Enteropathogenic and enterohemorrhagic *Escherichia coli* infections: Emerging themes in pathogenesis and prevention

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Enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E coli* (EHEC) are important causes of infectious diarrhea, particularly among pediatric populations. While EPEC is a significant health threat in the developing world, EHEC causes sporadic but deadly outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome in North America and other developed areas. The present review discusses emerging themes in the pathogenesis of EPEC and EHEC, including the discovery and characterization of novel bacterial proteins that are injected by the pathogen into host cells. Recent advances have also been made in the development of relevant animal models, while bacterial virulence factors are being investigated as potential vaccination targets for humans and animals. It is hoped that these new areas of study will not only further our knowledge of the pathogenesis of EPEC- and EHEC-induced disease but also provide opportunities for reducing infection rates and improving treatment options in the future.

Key Words: *Citrobacter rodentium*; *Escherichia coli*; *EPEC*; *EHEC*; *Diarrhea*; *Hemolytic-uremic syndrome*; *Infection*; *Pathogenicity island*

Infection à *Escherichia coli* entéropathogène et entérohémostatique : thèmes émergents en pathogénèse et prévention

RÉSUMÉ : Les espèces *Escherichia coli* entéropathogènes (EPEC) et entérohémostatiques (EHEC) sont d'importantes causes de diarrhée infectieuse, particulièrement auprès des populations pédiatriques. Bien que l'EPEC présente une menace significative pour la santé des pays en voie de développement, l'EHEC cause des éclosions sporadiques et mortelles de coliques hémorragiques et du syndrome hémolytique urémique en Amérique du Nord et dans d'autres régions industrialisées. Le présent rapport abordera les thèmes émergents touchant la pathogénèse de l'EPEC et de l'EHEC, y compris la découverte et la caractérisation de nouvelles protéines bactériennes qui sont injectées par l'agent pathogène dans les cellules de l'hôte. De récents progrès ont été réalisés dans le développement de modèles animaux pertinents alors que des facteurs liés à la virulence bactérienne font l'objet de recherche comme cible potentielle de l'assignation pour l'être humain et l'animal. Il est à espérer que ces nouveaux domaines d'étude feront non seulement avancer nos connaissances sur la pathogénèse des maladies causées par EPEC et EHEC, mais également permettront de réduire les taux d'infection et d'améliorer les options thérapeutiques à l'avenir.

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Over the past decade, interest in the field of bacterial pathogenesis has been rekindled. Bacterial pathogens pose a significant and ongoing health threat, and the prevalence of antibiotic-resistant bacteria is increasing. An emerging theme has been the realization that many bacterial pathogens actively subvert the cytoskeleton and signal transduction pathways of eukaryotic cells to infect their hosts and cause disease (1,2). Most bacterial pathogens have evolved strategies to bypass mucosal defences and invade their hosts (1). In contrast, enteropathogenic *Escherichia coli* (EPEC) and enterohemorrhagic *E coli* (EHEC) are noninvasive pathogens that remain extracellular throughout their interactions with their hosts (2-4).

EPEC infects the small bowel epithelium, whereas EHEC colonizes the large bowel. Both pathogens, however, are able to attach intimately to the surface of intestinal epithelial cells by exploiting the host cell's cytoskeleton, producing a characteristic pathology called the attaching and effacing (A/E) lesion (2,3,5). The A/E phenotype is characterized by focal degeneration of the brush border microvilli, intimate bacterial adhesion and host cytoskeletal reorganization, leading to the accumulation of polymerized actin (3). This results in the formation of a pedestal-like structure beneath the adherent bacterium (Figure 1). The A/E lesion anchors the bacterium to the host, which allows EPEC and EHEC to colonize their respective intestinal niches rapidly, where they remain for the duration of the infection.

In children and occasionally in adults, EPEC can produce an acute illness manifested by profuse watery diarrhea, dehydration and even death (3). In other cases, EPEC infection leads to persistent diarrhea (6,7). EPEC transmission occurs predominantly through fecal contamination of water supplies. Recent outbreaks in developing countries have resulted in a case-fatality rate of 30%, and EPEC is estimated to cause the deaths of several hundred thousand children each year (3). While EPEC has been recognized as a cause of human diarrheal disease since the 1940s, EHEC was not identified as a human pathogen until 1983 (3).

Despite its recent emergence, EHEC has rapidly achieved notoriety, causing sporadic diarrheal outbreaks across North America and elsewhere. EHEC is the causative agent of hemorrhagic colitis, more commonly known as hamburger disease, in both adults and children. This disease initially presents with severe abdominal pain, followed by watery and then bloody diarrhea, with little or no fever. Unlike EPEC, EHEC is a zoonotic pathogen that is carried asymptotically by various ruminants, especially cattle. Fecal contamination of meat or water supplies is the cause of most disease outbreaks. There are several different EHEC serotypes associated with disease, but the O157:H7 serotype is the one most frequently associated with outbreaks in North America (8). During the course of infection, EHEC releases a shiga toxin that damages intestinal tissues and, in some cases, causes renal failure, neurological disease, thrombocytopenia and hemolytic anemia. These severe complications are collectively known as hemolytic-

Figure 1 Transmission electron micrograph of the attaching/effacing (A/E) lesions found in rabbit intestinal tissues during infection by rabbit enteropathogenic *Escherichia coli* O103. Note the bacteria (B) resting atop pedestals (P) as well as the surrounding microvilli effacement on the surface of intestinal epithelial cells (EC). Adapted with permission from the National Academy of Sciences (2) (photo courtesy of Dr Ursula Heczko, Biotechnology Laboratory, University of British Columbia) (original magnification $\times 20,000$)

uremic syndrome (HUS), which mainly affects children. The involvement of the shiga toxin in HUS has been comprehensively reviewed elsewhere (9).

Surprisingly, the mechanisms whereby EPEC and other A/E pathogens cause diarrhea are still unknown (2,3). While the host inflammatory response undoubtedly contributes, most research has focused on the morphological and signalling changes that bacteria provoke in infected host cells (4,10). The ability of EPEC to create pedestals (part of the A/E lesion) both in vivo and in tissue culture has received the most attention. The A/E lesion has been shown to be essential to the pathogenicity of EPEC and EHEC (3,11,12). As with most Gram-negative bacterial pathogens, the genes responsible for the virulence of A/E pathogens are located within a distinct chromosomal region, termed a pathogenicity island. This region, called the locus of enterocyte effacement (LEE), is a 36 kb segment of the EPEC chromosome. It contains 41 genes, most of which are involved in the formation of the A/E lesion (13). The mechanisms of attachment are similar for EHEC and EPEC. After initially binding to the intestinal epithelial surface, both EPEC and EHEC use a type III secretion system (TTSS) (14,15) to inject virulence factors directly into the cytosol of host cells, where they interact with the cytoskeleton and signalling pathways (2,4,5).

EPEC'S TYPE III SECRETION SYSTEM

The LEE region is common to all A/E lesion-producing bacteria, including EPEC, EHEC, rabbit enteropathogenic *E coli* (REPEC) (16) and the mouse pathogen *Citrobacter*

rodentium (17). Sequencing of the EPEC LEE revealed 41 open reading frames that, when introduced into the non-pathogenic *E coli* K-12 strain, are able to confer the A/E phenotype (18). Variations in the orientation of the inserted LEE sequences in different organisms suggest that they were acquired independently by each pathogen during its evolution (16,17). The high degree of homology in the sequences, however, suggests that the mechanism for pedestals and A/E lesion formation is conserved in these species. Within the LEE pathogenicity island are the genes that code for the TTSS as well as for proteins that this system inserts into the host cell. Because the ability to form A/E lesions is required to induce disease, the TTSS plays a central role in the pathogenicity of these bacteria and is a potential therapeutic target. TTSS are also found in many other pathogenic bacteria, such as *Salmonella typhimurium*, *Shigella flexneri* and *Yersinia* species (19). The *Yersinia* TTSS was first identified in 1990 and is the best characterized of these systems (20). As a result, the putative roles of several of EPEC's type III proteins and genes have been assigned based on their sequence homologies to *Yersinia* TTSS components. The EPEC TTSS is made up of over 20 proteins, including EspA, EspB and EspD, which form a needle-like complex capable of creating a hole in the host cell membrane (Figure 2), allowing the secretion of EPEC-secreted proteins (Esp) into the host cell. The proposed roles of some of EPEC's TTSS components have been recently reviewed in detail (14,21,22).

Despite similarities to the *Yersinia* TTSS, the exact functions are known for only a few of the proteins that make up EPEC's TTSS needle complex assembly. As a result, several laboratories are actively investigating this system (15,23). Characterization of the TTSS of EPEC would probably help to elucidate the function of similar proteins in other pathogens. In fact, it is hoped that natural or synthetic compounds might be identified that could selectively block the assembly or function of these secretion systems. Such compounds might specifically impede bacterial pathogens without affecting the commensal flora (which lack type III systems) or inducing resistance (as seen with antibiotics). While the structure of the needle complex may be key to the function of the TTSS, researchers are also exploring how bacterial pathogens sense eukaryotic cells, how the TTSS apparatus is constructed, and how effector proteins are subsequently secreted and translocated (24-26).

EPEC'S TRANSLOCATED EFFECTOR PROTEINS

In many cases, the A/E pathogens appear to use unique effector proteins. These effectors control the local environment of the pathogen, exploiting the host cell to establish a secure and protected niche for the parasite (14). They are of key importance to our understanding of the pathogenesis of EPEC disease. A recent study reported that human neutrophil elastase actively degrades bacterial virulence factors 1000 times more readily than other bacterial proteins (27). This may explain why neutrophils, despite being one of the

Figure 2) Diagram showing the structure of enteropathogenic *Escherichia coli* (EPECs) type III secretion system translocon made up of several bacterial proteins including Esp A, B and D, as well as many more not shown. Note that the type III secretion system apparatus crosses both the inner and outer bacterial membranes and penetrates the host cell membrane, forming a pore to allow the bacteria (bottom) to secrete and translocate Esps such as Tir into the host cell (top), resulting in the subversion of host cell function

first lines of defense against bacterial infections, are so rarely infected themselves. It also indicates that the targeting of bacterial effector proteins is a useful approach to controlling bacterial infections.

Arguably, the most significant advance in EPEC pathogenesis in recent years was the discovery that EPEC does not bind to a host receptor during intimate attachment to the host cell, but rather inserts its own receptor, the Translocated intimin receptor (Tir), into the host cell membrane (25). All other bacteria that induce the A/E lesion produce Tir homologues, including EHEC (28), REPEC (12) and *C rodentium* (17). These pathogens are thus able to remain anchored extracellularly within the intestinal lumen, protected from the major host defenses, while exploiting the host cell machinery to mediate disease. Until recently, putative effectors were identified by the ability of mutations or deletions of their respective genes to abrogate EPEC's capacity to form pedestals. This functional

Figure 3) Pedestal formation during enteropathogenic *Escherichia coli* (EPEC) infection is dependent on the multifunctional nature of Tir. Tir not only acts as the translocated receptor for EPEC intimin, but Tir's tyrosine residue 474, at its C-terminus, becomes phosphorylated and directly binds to the host cell adaptor protein Nck. Nck binding is required for the recruitment of both N-WASP and the Arp2/3 complex and the subsequent polymerization of actin to form the bulk of the pedestal. Interestingly, alpha-actinin also binds Tir, but at its N-terminus, where it is thought to function in pedestal stabilization

approach demonstrated the existence of several proteins, such as Tir, EspA and EspB, that are involved in the formation of the TTSS translocon (11,29).

More recently, however, several new type III translocated effector proteins have been identified that have since been found not to be essential for pedestal formation. They include EspF and EspG, as well as the mitochondrial associated protein (MAP). EspF is translocated into the host cell cytoplasm (30) and appears to contribute to EPEC's ability to disrupt epithelial barrier function, at least in vitro. It may also play a role in triggering epithelial cell death (31). Based on its effects in vitro, EspF is thought to be important in triggering the diarrhea associated with EPEC infection, but this putative role has yet to be tested in an appropriate animal model. MAP first acts to form filopodia at the site of bacterial attachment (32), and is later transported to the host cell mitochondria for an as yet unknown reason (25). Lastly, EspG, a homologue of the *Shigella* effector VirA (33), is also translocated by the TTSS apparatus, but its role in EPEC pathogenesis has not yet been defined. In fact, of the three effectors mentioned, only EspG has been tested as a virulence factor in vivo. A REPEC strain that is deficient in EspG showed an impaired ability to colonize weanling rabbits (26).

Surprisingly, compared with *S typhimurium* and other bacterial pathogens, EPEC appears to possess relatively few translocated effector proteins, perhaps only half a dozen. It appears that EPEC effectors, such as MAP, are multifunctional (32). Tir not only acts as a receptor for intimate attachment but also initiates the cytoskeletal rearrangements seen in A/E lesion formation (34,35). While one can expect the rest of EPEC's effectors to be identified in the near future, far greater time and effort will be required to unravel the probably complex roles that these effectors play

in EPEC pathogenesis. Interestingly, there is a tendency for these bacterial proteins to localize at the site of pedestal formation, even among effectors that have no obvious role in pedestal formation (32, and BA Vallance, unpublished observations). This observation suggests that pedestals might play a more complex role in EPEC pathogenesis than simply being the sites of bacterial attachment to the host cell. Further studies are needed to illuminate the other bacterial-host interactions that occur at these sites.

PEDESTAL COMPONENTS

Over the past several years, the authors' laboratory and others have discovered the roles of both bacterial virulence factors and host proteins in the formation of pedestals (2,5). These studies have provided important information about the role of the pedestal in EPEC pathogenesis. Immunofluorescence studies have shown that EPEC-induced pedestals contain Tir at the pedestal tip, as well as filamentous actin, talin, alpha-actinin, ezrin and many other host proteins (5,36). Recent studies have identified the host factors that link Tir to the host cell cytoskeleton. Several years ago, Kalman et al (37) showed that both neuronal Wiskott-Aldrich syndrome protein (N-WASP) and the actin nucleating heptameric Arp 2/3 complex were essential for pedestal formation. More recently, Gruenheid et al (35) observed direct binding of the host adaptor protein Nck to the C-terminus of Tir (Figure 3), and demonstrated that this binding was essential for the recruitment of N-WASP and Arp 2/3 to the pedestal (35). Several other host proteins, including the aforementioned ezrin, alpha-actinin and talin, may bind to the N-terminus of the bacterial Tir protein (34).

Interestingly, while EPEC requires the recruitment of the host Nck protein for pedestal formation, EHEC does not. In fact, EHEC differs from EPEC in that it lacks the Nck binding site, a 12-amino acid region of Tir surrounding the key phosphorylated tyrosine residue 474 (35). How EHEC forms pedestals in the absence of Nck recruitment is unclear, but this apparent divergence in signalling requirements suggests that EPEC and EHEC differ in how they subvert host cell function (38,39). Nevertheless, the ability to form pedestals appears to be necessary for virulence in both cases, although it is still not known how pedestals contribute to EPEC- and EHEC-induced diarrhea and other symptoms of disease. This is due, in part, to the limitations of in vitro tissue culture models. The authors are examining the role of pedestal formation in the *C. rodentium* and REPEC infection models, as well as the precise composition of EPEC- and EHEC-induced pedestals. Proteomic analysis may be able to identify bacterial proteins and novel host proteins within pedestals, and help to further define their roles in pedestal formation.

ANIMAL MODELS OF EPEC-MEDIATED DISEASE

While EPEC and EHEC readily infect the cells of most animal species in tissue culture, it has been more difficult to establish suitable animal models (2). While piglets and

mice have been employed with varying degrees of success, the most successful animal model of EPEC-mediated diarrheal disease has been the infection of weanling rabbits with the rabbit pathogen REPEC – an infection that leads to both diarrhea and weight loss (12,26). This model has proved to be very useful in confirming that Tir, EspA and EspB are important virulence factors (13,41). Unfortunately, the lack of genetic and immunological tools for rabbits are significant host limitations to the REPEC model.

Because of these limitations, several groups have begun to use *C. rodentium*, which is a natural pathogen for mice (41,42). It colonizes the large bowel, where it produces A/E lesions on the apical surface of colonic epithelial cells. Infection results in a strong T helper 1 immune response, colonic epithelial cell hyperplasia and mild diarrhea (42-44). So far, based on both in vivo and in vitro studies, it appears that EPEC, EHEC and *C. rodentium* share the same virulence factors for the formation of the A/E lesion. The ease and relatively low cost of using a mouse model seems to make *C. rodentium* the best choice to screen for additional virulence factors within the LEE (17). In particular, this model should prove useful in clarifying the pathogenic roles of translocated effectors, such as EspF, EspG and MAP, which are not critical for pedestal formation. The wide array of immunological and genetic reagents available for mice makes this model ideal for studying the innate and acquired immune responses to A/E pathogens. There are limitations to the murine model, however, including that only mild diarrhea is produced, which might interfere with the analysis of how effector proteins induce this symptom.

EPEC and its relatives are unusual in that they dwell within the intestinal lumen. While there are significant data outlining the host response to invasive bacterial pathogens, there is a paucity of studies that address how hosts deal with luminal pathogens. Such organisms are relatively protected against the antimicrobial actions of macrophages and neutrophils (45). Because *C. rodentium* is a natural murine pathogen, this model has the added advantage that the bacteria directly infect mouse intestinal epithelial cells (42). Therefore, it may represent the most relevant model of bacteria-induced gastroenteritis available. In this regard, there is in vitro evidence that epithelial cells could play an active role in the host defence against bacterial pathogens (46), yet this role has not been adequately addressed in animal models. Interestingly, *C. rodentium* appears to provoke colonocytes to express inflammatory markers (45,47). Studies are underway to determine what role these activated epithelial cells play in host defence. As for the acquired immune response, preliminary studies have found that both T and B lymphocytes are required for the clearance of *C. rodentium* from the mouse gut (41), but the exact mechanisms remain to be determined. Furthermore, studies involving both mice (Dr Wanyin Deng, unpublished observations) and human patients who are convalescing from EHEC infection have found that the host produces antibodies against Tir and other secreted or translocated bacterial proteins (48). The

Figure 4) Outline of the routes of enteropathogenic *Escherichia coli* (EPEC) transmission into food and water supplies from its cattle reservoir, as well as potential sites for future intervention and study. Vaccination of cattle against EHEC O157:H7 should reduce transmission, while proper handling and treatment of food and water can diminish exposure to the pathogen. While prevention of infection is the ultimate goal, if it does occur, type III secretion inhibitors could prove to be useful agents in the treatment of infection

antigens that are recognized by the host during the clearance of A/E pathogens might be suitable targets for vaccines.

APPROACHES TO BLOCK PATHOGEN TRANSMISSION

The ultimate goal of research into EPEC and EHEC is to reduce the amount of suffering that these organisms inflict on human populations worldwide. There is no substitute for the proper handling of meat and other food products to prevent contamination with pathogenic bacteria. Similarly, proper water treatment should also limit the transmission of these organisms (Figure 4). A complementary approach would be to limit the contamination of food or water supplies by reducing the transmission of pathogens through their natural hosts. Based on the observations that infected human and animal hosts develop antibodies against bacterial surface proteins and secreted virulence factors (48), vaccination against these agents might be feasible. Several groups are examining potential vaccines against EPEC for children in the third world (49,50). In the case of EHEC, vaccination of its animal reservoirs, especially cattle and other ruminants, is being considered to reduce the contamination of food and water supplies. Studies of vaccines against bacterial proteins are already underway, with prom-

ising initial results (BB Finlay, unpublished observations).

Aside from harnessing the host's immune system to prevent transmission, it might be possible to develop therapeutic agents that are specifically aimed at pathogenic bacteria such as EPEC and EHEC. As previously mentioned, plants and marine animals might possess natural antimicrobials that reduce the virulence of pathogenic bacteria by interfering with the function of the TTSS apparatus. Once these are identified, synthetic TTSS inhibitors could be created that act against a broad spectrum of pathogenic bacteria, including EPEC and EHEC, without harming the normal gastrointestinal flora. Such TTSS inhibitors could also provide microbiologists with tools to better characterize and dissect the complex pathways involved in bacterial pathogenesis.

POSTINFECTIOUS SEQUELAE AND RELEVANCE TO CHRONIC INTESTINAL DISEASES

It is undeniable that A/E pathogens inflict a heavy cost in human lives and suffering, particularly in developing countries, where infant mortality is at epidemic proportions. The health care burden in developed countries is not as great, because these pathogens generally cause self-limited gastroenteritis. The incidence of renal failure and death due to EHEC-induced HUS, however, appears to be increasing.

Interestingly, there is a growing consensus that these infections might have long term consequences for the gastrointestinal tract. Several investigators have reported that irritable bowel syndrome (IBS) can be precipitated by an acute enteric infection, and have proposed the term 'post-infectious IBS' for a subset of IBS patients (51). Moreover, biopsy samples from some IBS patients reveal evidence of activation of the mucosal immune system (52). These observations have led to speculation that inflammation induced by enteric infection might underlie IBS (51,53). This immunological activation does not appear to be due to chronic infection per se, but instead reflects an enhanced response to noxious stimuli. It is not known how intestinal immune cells cause alterations of gut physiology and IBS symptoms, but answers may be found from work with animal models of EPEC and other enteric pathogens.

While the recognition that enteric infection might indirectly lead to IBS has been fairly recent, bacterial involvement has long been suspected in the pathogenesis of inflammatory bowel diseases (IBD) (54). Even so, there is no evidence that either EPEC or EHEC triggers the onset of IBD. In some ways, however, the pathology of *C. rodentium* infection, an animal model of EPEC-induced disease, resembles that seen in IBD (43). Both bacterial infection and idiopathic IBD exhibit activation of the mucosal immune system and intestinal epithelial cells (45,55), recruitment of neutrophils, villus atrophy, crypt hyperplasia and, in some cases, mucosal ulceration. The immunological features of IBD are similar to the response to infection, but occur in an inappropriate context, ie, in the absence of an identifiable pathogen. Therefore, exploration of the host

response to enteric infections should help us to understand not only gastrointestinal infections, but also the possible contribution of bacterial infection to the development of IBD.

CONCLUSIONS

Globally, the A/E family of bacterial pathogens is among the most important bacterial causes of diarrheal disease, particularly in children. Unfortunately, despite their impact on human health, very little is known about how these bacteria infect their hosts, let alone how they cause disease. It is known that pedestal formation is necessary for the pathogenic effects of these microorganisms, but a major challenge facing researchers is to determine how these pathogens affect host cell function. This will require bringing the study of EPEC pathogenesis out of the tissue culture dish and into the intact host. Animal models will soon illuminate the roles of translocated effectors, such as EspF, as well as of the host inflammatory and immune responses, in causing diarrhea. Another key focus of investigations will be determining how these pathogens either suppress or evade host defences.

It should also be noted that treatment options for these infections are limited, particularly for EHEC infection. Antibiotics appear to trigger the release of shiga toxin from lysed bacteria, which actually results in a worsening of intestinal symptoms. Thus, the purpose of studies in this field is not only to increase our knowledge of the pathogenesis of EHEC-induced disease, but also to devise improved means of treating and limiting the transmission of this organism. While there is no substitute for the proper treatment of food and water supplies, one could also reduce contamination by inhibiting the transmission of pathogens through human and animal reservoirs. We believe that advances in this field will not only affect the course of infections caused by bacteria that cause A/E lesions, but also provide clues about the etiology of other gastrointestinal diseases.

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