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Pathogenesis of *Clostridium difficile* infection

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Clostridium difficile **produces two major toxins referred to as toxins A and B. These are thought to be primarily responsible for the virulence of the bacterium and the major contributors to the pathogenesis of antibiotic-associated gastrointestinal disease. The molecular organization and control of expression of toxins A and B is now starting to be understood, and the cellular mechanism of action of both toxins, glucosylation of Rho family proteins, has been discovered. Other factors, such as production of proteolytic and hydrolytic enzymes, expression of fimbriae and flagella, chemotaxis and adhesion to gut receptors, and production of capsule, may all play a part in pathogenesis by facilitating colonization or by directly contributing to tissue damage, or both. Differential expression between strains of various combinations of these colonization and virulence factors may explain the apparent variability in virulence of *C. difficile* strains.**

Introduction

The outcome of any infection is the end-product of a complex set of interactions between the host and the pathogen. For *Clostridium difficile* this picture is further complicated by the role of the normal gut microbiota (microflora), which is an important first-line defence to infection. The disease process will be presented by describing the pathology and then the colonization and virulence factors of *C. difficile* and their interactions with the host which lead to disease.

Clinical manifestations and pathology

Infection is most commonly seen in the hospitalized elderly[1,2] and is almost always associated with recent or current administration of antibiotics. However, although rare, cases can occur in children[3] and infants.[4–7] Disease may be associated with a spectrum of severity, ranging from mild diarrhoea, through moderately severe disease with watery diarrhoea, abdominal pain and systemic upset, to life-threatening and sometimes fatal pseudomembranous colitis (PMC). This may be accompanied by toxic megacolon (rare), electrolyte imbalance and occasional bowel perforation. The onset of symptoms is frequently abrupt, with explosive, watery, foul-smelling diarrhoea[8,9] accompanied by abdominal pain, the absence of frank blood in the stool in most cases, elevated white blood cell count and, in some cases, fever.

PMC is well defined histopathologically[10–12] and is almost invariably restricted to the colon and rectum, though there may be rectal sparing.[13] As disease progresses, the mucosa becomes necrotic with the formation in fulminating cases of an exudative membrane. This pseudomembrane, which may become confluent, is formed in multiple, friable yellow/white plaques varying in diameter from a few millimetres to about 2 cm, attached to the mucosal surface. Between these plaques the mucosa may appear to be normal or only congested. Microscopically, the membrane is seen as a fibrinous exudate containing leucocytes, epithelial cells and mucin. The underlying intestinal submucosa shows a varying degree of necrosis and inflammatory reaction. In the earliest stages of the disease, tiny superficial intercryptal erosions (summit lesions) may be found.[11]

Colonization resistance

Infection is associated with antibiotic use because the normal stable gut microbiota has to be disrupted before *C. difficile* can become established and produce toxins. This protective effect of the normal microbiota is frequently referred to as 'colonization resistance'. This barrier effect to infection with *C. difficile* and its disruption by antibiotics has been demonstrated in animals[14,15] and *in vitro* with human and animal faecal or caecal material.[16,17] A variety of antibiotics can predispose to *C. difficile*

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infection.[1,18,19] The frequency of association is likely to be related to frequency of use, the route of administration and impact on the bacterial components of the gut microbiota. Frequency of association does not necessarily correlate with relative ability to predispose to infection. Although difficult to extrapolate from frequency of association of particular antibiotics to the relative risk of infection, there is evidence in the hamster model of disease that the major difference between oral antibiotics is the duration, rather than the degree, of susceptibility induced.[15] It is possible that the apparent higher incidence of infection in the elderly is in part the result of their poorer colonization resistance,[16] a smaller effect being required by antibiotics to induce susceptibility to colonization. The other age group in which there is a poor barrier effect is neonates, as they have still to develop a complex gut microbiota that can exclude *C. difficile*; this age group commonly has *C. difficile* present in the faeces,[20,21] as well as *C. difficile* toxins.[22,23]

Virulence factors of *C. difficile*

C. difficile produces a number of factors that contribute to its virulence. Some are true aggressins, i.e. directly contribute to the pathology associated with infection, while others enable *C. difficile* to colonize and produce these aggressins within the human host. As with all pathogens, not all strains are equally virulent. A comparison of the ability of different strains of *C. difficile* to cause disease in the hamster model was undertaken in 1987; this clearly demonstrated differences in virulence between strains of *C. difficile*, and showed that strains that were more virulent produced more toxin A *in vivo* than less virulent strains.[24] This spectrum of virulence was confirmed by Delmée & Avensani[25] who showed that particular serotypes were associated with reduced virulence.

C. difficile has been studied in order to understand its virulence and also to try to determine what accounts for the differing virulence between strains. Findings on the colonization factors will be discussed first and then the two major aggressins of *C. difficile*, toxins A and B.

Adhesion

Adhesion to host tissue is important for full expression of virulence for many pathogens. The first indication that *C. difficile* adhered to human gut was obtained in 1979 following its recovery from a washed biopsy specimen from a patient with PMC.[22] In a hamster model of the disease, a highly virulent toxigenic strain adhered better than a poorly virulent strain, and both strains adhered better than an avirulent non-toxigenic strain.[26] In all cases adherence was most pronounced in the terminal ileum and caecum, in keeping with the pathology of ileocaecitis. A further observation from the same study was that co-administration of toxin A with the non-toxigenic strain raised adhesion by the latter strain to the same level as that seen for the highly virulent toxigenic strain. This implies either that adhesion is facilitated by toxin A-mediated damage or that toxin A is directly involved in binding *C. difficile* to the gut, and could contribute to other adhesion mechanisms. A number of factors can be involved in binding to mucus and cells. There have been several attempts to identify adhesins of *C. difficile*. Fimbriae were detected in 1988 and shown to be polar, 4–9 nm in diameter and 6 μm in length.[27] However, their role in colonization is unclear, especially as no correlation could be found between presence of fimbriae and the relative ability of different strains of *C. difficile* to adhere to hamster gut mucus. Many strains of *C. difficile* are motile and have flagella (see below), but it is not known whether these flagella also serve as adhesins. In studies on adhesion to human gut cells (Caco-2 and HT-29MTXC) *in vitro*, Eveillard *et al.*[28] and Karjalainen *et al.*[29] identified heat-stimulated 27 kDa and 40 kDa proteins that appear to be involved in adhesion and which, when cloned and expressed, promoted adhesion in *Escherichia coli*.

Physicochemical properties of microorganisms may also contribute to adhesion. *C. difficile* cell surfaces are moderately hydrophobic, even when grown in ex-vivo conditions, and carry a net positive charge.[30] This charge is evenly distributed and resides predominantly in the cell wall.[30] Charge interactions with negatively charged host cells may contribute to gut colonization. Interestingly, the ability of *C. difficile* to agglutinate various human, sheep, rabbit and horse red blood cells noted by Karjalainin *et al.*[29] was not seen by Krishna *et al.*[30] Although possible reasons for this discrepancy were speculated on,[30] the actual reasons remain unknown.

Chemotaxis

The ability of an enteric bacterial pathogen to move from the lumen to the gut mucus would enhance its chances of adhering to gut receptors. Recent work (Borriello & Bhatt,[31] and R. Bhatt and S. P. Borriello, unpublished) has shown that gut mucus of different animals and humans serves as a chemoattractant for *C. difficile*. The taxin (attractant) is heat-stable and resistant to proteolysis. The degree of chemotaxis correlated positively with the relative virulence of the strains examined in a hamster model.[31]

Chemotaxis is impossible without motility. For the vast majority of bacteria, motility is mediated by flagella. Little was known about flagella of *C. difficile* until very recently, when a joint French–British team purified the flagellin of different strains of *C. difficile*. The flagellin has a molecular mass of about 39 kDa,[32] and the same group has now cloned and sequenced the gene encoding this flagellin and shown it to have 60% similarity to the *hag* flagellin gene of *Bacillus subtilis* (P. Dodson and M.-C. Barc, unpublished).

Capsule

C. difficile requires opsonization for significant phagocytosis, suggesting that there might be an anti-phagocytic factor on its cell surface.[33] Removal of the cell-surface carbohydrates did not affect the degree of phagocytosis, suggesting that *C. difficile* may have a polysaccharide capsule. This was subsequently shown to be the case.[34]

The accumulation of polymorphonuclear cells in gut tissue in PMC may in part be due to toxin A-induced recruitment and, although they appear to have little effect on *C. difficile*, they may contribute to the tissue damage characteristic of this infection.[35]

Hydrolytic enzymes

There has been sporadic work on the production of hydrolytic enzymes by *C. difficile*. Hafiz & Oakley[36] found that all of 21 isolates examined were positive for hyaluronidase activity, though the amount produced was variable. Popoff & Dodin[37] screened 25 isolates from infants for neuraminidase activity, finding none of them positive. Steffen & Hentges[38] examined one isolate of *C. difficile* in a study of hydrolytic enzyme production by anaerobes from human infections. This isolate was positive for hyaluronidase, chondroitin-4-sulphatase, gelatinase and collagenase, but negative for heparinase, fibrinolysin, lecithinase and lipase.

The most detailed study of hydrolytic enzymes that may be involved in breakdown of connective tissue was undertaken by Seddon and colleagues,[39] who showed that most of the strains examined had hyaluronidase, chondroitin-4-sulphatase and heparinase activity, though the heparinase activity was generally weak. Highly virulent strains were more active than less virulent strains. Collagenase activity was also present, but was generally weak and more restricted to highly virulent strains.

It is possible that some of these tissue degradative enzymes contribute to the observed pathology and help to compromise further gut integrity and subsequent fluid accumulation. It is also likely that *C. difficile* derives nutritional benefit from such activity. For example, it is known that *C. difficile* utilizes *N*-acetylglucosamine, but cannot derive this saccharide by cleavage of mucus glycoproteins.[40] However, it is the end-product of hydrolysis of hyaluronic acid, indicating that this is the utilized product of *C. difficile* hyaluronidase activity.

Toxins

To date, five toxic factors of *C. difficile* have been described (reviewed by Borriello *et al.*[41]), though only two of these, toxins A and B, have been studied in any detail with good evidence of involvement in disease. The other three factors are a second, unstable, enterotoxic protein,[42–44] a high molecular weight protein that causes changes in electrical potential in isolated segments of rabbit intestine,[45] and an actin-specific ADP–ribosyltransferase.[46] The apparent second enterotoxin, which does not cause haemorrhage, has not been purified and studied. The unstable nature of this molecule, its sporadic production and the inability of other workers to confirm its presence make it difficult to determine its role, if any, in disease. The ADP–ribosyltransferase, which acts in a similar manner to *Clostridium botulinum* C2 toxin light chain, is found in only a few strains of *C. difficile* and so probably plays little, if any, part in the usual disease process.

The genes for both toxins A and B have been sequenced.[47,48] The estimated molecular masses derived from these sequences are 308 kDa and 269 kDa for toxins A and B, respectively. These toxin genes are arranged on a 19.6 kb toxicon and have their own promoters and ribosome binding sites, although they can been expressed from a single common promoter.[49–51] A diagrammatic representation of the arrangement of the genes on the toxicon is shown in the Figure. In non-toxigenic strains the toxicon is replaced with 127 bases of non-coding DNA.

Recent studies on the regulation of the toxin genes in this toxicon have shown that the small open reading frame upstream of toxin B (orftxe1 in the Figure) is involved in regulation of expression of that toxin.[51] This small open reading frame codes for a 22 kDa protein which contains a helix–turn–helix motif with sequence identity to DNA-binding regulatory proteins. Of particular interest is its 21% identity with a putative positive regulator of *C. botulinum* neurotoxin. Further, this open reading frame, which has been designated *txe*R, can also positively regulate expression of the toxin A gene.[51] The extent to which *orftxe*2 can positively regulate toxin A, and the extent to which *orftxe*3, which is transcribed in the opposite direction, can down-regulate either toxin, remain to be determined.

These large protein toxins have a range of biological activities.[41,52] They are both cytotoxic to a very large number of different cell types, both cause increased

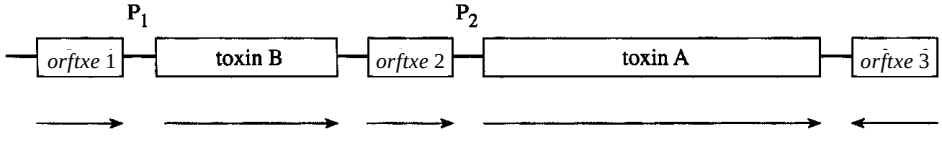

Figure. Diagrammatic representation of the 19.6 kb toxicon of *C. difficile* strain VPI 10463 (not to scale). *orftxe* 1, 2 and 3 are small open reading frames. P_1 and P_2 are toxin B and A promoters respectively. The arrows indicate the direction of transcription.

vascular permeability, and both cause haemorrhage (toxin A more so than toxin B). The biggest difference between the toxins is that toxin A causes fluid accumulation in various animal models, whereas toxin B does not. That toxin A alone may be responsible for the enterocolitis in the hamster model was suggested by the observations of Lyerly *et al.*[53] In a series of animal feeding experiments, these authors showed that culture filtrates from a strain of *C. difficile* caused intestinal lesions and diarrhoea, but that filtrate from which toxin A had been removed was inactive, as was toxin B alone. Moreover, the two toxins appeared to act synergically. Recent studies[34,43,54,55] showed that toxin A had both histotoxic and enterotoxic activity, and that the toxin was important in the causation of both tissue damage and accumulation of luminal fluid in experimental animals.

Mitchell and colleagues[54] showed that rabbit ileum was more susceptible to the action of toxin A than was the colon. Gross haemorrhages occurred in the ileal tissues and the architecture of the villus was severely damaged; giving rise to a bloody, protein-rich luminal fluid. In the colon, there was damage to the surface epithelium, with interstitial haemorrhages and a watery luminal fluid containing little protein. They also demonstrated an apparently continuous cycle of tissue damage and new cell exposure, the toxin being less able to penetrate to deeper tissue in the colon than the ileum. Similar differential effects of toxin A on rabbit ileum and colon were also shown by Lima *et al.*[56] The accumulation of protein-rich fluid is indicative of an increased epithelial cell layer permeability. A direct demonstration of this increase in permeability by toxin A has been demonstrated with rabbit ileum by use of [^{3}H]mannitol.[31] Interestingly, these same workers provided evidence that there was a requirement for recruited neutrophils in order to generate tissue damage.[31] A possible mechanism for this includes an apparent toxin A-stimulated macrophage cytokine production (tumour necrosis factor alpha, interleukin 1β and leukotrienes) that stimulates neutrophil migration (infiltration) and consequent inflammation.[57]

A recent study[58] of the effect of toxin A on established human intestinal epithelial cells (Caco2, HT29 and T84 cells) and primary epithelial cells in organ cultures of human colonic biopsy specimens showed that toxin A induced interleukin 8 production, cell detachment and consequent apoptosis of isolated cells. Interestingly, no morphological changes were seen in mucosal tissues from one of five subjects, despite exposure of the tissue to 1000 ng/mL of toxin A for 24 h. The reasons for this resistance are unknown, but the results indicate that there may be differences between subjects on susceptibility to the effects of toxin A.

The above evidence that only toxin A induces the extensive tissue damage and fluid response seen in experimental animal models[42,54,59] and the observation that the severity of symptoms in the hamster model is more closely related to the degree of production of toxin A *in vivo* than to the production of toxin B[24] is indicative of a more critical role for toxin A than toxin B. However, there is experimental evidence that questions this assumption: (i) toxins A and B can act synergically;[53] (ii) it has been reported that toxin B is more damaging than toxin A to human colonic epithelium *in vitro*;[60] (iii) an apparently toxin A-negative, toxin B-positive strain of *C. difficile* which causes full pathology in animal model systems has been described.[1,62] The most recent work on this strain shows that it has part of the 5′ end of the toxin A gene, which is transcribed though not translated, but lacks all of the 3′ end, which contains the multiple repeats involved in cell binding (P. Dodson and S. P. Borriello, unpublished). This strain is not unique in having polymorphism of its toxin A gene, as others have recently been described at the molecular level[63] and toxin A-negative, toxin B-positive strains identified phenotypically have been described and are of a common 'PCR genotype'.[64]

Whatever the relative roles of the toxins, it is likely that activity *in vivo* is mediated by a specific receptor(s). What this may be for toxin B is completely unknown. However, Lewis, X, Y and I antigens can bind to toxin A[65] and may serve as functional receptors in the gut. However, these receptors are not universally expressed by colonic epithelial cells. Toxin A also non-specifically binds monoclonal antibodies[66] and both toxins bind nucleic acid[67,68] nucleosides and nucleotides.[69,70] The significance of these functions remains to be determined. Interestingly, the non-specific binding to monoclonal antibodies has been shown to be mediated by the C-terminal repeat component of toxin A, which binds uniquely the Fab component of the immunoglobulin.[71]

Most recent work has concentrated on the cellular mechanism of action of the toxins, and represents some of the most exciting work to date.[72–74] Both toxins A and B have monoglucosyltransferase activity which catalyses incorporation of glucose into a variety of substrate proteins. These include the small GTP-binding proteins (Rho, Rac and Cdc42Hs) which are involved in regulation of the actin cytoskeleton, specifically in the formation of actin stress fibres and focal adhesions. Both toxins glucosylate a threonine at position 37 on Rho. Disruption of the activities of these proteins leads to the shift from F-actin to G-actin and the resultant cell rounding which is characteristic of the toxin-induced cytopathic effects. For both toxins this activity is located towards the N-terminus.

Conclusions

A likely sequence of events that may lead from antibiotic exposure to fulminant PMC is presented. Following most antibiotic treatment there will be a point at which the impact on the normal gut microbiota depresses

colonization resistance to *C. difficile*, and at which residual antibiotic is inactive against *C. difficile*. Exposure of the patient to *C. difficile* at this time may lead to colonization. The ingested spores germinate in the terminal ileum and multiply in the colonic lumen. Toxins A and B will be produced, inducing damage to gut tissue and disrupting cell–cell tight junctions. *C. difficile* may swim towards gut mucus and adhere to the colon, producing and releasing toxin locally, as well as enzymes that degrade connective tissue. Direct damage is further exacerbated by activity of recruited neutrophils. The serum albumin-rich fluid being lost into the lumen (possibly by the toxin-induced increase in permeability of capillaries) may compete with host proteases and protect the toxins from proteolytic degradation. Released nutrients, and possibly locally induced anoxia, may in turn stimulate *C. difficile* growth and toxin production. Successive cycles of pathogen replication, toxin production and neutrophil recruitment would ultimately lead to cell detachment and apoptosis, local necrosis and the pseudomembranes associated with the diarrhoea seen in this infection.

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