, followed by the movement of water in an attempt to restore the appropriate ion concentrations. Often, this imbalance is caused by the presence of bacteria that secrete toxins that disturb the organization of the epithelium. Diarrhoea benefits enteric pathogens by facilitating their rapid dissemination into the environment and, consequently, the infection of new hosts. Additionally, host passage increases the virulence of some bacterial pathogens⁴⁻⁶. Although diarrhoea probably reduces bacterial burden in the host, any potential benefit is negated by the fatality that is associated with the concurrent loss of fluid and electrolytes. In a study with the murine enteric pathogen *Citrobacter rodentium*, the mortality of susceptible strains of mice was attributed to fluid loss; subcutaneous administration of fluids fully protected the infected animals without affecting bacterial shedding, body weight alterations or the magnitude of colonic lesions⁷.

The intestine absorbs nutrients while simultaneously forming a barrier to noxious substances and bacteria. Along its 7 metre length, the human intestine displays regional specialization, which is further marked by the presence of distinct cell types in different areas. There is also a gradient distribution of commensal bacterial flora along the length of the intestine, with maximal numbers being present in the colon. A single layer of polarized columnar epithelial cells resting on a basement membrane separates the intestinal lumen from the underlying tissue (FIG. 1). Below the epithelial monolayer are the supporting stroma, the lamina propria and a layer of smooth muscle. The undulating ridges of the intestinal submucosa, the folds of the lamina propria forming the villi and crypts, and the finger-like microvilli on epithelial cells maximize the absorptive surface of the small intestine. The apical part of the lateral membrane of intestinal epithelial cells contains the junctional complexes, which comprise tight junctions, adherens junctions and desmosomes. Desmosomes and adherens junctions mechanically connect adjacent cells, and tight junctions form a regulatable barrier between the luminal and serosal sides of the epithelial monolayer (barrier function). In addition, by restricting the free movement of apical and basolateral components of epithelial cell membranes, tight junctions maintain polarity and thereby aid the directional movement of water, electrolytes and nutrients (fence function).

Recent studies have greatly advanced our understanding of the complex and dynamic regulation of ion transporters and channels⁸, the mechanisms regulating the integrity of the epithelial monolayer⁹, the dynamic nature of tight junctions and the highly sensitive and rapid process of wound healing¹⁰. In the field of innate immunity, great strides have been made in uncovering the roles of Toll-like receptors (TLRs) and NOD (nucleotide-binding oligomerization domain-containing)-like receptors (NLRs) in the recognition of the molecular patterns that are associated with microbes, both pathogenic and commensal¹¹. The role of the gut microbiome in physiological and pathological contexts is now realized and is beginning to be explored¹². The education and development of the adaptive immune system within this complex milieu has also been studied¹³.

Despite significant advances, the precise mechanisms by which bacterial infection leads to diarrhoea are not entirely clear. It is now evident that diarrhoeagenic bacteria actively communicate with other bacteria and with host cells¹⁴, that they use multiple adhesins to attach to enterocytes¹⁵ and that they produce and secrete a plethora of protein molecules that manipulate epithelial cell physiology both from the outside and within host cells¹⁶. Correspondingly, bacterium-induced fluid and electrolyte loss is multifactorial, involving an array of virulence factors and host signalling pathways (FIG. 2).

This Review focuses on the mechanisms by which enteric bacterial pathogens and, in some instances, commensals, cause diarrhoea, with an emphasis on the effects on intestinal epithelial cells. Specifically, an attempt will be made to link the effects of specific bacterial

molecules on intestinal epithelial cells to the eventual loss of fluids and electrolytes. In the mammalian intestine, components of the adaptive immune system and the enteric nervous system¹⁷, specialized cells such as mast cells and neutrophils^{18–20}, and pro-inflammatory molecules²¹ also influence intestinal epithelial cell function and the fluid and electrolyte balance. The specific contributions of these systems to bacterium-induced diarrhoea, however, are not addressed here. Broader coverage of the epidemiology, host susceptibility and morbidities associated with diarrhoea is available in a recent review by Petri and colleagues³.

Ion transport and diarrhoea

Although a number of factors, including genetic mutations, hormonal alterations, radiation injury and malabsorption, can cause diarrhoea, the most common cause is infection by bacterial pathogens and the subsequent release of bacterial toxins. Such toxins typically trigger signalling molecules such as cyclic AMP or intracellular Ca^{2+} , which, in turn, activate cellular Cl^- channels leading to an increase in secretion of Cl^- and consequently water (FIG. 2a, 3). Ca^{2+} activates the calcium-dependent chloride channel CLCA (chloride channel, calcium activated) and cAMP activates the cystic fibrosis transmembrane regulator (CFTR), a Cl^- channel that was originally identified through the presence of mutations in cystic fibrosis patients²². Cholera toxin ADP-ribosylates adenylate cyclase, resulting in increased cAMP production and activation of protein kinase A, which in turn phosphorylates the regulatory domain of CFTR to activate Cl^- secretion^{23,24}. Cholera toxin entry and the activation of Cl^- secretion is shown in more detail in BOX 1. The thermostable direct haemolysin (TDH) of *Vibrio parahaemolyticus*, however, activates the Ca^{2+} pathway²⁵. The molecular pathways involved in Ca^{2+} -dependent Cl^- secretion are less clear, as the CLCA family was recognized far more recently than CFTR²⁶. TDH causes an increase in intracellular Ca^{2+} in a protein kinase C (PKC)-dependent manner, but direct regulation of CLCA activity by PKC has not been studied. Cholera toxin activation of CFTR through cAMP and protein kinase A has been well described²⁷, and recent studies have focused on cholera toxin retrograde trafficking through the cell cytosol, a process that is important for the activation of this potent diarrhoeagenic mechanism (BOX 1).

The internalization and toxicity of cholera toxin is dependent on cell type, shifting from clathrin- to caveolin- mediated uptake as cells mature²⁸. One explanation for the increased sensitivity of neonates to cholera toxin-induced diarrhoea is development-associated alterations in membrane phospholipids and consequent changes in endocytosis and signalling pathways. In intestinal epithelial cells, maturation of membrane phospholipids and association of membrane proteins with lipid rafts shifts the endocytic process from a rapid clathrin-dependent mechanism to the slow and regulated caveola-mediated pathway²⁹. Thus, clathrin-mediated uptake is the predominant means of internalization in human fetal cell lines and xenografts. In the adult cell line T84 and hydrocortisone-treated fetal cells, however, uptake is mediated by caveolae and shows dependence on GM1 and lipid rafts²⁹. Compounds, such as glucocorticoids, that contribute to the growth and development of immature enterocytes alter cholera toxin endocytosis, probably by altering membrane lipid composition²⁹.

Reduced Cl^- absorption

Although CFTR and CLCA are the most recognized targets of bacterial diarrhoeal pathogens, Cl^- absorption can also be affected (FIG. 2a). Increased secretion and decreased absorption of Cl^- have the same net effects on luminal Cl^- concentrations. Congenital chloride diarrhoea (CLD), a recessive disorder characterized by lifelong diarrhoea, is caused by a number of mutations in an apical Cl^-/OH^- exchanger called downregulated in adenoma

(DRA)³⁰. Interestingly, enteropathogenic *Escherichia coli* (EPEC) decreases the cell surface levels of DRA, ultimately inhibiting the absorption of Cl^- (REF. 31) (FIG. 3). This process depends not on a secreted toxin but on the injection of effector proteins into the host cytosol by a type III secretion system (T3SS). This needle-like complex allows EPEC to bypass the complicated retrotranslocation pathway that cholera toxin and other AB_5 toxins require to access the cytosol. The EPEC T3SS, a multiprotein secretion complex spanning the inner and outer bacterial membranes, communicates with a translocon comprising the external filamentous appendage of EspA monomers and the pore-forming complex of EspB and EspD proteins inserted in the host cell plasma membrane. The bacterial ATPase EscN drives protein translocation across the T3SS and into the host cytosol. Deletion of *escN*, *espA*, *espB* or *espD* effectively disrupts the T3SS and prevents the decrease in DRA activity. Characterization of a series of mutants identified two homologous proteins, EspG and EspG2, that are required to decrease Cl^- absorption. These proteins degrade tubulin, directly or indirectly, resulting in disruption of the host microtubule network³². Although the specific mechanism involved is yet to be elucidated, the result is a dramatic internalization of DRA into the sub-apical vesicle pool, thus reducing surface Cl^-/OH^- exchange³¹.

Microtubule disruption can also be achieved by treatment with the drug colchicine; interestingly, treatment of cultured intestinal epithelial cells with colchicine *in vitro* causes a decrease in DRA activity similar to that of EPEC infection. Several ion exchangers are regulated through apical recycling into endosomes, creating a pool of exchangers at the cell surface and just below the surface in membrane vesicles, so it is possible that EPEC-induced disruption of micro-tubules influences the surface concentration, and hence activity, of other transporters as well

Decreased sodium uptake

In addition to changes in Cl^- secretion or absorption, attenuation of Na^+ absorption can also cause diarrhoea (FIG. 2b). Intestinal Na^+ absorption is primarily mediated by Na^+/H^+ exchangers (NHEs). In addition to its effects on Cl^- secretion, cholera toxin has long been known to modulate Na^+ absorption. It has recently been shown that both apical intestinal NHE isoforms, NHE2 and NHE3, are suppressed in response to cholera toxin. However, effects on NHE2 are post-translational whereas effects on NHE3 are post-transcriptional³³. In fact, as cAMP decreases NHE2 and NHE3 activity, all pathogens that stimulate Cl^- secretion through cAMP — including heat-labile enterotoxin-producing strains of *E. coli* — presumably also inhibit Na^+ uptake by NHE2 and NHE3. In general, movement of Na^+ and Cl^- ions is coupled either directly or indirectly. An example of indirect coupling is the effect of cAMP that is induced by cholera toxin. cAMP inhibits Na^+ absorption through NHE2 and NHE3 but increases Cl^- secretion through CFTR, resulting in net accumulation of NaCl at the epithelial surface. Alternatively, some transporters, such as NHE3 and DRA, are directly coupled through protein–protein interactions. The NHE regulatory factor (NHERF2) binds to both NHE3 and DRA; in addition, NHERF2 can bind to other NHERF2 proteins and form a large complex containing clustered NHE3 and DRA proteins³⁴.

Interestingly, although EPEC alters the activity of NHEs and DRA, these effects are not linked³¹. Whereas cholera toxin suppresses all apical Na^+ uptake, EPEC increases the activity of NHE2 by about twofold and decreases NHE3 activity by half in *in vitro* models³⁵ (FIG. 4). Although both NHE2 and NHE3 are involved in apical Na^+ -absorptive pathways, NHE3 has a more important role in diarrhoeal disease³⁶. A functional T3SS is required for alteration of NHE activity, but EspG1 and EspG2, the effectors responsible for the alteration of DRA activity, are not involved. In fact, EspF has recently been shown to be responsible for decreasing NHE3 activity in EPEC-infected cells^{37,38}. Although the effect of EPEC on NHE3 is under investigation, it is known that changes in NHE2 activity rely on cellular

signal transduction. Stimulation of NHE2 by EPEC involves activation of phospholipase C and Ca^{2+} , which in turn triggers PKC α and PKC ϵ ³⁷. PKC has been shown to regulate NHE2 and NHE3 in the differential manner seen with EPEC infection, that is, an increase in NHE2 activity and a decrease in NHE3 activity. However, the role of PKC in EPEC-mediated alteration of NHE3 has not been investigated.

In addition to the effects on NHE activity, EPEC alters another route of Na^+ uptake: the Na^+ /glucose cotransporter SGLT1 (REF. 39) (FIGS 2c,4a). SGLT1 is active only in the presence of glucose. Oral rehydration solutions rely on the presence of glucose and SGLT1 to drive Na^+ into cells and restore fluid balance. EPEC-mediated diarrhoea is typically less severe than that caused by cholera, but is refractory to oral rehydration therapy⁴⁰. EPEC alters SGLT1 activity by two distinct mechanisms³⁹. The first is associated with the characteristic phenotype of EPEC infection: the formation of attaching and effacing lesions by the bacterial microcolonies leading to the effacement of microvilli and the consequent decrease in epithelial cell surface area. Both the destruction of microvilli and the loss of SGLT1 activity 6 hours after infection are dependent on a number of secreted effector proteins, including translocated intimin receptor (Tir), EspF and Map, in addition to intimin, the EPEC outer membrane binding partner for Tir³⁹. The second means of altering SGLT1 is specific and occurs more rapidly. Within 30 minutes of EPEC infection of Caco-2 human intestinal epithelial cells, SGLT1 activity is reduced by 25–85%, depending on the multiplicity of infection³⁹. The mechanism by which this occurs, however, is unknown (FIG. 4a).

Altered water transport

The controversy over the mode of transepithelial water transport was resolved by the discovery of water channels known as aquaporins⁴¹, but the role of aquaporins in the pathophysiology of diarrhoea is unclear (FIG. 2d). Recently, correlation between the internalization of aquaporins 2 and 3 with peak fluidity of stool was demonstrated⁴². Infection of mice for 7 days with *C. rodentium*, a murine pathogen closely related to EPEC and EHEC⁴², resulted in decreased aquaporin staining at the apical and lateral membranes, presumably causing a decrease in water absorption. The secreted effector proteins EspF and EspG were implicated in this phenotype. Although it is difficult to compare with mouse infection, human volunteer studies with EPEC showed a rapid onset of diarrhoea at 4 hours after infection. Thus, the role of aquaporins in the early stages of EPEC infection remains to be determined. One would predict that loss of aquaporins would exacerbate diarrhoea, but the loss of a single aquaporin gene has not been correlated with net changes in water movement. Despite this, they are of great interest and will no doubt be the basis of future work.

In a recent study, Borenshtein *et al.* performed a genome-wide transcriptional analysis of two closely related mouse strains that demonstrate differential susceptibility to *C. rodentium* infection⁴³. In response to *C. rodentium* infection, inbred FVB/N mice develop more severe colitis and display higher mortality than resistant outbred Swiss Webster mice. During the initial phase of infection (4 days) the two strains demonstrate comparable changes in body weight, shed bacteria in similar numbers and have few colonic lesions. Greater than twofold difference in expression between the two strains was observed in 462 loci before infection; the number increases to 5,123 differentially expressed genes following *C. rodentium* infection of the two strains. More genes related to water and ion transport are differentially regulated than even immune response-associated genes. *C. rodentium* infection of the susceptible mouse strains for 9 days results in downregulation of DRA (1,100-fold) and aquaporin 8 (268-fold), but has a less dramatic effect on these genes in the resistant strains (<3-fold decrease in the expression of the same genes). Consistent with the hypothesis that

diarrhoea contributes significantly to death, the susceptible strains are completely protected from mortality by fluid therapy, although no significant changes are seen in other disease parameters or the severity of colonic lesions^{7,43}.

Epithelial barrier function and diarrhoea

A single layer of contiguous cells executes the vectorial transport of ions and solutes across the apical and basolateral surfaces of the intestinal epithelium. Gross alterations in homeostatic functioning of the intestinal epithelium, such as loss of the epithelial monolayer by mechanisms including apoptosis and delayed wound repair, are likely to result in unregulated fluid loss and consequent diarrhoea. More subtly, enteric pathogens and their toxins can interfere with ion and solute transporters and cause diarrhoea without necessarily compromising monolayer integrity. Tight junctions provide the barrier required for the maintenance of the electrochemical gradients that are necessary for efficient transcellular transport⁴⁴. The tight junction is a multi-protein complex composed of transmembrane proteins, such as occludin and members of the claudin family, cytoplasmic scaffolding proteins, including the zonula occludens family (ZO1, ZO2 and ZO3), and signalling molecules⁴⁴. A key mechanism controlling permeability across this barrier involves the contractile actomyosin ring that is positioned just below, and interacts with, tight junctions. This is achieved by the myosin light chain kinase (MLCK)-dependent phosphorylation of the regulatory subunit of the myosin light chain (MLC) (FIG. 2e). Various pathogens, including EPEC and *Giardia lamblia*, stimulate intestinal epithelial MLC phosphorylation⁴⁵. An increase in phospho-MLC could result from activation of MLCK, or the inhibition of MLC phosphatase. Although the precise mechanism for the infection-mediated increase in phospho-MLC is not known in many instances, EPEC stimulates an increase in intracellular Ca^{2+} and the accumulation of Ca^{2+} -calmodulin complexes that induce MLCK activation. Correspondingly, Ca^{2+} chelators and MLCK inhibitors blocked EPEC-induced disruption of the epithelial barrier^{46,47}.

Although MLC-dependent disruption of the epithelial barrier by intestinal pathogens has been demonstrated, direct evidence for the contribution of barrier disruption to diarrhoea derives from studies involving the pro-inflammatory cytokine, tumour necrosis factor- α (TNF α)^{9,48} (FIG. 2e). Systemic T-cell activation using antibodies that are specific for the CD3 receptor results in a TNF α -dependent, self-limited diarrhoea in mice and humans. However, this treatment fails to disrupt the epithelial barrier or cause diarrhoea in MLCK (long isoform)-knockout mice, despite inducing an increase in TNF α . Furthermore, a permeable inhibitor of MLCK that accesses only epithelial cells prevents diarrhoea in anti-CD3 antibody-treated mice. These studies suggest that infection-dependent MLC phosphorylation could similarly contribute significantly to diarrhoea. Intestinal pathogens can also disrupt the barrier by directly altering the distribution or phosphorylation status of tight junction proteins. For example, EPEC, *Shigella flexneri* and *Salmonella enterica* serovar Typhimurium alter the phosphorylation status and distribution of occludin and ZO1, and thereby disrupt epithelial barrier function^{49–52} (FIG. 2f).

Tight junctions also assist in maintaining the polarized distribution of membrane proteins on the basal and apical aspects of epithelial cells, also known as fence function. Obviously, loss of fence function owing to tight junction disruption will interfere with vectorial absorption and secretion by the intestinal epithelium⁴⁴. In the context of an infection, the redistribution of apical and basal proteins can expose new attachment sites for the bacteria (FIG. 2f). EPEC intimin and *Yersinia* spp. invasins are adhesins that bind to $\beta 1$ integrin, a membrane protein that is predominantly localized to the basolateral sides of intestinal epithelial cells^{53,54}. EPEC-induced tight junction disruption and the consequent loss of fence function

results in the positioning of $\beta 1$ integrin on the apical side of enterocytes, thereby increasing the potential adherence sites for this pathogen⁵³.

Innate immunity and diarrhoea

The innate immune response has a significant role in the development of diarrhoea: First, the innate immune response is important for the maintenance of intestinal homeostasis and controlling the commensal flora⁵⁵. Second, innate immune responses interfere with colonization by pathogenic organisms^{56,57}. Thus, compared with wild-type animals, mice lacking the p50 subunit of the pro-inflammatory transcription factor nuclear factor- κ B (NF- κ B) have diminished inflammatory infiltration in the intestines when infected with *C. rodentium* and fail to clear the bacteria⁵⁷. Infected p50^{-/-} mice also display greater colonic hyperplasia and cytokine levels in the colon than infected wild-type mice. Third, several pathogens stimulate the recruitment and transmigration of neutrophils into the intestinal lumen^{58,59}. Neutrophils attached to the apical side of epithelial cells release 5'-AMP, which is converted to adenosine in the lumen (FIG. 2g). The interaction of adenosine with A2b receptors results in cAMP-dependent vectorial Cl⁻ secretion⁶⁰. Although this has not been extensively explored in the context of infection, adenosine release following EPEC infection contributes to Cl⁻ secretion^{61,62}. Finally, as discussed above, pro-inflammatory cytokines disrupt epithelial barrier function and thereby contribute to diarrhoea.

TLRs and NLRs are membrane-associated and cytosolic proteins, respectively, that act as sentinels to monitor the presence of pathogenic organisms in the intestine⁶³. These pattern recognition receptors detect microbe-associated molecules such as lipopolysaccharide, lipoteichoic acid and flagellin. Following ligand binding, TLRs signal through adaptor proteins such as myeloid differentiation factor 88 (MyD88) to eventually activate the transcription factor NF- κ B (BOX 2).

Many interactions between TLRs and NLRs and their ligands activate the IKK (inhibitor of NF- κ B (I κ B) kinase) complex, which phosphorylates I κ B^{63,64}. The consequent ubiquitylation and degradation of I κ B frees the transcription factor NF- κ B to enter the nucleus. NF- κ B promotes the transcription of many inflammatory response genes and pro-survival pathways. Epithelial NF- κ B has a key role in maintaining intestinal homeostasis. Selective blockage of NF- κ B activation in mouse intestinal epithelial cells (NEMO^{IEC-KO}) by ablation of IKK γ (also known as NEMO) results in runting, severe chronic inflammation, diarrhoea and rectal bleeding⁶⁵. Similarly, mice with epithelial cell-specific ablation of both IKK1 and IKK2 develop diarrhoea, colitis and rectal bleeding⁶⁵. These pathologies are due to TNF α -induced apoptosis of epithelial cells, reduced production of antimicrobial peptides, loss of epithelial integrity and translocation of bacteria into the mucosa. When TNF α signalling is disrupted in NEMO^{IEC-KO}/TNF receptor 1 double-knockout mice, they display no signs of colitis. Thus, a calibrated TLR-mediated NF- κ B activation in response to commensals and pathogens can be protective. Inappropriate NF- κ B activation, however, can result in an exaggerated inflammatory response. For instance, commensal bacteria cause colitis in the susceptible interleukin 10 (IL-10)^{-/-} mouse by inducing uncontrolled TLR-mediated NF- κ B signalling⁶⁶.

The heightened sensitivity of TLR2-, TLR4- and MyD88-knockout mice to dextran sulphate sodium-induced colitis suggests a general role for TLRs in intestinal health⁶⁷. Unlike adult immunocompetent mice, animals lacking MyD88, which is required for most TLR signalling, are susceptible to infection by the gut pathogen *Campylobacter jejuni*⁶⁸. These mice display a greater bacterial burden, barrier dysfunction, bacteraemia, severe colitis and a deficiency for epithelial repair responses, and die in significant numbers by day 7, whereas wild-type mice survive beyond 8 days after infection^{69,70}.

The various TLRs can also have specific and non-redundant roles. TLR5-knockout mice uniformly harbour increased bacterial burdens in the colon and a subset (41%) develop severe colitis, even in the absence of additional stimuli⁷¹. Unlike the TLR5 knockouts, a TLR5 and TLR4 double-knockout fails to develop intestinal bleeding or rectal prolapse despite harbouring increased bacterial loads, implicating a role for TLR4 in the pathology that is seen in TLR5^{-/-} mice. These results indicate that TLR5 signalling controls colonic bacterial populations and maintains intestinal homeostasis. Infection of streptomycin-treated wild-type animals with flagellin-deficient *Salmonella enterica* subsp. *enterica* serovar Typhimurium, or infection of TLR5-knockout mice with wild-type parent strain, causes more severe gut pathology and outcome, confirming the protective role of TLR5 signalling in promoting host cell survival^{72,73}.

Not unexpectedly, bacteria have developed various strategies to escape detection by TLRs. Such strategies include the production of flagellin variants that are not recognized by TLRs, masking of flagellar structures and molecular changes in lipopolysaccharide that result in hyporesponsiveness⁷⁴. Interestingly, effector protein(s) secreted by EPEC into host cells dampen inflammation before infection-induced barrier disruption⁷⁵. Thus, when EPEC flagellin crosses the breached barrier and engages the predominantly basolateral TLR5, the downstream signalling is already blocked. EPEC inhibits multiple pro-inflammatory signalling pathways, including the activation of IKK, MAP kinases and PI3K, by a T3SS-dependent mechanism⁷⁵. Although this study ruled out the involvement of several known EPEC effectors in this phenotype, the responsible molecule(s) and its mechanism of action were not determined. Whereas pathogenic organisms can interfere with TLR signalling⁷⁶, some bacterial virulence factors, such as *Salmonella enterica* serovar Enteritidis TlpA, *E. coli* TcpC and *Brucella mellitensis* TcpB, contain the TIR (Toll-IL-1 receptor) domain that is typical of TLRs^{77,78}. *E. coli* TcpC is secreted into the medium by an unknown mechanism, and the secreted protein is taken up by host cells⁷⁸. The secretion, delivery and localization of *S. Enteritidis* TlpA and *B. mellitensis* TcpB remains to be confirmed. These proteins inhibit TLR-dependent NF-κB activation. TcpB and TcpC, in addition, were shown to interact directly with MyD88 (REF. 78). Mice infected orally with a lethal dose of wild-type *S. Enteritidis* survived for an average of 4 days, whereas animals infected with a *tlpA* deletion strain survived for close to 8 days, confirming a role for TlpA in pathogenesis⁷⁷. Similarly, mouse experiments demonstrated a crucial role for TcpC in promoting bacterial numbers in the urinary tract and kidney damage. The work with NEMO knockouts discussed above suggests that interference with TLR signalling or NF-κB activation by bacteria could contribute to, or cause, diarrhoea.

The role of the host flora in homeostasis

The composition of the intestinal bacterial milieu or the microbiome is an important factor in the development of infectious diarrhoea. The human gut microbiota, which is estimated to contain 18,000 genera and 15,000–36,000 species, can be classified into two main phyla: the Bacteroidetes and Firmicutes⁷⁹. Aside from direct competitive exclusion of binding sites for potentially pathogenic organisms, commensal bacteria shape the intestinal ecology by their complex and dynamic interactions with the host immune system. Exposure of the infant gut to appropriate microbes at critical times is essential for proper development of the gut — particularly the gut-associated and systemic immune components — and for education of the immune system⁸⁰. It has been known for nearly half a century that antibiotic-mediated clearance of the gut flora sensitizes mice to infections by various gut pathogens. In the clinical setting, antibiotic treatment predisposes patients to *Clostridium difficile*-associated disease⁸¹, which is of increasing concern in light of the emergence of novel epidemic strains of this pathogen.

Host genetic factors, diet and age probably influence the composition of the microbiota; these relationships are just beginning to be explored. Metagenomic studies reveal changes in the composition of the microbial populations in disease states such as Crohn's disease and obesity, although the contribution of such alterations to pathogenesis is presently unclear⁸². RAG1-knockout mice (that is, mice lacking both T and B cells) that are deficient for the transcription factor T-bet (also known as TBX21; "TRUC mice") develop spontaneous colitis that is dependent on increased TNF α production⁸³. Most intriguingly, the mice develop a colitigenic gut flora, and transmission of this flora to wild-type mice by cross-fostering the animals results in colitis in the latter group. This study clearly shows that disease states alter the composition of the gut flora and, more importantly, that such alterations can play an important part in pathogenesis.

These investigations suggest that directed alteration of the gut flora could effectively counter intestinal disorders such as inflammatory bowel disease or infectious diarrhoea. In recent years, various groups have attempted to understand, refine and validate the therapeutic use of bacteria ('probiotics') to prevent or limit intestinal infections. Probiotics are defined functionally, as microorganisms that confer health benefits, and the organisms in question are often phylogenetically unrelated. It is, therefore, difficult to present a comprehensive explanation for their mechanism of action. The range of effects include maintenance of normal gut function, interference with pathogen attachment and/or virulence gene expression, modulation of innate and adaptive immune responses and preservation of epithelial barrier function⁸⁴. Bacteria have also been engineered to express specific molecules that mimic host oligosaccharide receptors of different bacterial toxins⁸⁵. For instance, the B subunit of Shiga toxins produced by *Shigella dysenteriae* and STEC (Shiga toxin-producing *E. coli*) interacts with Gb3 (ganglioside receptor for Shiga toxin) on target cells and facilitate the internalization of the enzymatically active A subunit. 'Designer probiotics' expressing Gb3 effectively prevent disease in mice infected with STEC⁸⁵. Understandably, regulatory and safety concerns will have to be addressed before such approaches are used in human and veterinary populations. Interestingly, formaldehyde-killed bacteria (expressing Gb3 mimics) also protect mice against challenge with STEC⁸⁵.

Given their low cost, efficacy and relatively limited side effects, the potential for probiotics to be used as therapeutic agents against diarrhoea is particularly attractive in developing countries where the disease burden is considerable³. A field trial (Phase III) in India involving 4,000 children (ages 1–5) is presently exploring the ability of lactobacillus (15 billion organisms daily for 12 weeks) to reduce the incidence of diarrhoea. Results are awaited in another Phase III trial in the United States on the ability of *Lactobacillus casei* to reduce the cumulative rate of gastrointestinal infections and diarrhoea in children aged 3–6.

Malnutrition, an underlying risk factor for diarrhoeal disease, is thought to be responsible for over 60% of diarrhoeal deaths worldwide. Vitamin A and zinc supplementation, in particular, have been shown in large-scale trials to be effective in combating diarrhoea⁸⁶. Twice-yearly supplementation of vitamin A reduced the diarrhoea-associated mortality of children 6–59 months of age by 32% (REF. 86). Vitamin A appears to decrease mortality by reducing the proportion of incidences that develop into more severe disease. However, it is not effective as a treatment for reducing diarrhoeal morbidity or the duration of a diarrhoeal episode. Zinc, a micronutrient that is essential for growth, development and immunity, is involved in various metabolic processes. Zinc has multiple effects in the intestine, including the ability to block basolateral K⁺ channels and thereby reduce cAMP-mediated Cl⁻ secretion. Daily zinc supplementation for children below 5 years of age reduces the incidence of diarrhoea, shortens the duration of diarrhoeal episodes (by up to 24%) and reduces the severity of such episodes^{86,87}. Although the therapeutic benefits of nutritional supplements and foods (such as yogurt) with live microorganisms have been recognized for

centuries, exciting discoveries in the areas of gastrointestinal physiology, adaptive and innate immune responses in the gut, and metagenomic and metabolomic studies of the microbiome are poised to place these approaches on a sound scientific footing.

Conclusion

Recent advances in genomics and proteomics have made the identification of bacterial virulence factors considerably easier. Moreover, the experimental tools to evaluate the composition of the intestinal micro-biome and its contribution to health and disease are now available. Also, recent studies on innate immunity and epithelial integrity highlight the complexity of intestinal function.

Bacterium-induced diarrhoea is multifactorial. The challenge for the future, therefore, is to understand the interplay between different aspects of intestinal physiology, such as the relationship between inflammation and secretion, and the connection between tight junctions and secretion. In the past 2 decades, research on bacterial toxins has proved instrumental in developing an understanding of normal intestinal function. For example, study of the heat-stable enterotoxins of enterotoxigenic *Escherichia coli* led to the discovery of endogenous mammalian ligands of guanylyl cyclase⁸⁸. Unravelling how newly identified bacterial virulence factors function will also provide insights into intestinal physiology and new therapeutic approaches for the management of diarrhoea.

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Glossary

Diarrhoea	A disorder manifested by frequent evacuation of excessively fluid faeces
Enteric pathogen	A pathogen whose primary target is the gastrointestinal tissue
Commensal	An organism that derives food or other benefits from another organism without harming it
Lamina propria	The layer of mucosal tissue directly below the epithelial cell monolayer; various cell types including those involved in immunity reside here
Tight junctions	Also known as kissing junctions, these lipid–protein complexes at the apical junctions of epithelial cells form a regulatable barrier, selectively allowing the passage of ions and electrolytes
Adherens junctions	Present at the contact point between epithelial cells, these serve as anchor points for cytoskeletal filaments made of actin (microfilaments)
Desmosomes	Intercellular junctions typically present in tissues subject to mechanical stress. The adhesion molecules in this region serve as a tether for cytoskeletal filaments known as intermediate filaments (for example, cytokeratins)
Microbiome	The totality of microbial species living in a specific organism

AB₅ toxins	Bacterial toxins that have the structural composition of a single catalytically active A subunit and a pentamer of B subunits. The B subunits are involved in receptor binding and deliver the A subunit to the target cells
Attaching and effacing	Attaching and effacing pathogens are a group of enteric pathogens that includes enteropathogenic <i>Escherichia coli</i> , enterohaemorrhagic <i>E. coli</i> and the mouse pathogen <i>Citrobacter rodentium</i> . These bacteria attach intimately to intestinal epithelial cells and cause the effacement of the brush border microvilli. Brush border microvilli are finger-like projections on epithelial cells that facilitate nutrient absorption
Colitis	Inflammation of the colon observed in various disease states
Runting	The birth or development of animals that are smaller than the average size for the species or strain

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Box 1**Retrograde trafficking of cholera toxin**

After entering the cell, cholera toxin (CT) is routed in a retrograde manner through the Golgi apparatus into the endoplasmic reticulum (ER)^{89–91} (see the figure). A specific amino acid sequence, KDEL, which is located within the A2 subunit of the toxin, mirrors an epitope that is present in proteins that are typically retained in the ER and results in CT translocation from the Golgi to the ER by a shuttle protein known as ERD2 (REFS 92,93). Cholera toxin co-opts the ER-associated degradation (ERAD) pathway to subsequently gain entry into the host cell cytosol. The ERAD pathway ensures that proteins transiting through the ER for secretion are properly folded. Cholera toxin mimics a misfolded protein and is retrotranslocated into the cytosol, where typical ERAD targets would be degraded by the proteasome. Instead, the A1 peptide of cholera toxin goes on to ADP-ribosylate adenylate cyclase, leading to production of cyclic AMP (cAMP), which activates protein kinase A. Protein kinase A (PKA) then phosphorylates cystic fibrosis transmembrane regulator (CFTR), leading to Cl^- secretion.

Box 2**Toll-like receptors**

Thirteen Toll-like receptors (TLRs) and 23 NOD (nucleotide-binding oligomerization domain-containing)-like receptors (NLRs) have been identified in mammals. The cytoplasmic Toll–interleukin 1 (IL-1) receptor (TIR) domain, common to all TLRs, interacts with TIR domains on various adaptor proteins such as myeloid differentiation factor 88 (MyD88), MAL and TRIF. Ligand specificities and signalling pathways for many of the TLRs and NLRs are known, and there is active investigation for others. Microbe-derived ligands engage TLRs (except TLR3), leading to recruitment of MyD88, a key adaptor protein. MyD88 then associates with IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1 through homotypic death domain interactions, resulting in the recruitment, oligomerization and activation of tumour necrosis factor-associated factor 6 (TRAF6). Subsequent recruitment of TAB2 to TRAF6 activates TAB2-associated kinase 1 (TAK1). TAK1-dependent activation of the IKK (inhibitor of nuclear factor- κ B (NF- κ B) kinase) complex results in activation of the pro-inflammatory and pro-survival transcription factor NF- κ B. For a more detailed discussion of TLR signalling see the recent review by Ishii and colleagues¹¹.

Figure 1. The 7 metre-long human intestine absorbs nutrients and forms a barrier to luminal contents

The absorptive surface of the intestine is maximized by the presence of undulating ridges of the intestinal submucosa, organization of the mucosa into villi and crypts, and the elaboration of finger-like microvilli on epithelial cells. A single layer of polarized columnar epithelial cells rests on a basement membrane, below which is the supporting thin layer of vascular connective tissue known as the lamina propria, and a layer of smooth muscle. The apical part of the lateral membrane of intestinal epithelial cells contains the tight junctions, which serve as a regulatable barrier between the luminal and serosal sides of the epithelial monolayer (barrier function). In addition, by restricting the free movement of apical and basolateral components of epithelial cell membranes (fence function), tight junctions maintain polarity and thereby aid the directional movement of water, electrolytes and nutrients. JAM-A, junction adhesion molecule A.

Figure 2. Mechanisms by which enteric pathogens cause diarrhoea

These mechanisms interfere with the normal absorptive and secretory functions of the intestinal epithelial monolayer. **a** | Increased Cl^- secretion through the cystic fibrosis transmembrane receptor (CFTR; as by cholera toxin) or decreased Cl^- absorption owing to inhibition of downregulated in adenoma (DRA). **b** | Decreased Na^+ absorption owing to inhibition of Na^+/H^+ exchanger 3 (NHE3). **c** | Downregulation of the $\text{Na}^+/\text{glucose}$ cotransporter (SGLT1). **d** | Direct inhibition of water channels or aquaporins (AQPs). **e** | Disruption of epithelial barrier function by promoting myosin light chain (MLC) phosphorylation (possibly by stimulating tumour necrosis factor- α (TNF α) production), leading to contraction of the peri-junctional actomyosin ring and opening of the tight junctions. **f,g** | Direct alteration of tight junction (TJ) protein localization leading to disruption of barrier and fence functions and pro-inflammatory signalling through engagement of Toll-like receptors (TLRs) by bacterial ligands, eventually leading to neutrophil transmigration into the lumen, and release of 5'-AMP and its conversion to adenosine, which subsequently causes cyclic AMP (cAMP)-dependent Cl^- secretion. MLCK, MLC kinase; NF- κ B; nuclear factor- κ B; PMN, polymorphonuclear leukocyte.

Figure 3. Cl^- secretion by intestinal epithelial cells is stimulated through the activation of cyclic AMP or Ca^{2+}

A number of bacterial toxins activate these signaling molecules in different ways. Thermostable direct haemolysin (TDH) from *Vibrio parahaemolyticus* activates Ca^{2+} , whereas cholera toxin (CT) from *Vibrio cholerae* increases the cAMP concentration by ADP-ribosylating adenylate cyclase. Activation of Ca^{2+} leads to Cl^- secretion by the calcium-activated chloride channels (CLCAs). Cholera toxin activates cAMP through ADP-ribosylation of adenylate cyclase. cAMP then activates protein kinase A (PKA), which phosphorylates the cystic fibrosis transmembrane receptor (CFTR) leading to Cl^- secretion.

Figure 4. Enteropathogenic *Escherichia coli* (EPEC) alter apical Na⁺, Cl⁻ and glucose absorption

a | Surface expression of the Na⁺/glucose cotransporter SGLT1 is decreased by two distinct mechanisms, both of which depend on the injection of effector proteins. The first mechanism is rapid and causes the movement of apical SGLT1 into intracellular vesicles. The second, and slower, pathway results from a loss of microvilli and hence SGLT1 on host cells, a process that is dependent on two secreted effector proteins, Map and EspF. **b** | The primary routes of apical Na⁺ uptake are the Na⁺/H⁺ exchangers (NHEs). EPEC differentially regulate the two apical NHE isoforms, increasing the activity of NHE2 through the activation of phospholipase C (PLC), which leads to activation of Ca²⁺ and protein kinase Cs (PKCs), whereas NHE3 activity is decreased through an effect of EspF. **c** | In addition to changes in Na⁺ uptake, apical Cl⁻ absorption is also altered. The injected effector proteins EspG and EspG2 disrupt microtubules, leading to internalization of the apical Cl⁻-OH⁻ exchanger downregulated in adenoma (DRA). In EPEC-infected cells DRA is displaced from the cell surface into subapical membrane vesicles. This blocks one route of apical Cl⁻ absorption, which has been previously implicated in congenital Cl⁻ diarrhoea.