The pathogenicity of *Clostridium difficile*

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It is now well established that the major virulence factors of *C. difficile* are the two toxins A and B. However, the organism possesses an array of other putative virulence factors that may be important for localisation within the colon, and in evasion of the immune system. It has been observed that certain types of *C. difficile* are more commonly found causing disease than others, and this seems to be independent of toxin production. Is this simply a reflection of their abundance in the hospital environment, or is it due to their virulence determinants? This review covers our current knowledge of the modes of action of toxins A and B at the cellular and molecular level. Many unanswered questions are posed that require answers before we can fully understand the pathogenic mechanisms of the organism and be in a position to manage better the spectrum of diseases it causes.

Keywords Toxin A, toxin B, antibiotic-associated colitis, *Clostridium difficile*, pathogenicity

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INTRODUCTION

The spectrum of disease and its pathology

Clostridium difficile is a commonly isolated organism from fecal specimens obtained from neonates and the elderly. Often its carriage is asymptomatic, and this is especially true in the neonate. However, in the elderly, it is often associated with disease symptoms that range from mild self-limiting diarrhea to serious diarrhea, with or without pseudomembrane formation (pseudomembranous colitis; Figure 1), and with the possibility of life-threatening complications such as toxic megacolon, perforation and peritonitis.

Antibiotics and the normal gastrointestinal microbiota

The proposed sequence of events that precipitate *C. difficile* disease are as follows: on exposure of the gut to antibiotics, the microbiota becomes disrupted and colonisation resistance is compromised. The gut is then susceptible to colonisation by +*C. difficile*. The organism is acquired in most cases from an exogenous source – either from an infected individual, from a contaminated health care worker, or indirectly from a contaminated environment. Once ingested *C. difficile* evades immune responses, multiplies in the colon and produces toxins A and B. The characteristic pathology then results.

Virulence factors of *C. difficile*

C. difficile is typical of its genus: it is an anaerobic, Gram-positive, spore-forming bacillus that produces toxins. The two toxins A and B are commonly referred to as the enterotoxin (toxin A) and the cytotoxin (toxin B). This terminology originated from the observed actions of these toxins: demonstration of fluid accumulation in intestinal loop models and the cytopathic effects on tissue culture monolayers, respectively. These investigations were done before the action at the molecular level was well understood. However, as is described below, both toxins have a great deal in common.

Other toxins have been identified, in particular the bipartite, ADP-ribosylating toxin, which is described in more detail in an accompanying review [1].

The role of other virulence factors is much more speculative. Adhesins have been proposed as being important but their relevance in the colon, and their identity, is still not assured. Several extracellular enzymes are produced that do have effects in vitro, but their role in pathogenesis is not well defined. Presumably these enzymes do have a role in the normal physiological processes in the gastrointestinal (GI) tract and may be crucial for the normal survival of the organism, giving it an advantage when the normal GI microbiota have been disturbed following antibiotic usage.

C. difficile is somewhat unusual in that it has an outer cell coat termed the S-layer. This consists of two polypeptides that together form a regular, crystalline array over the whole surface

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Figure 1 Pseudomembranous colitis, post-mortem specimen.

of the bacterium. The S-layer is proposed to be a virulence factor and is discussed in more detail later.

THE TOXINS AND HOW THEY WORK

The majority of toxigenic strains produce both the A and B toxins. In summary, their mode of action is similar: they are endocytosed by the cell, they affect the actin cytoskeleton and they result in cell death. They also induce the production of tumor necrosis factor-alpha (TNFα) and proinflammatory interleukins (ILs) which contribute to the associated inflammatory response and pseudomembrane formation.

Toxin A causes necrosis, increased intestinal permeability and inhibition of protein synthesis. Toxin A also affects phospholipase A2, thereby producing prostaglandins and leukotrienes. Toxin A damages villous tips and brush border membranes and complete erosion of the mucosa may result. A viscous, bloody fluid is produced in response to this tissue damage. However, in the case of toxin B, there is no noticeable enterotoxic activity but it is lethal to cells in vitro. Therefore Toxin B is thought to become effective once the gut wall has been damaged.

The structure of toxins A and B

The two major toxins are coded on a pathogenicity locus (Figure 2). The products of transcription and translation are extremely large single-chain peptides with molecular masses of 308 kDa for toxin A and 270 kDa for toxin B [3]. There are three functional domains to these toxins (Figure 3). The toxins

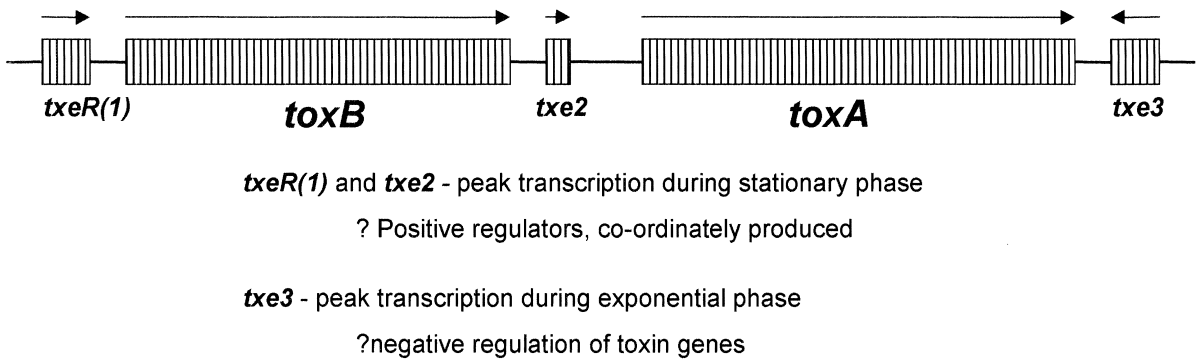

Figure 2 The pathogenicity locus of *Clostridium difficile* (modified from ref. 2).

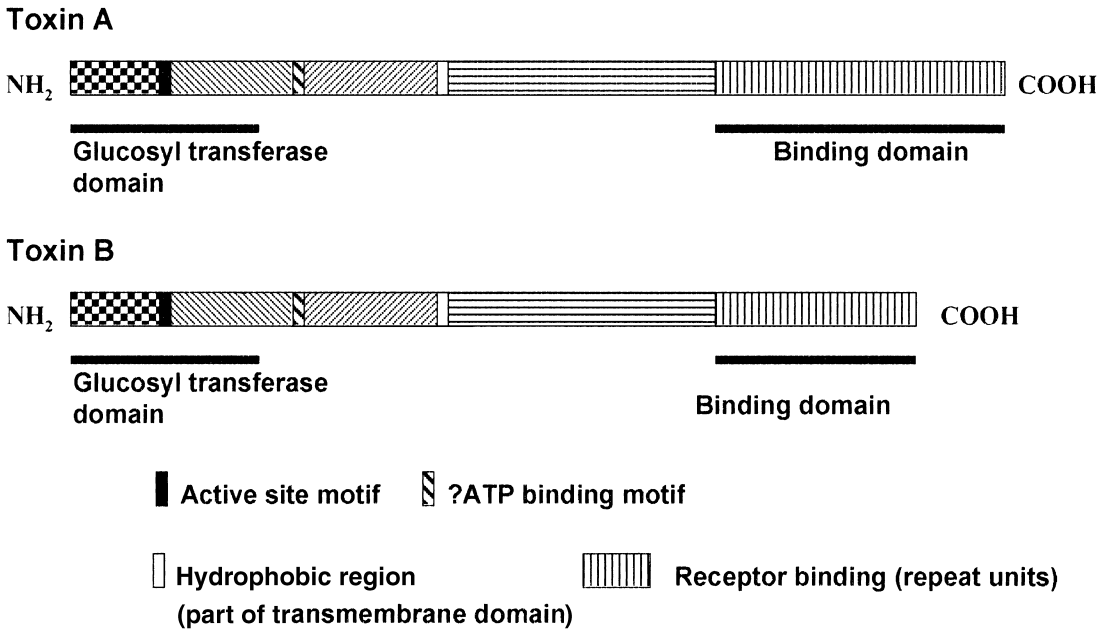

Figure 3 The structure of *Clostridium difficile* toxins A and B (modified from ref. 2).

are 50% identical at the amino acid level and have similar primary structures. Hoffman et al. [4] noted that the enzyme and cytotoxic activity of toxin B was to be found at the toxin's N-terminus, which also holds the enzyme and cytotoxic activity of toxin A. The middle section of both toxins includes a transmembrane domain, which is thought to encode for the translocation of the toxin into the cytosol, but this has yet to be proven [5]. The C-terminal of the toxin encompasses the receptor-binding domain and is constructed of repetitive peptide elements.

Action of toxins A and B

The carboxy terminal of toxin A forms binding domains for carbohydrate structures that occur on the surface of the epithelium. Toxin B binds to cells that are not covered by a thick carbohydrate matrix. They then enter the cell by endocytosis [6]. Both toxins require passage through an acidic intracellular compartment in order to intoxicate cells. This route is not known for toxin A, but toxin B is believed to be delivered by lysosomes and is then released into the cytosol.

The major effect of toxins A and B is the disruption of the actin cytoskeleton. Cells intoxicated by these proteins show a retraction of cell processes and a rounding of the cell body. This is due to the disassembly of filamentous F-actin and an increase in G-actin prior to cell rounding [7]. Very few toxin molecules are required to produce cell rounding. It has been proposed that *C. difficile* toxins act enzymatically within cells, modifying proteins that regulate actin polymerisation and fiber assembly. These proteins are known as the Rho proteins, a subfamily of the Ras-family of GTPases [7]. The mechanisms of action of either toxin are summarised in Figure 4.

In the diseased state, the colonic epithelium is the major target of *C. difficile* toxins. They cause disruption of the barrier function by opening the tight junctions. This effect is not merely caused by the breakdown of actin filaments but by the inactivation of the Rho function to regulate tight junction complexes. These barrier-disrupting effects of toxin A and B increase the colonic permeability, the basis of watery diarrhea, which is a typical feature of *C. difficile* antibiotic-associated diarrhea (Figure 5).

Apoptosis of enterocytes

A study by Fiorentini et al. [8] provided the first experimental evidence that cultured intestinal cells exposed to toxin B showed all the features of apoptosis. A study by Mahida et al. [9] showed the same effect being caused by toxin A. All cells undergo apoptosis at some point, as this controlled cell death is an important feature of tissue development and homeostasis, keeping the number of functional cells in balance in the body. Apoptosis can be identified as being different from cell necrosis by distinct morphological alterations. These alterations come in the form of nuclear condensation and fragmentation, cell shrinkage and the absence of inflammation [8]. In monolayers of cell cultures, apoptosis can be induced by inhibition of cell adhesion and of anchorage-dependent cell spreading. Toxin B is capable of both inhibition of anchorage and cell spreading.

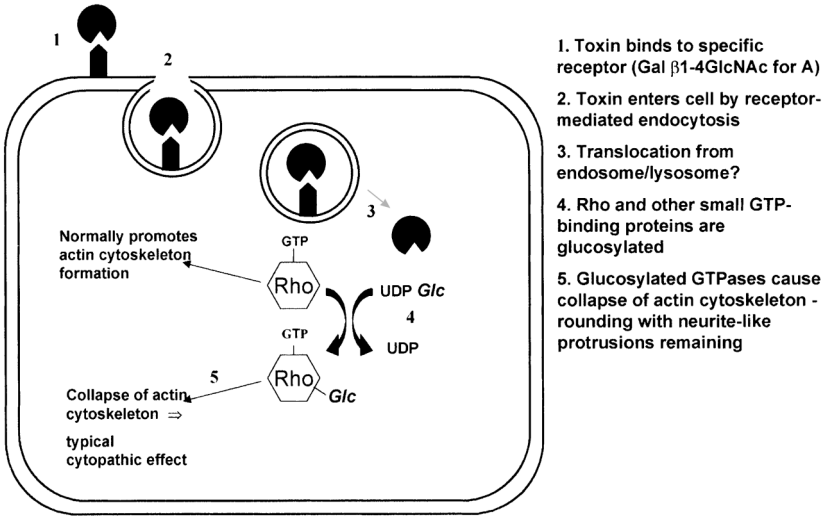

Figure 4 Simplified scheme for the action of *Clostridium difficile* toxins on cells (modified from ref. 6).

However, it was found that apoptosis was not only caused by toxin B inhibiting cell adhesion due to actin depolymerisation but that Rho proteins themselves may play an important role in the regulation of apoptosis under normal conditions [8]. Toxin B, therefore, can be seen as an inducer and not as the cause of apoptosis. It is the effects of toxin B on the Rho proteins that cause the abnormal activation of the apoptotic system. With toxin A, it is thought that apoptosis occurs because the epithelial cells are denied anchorage to the basement membrane.

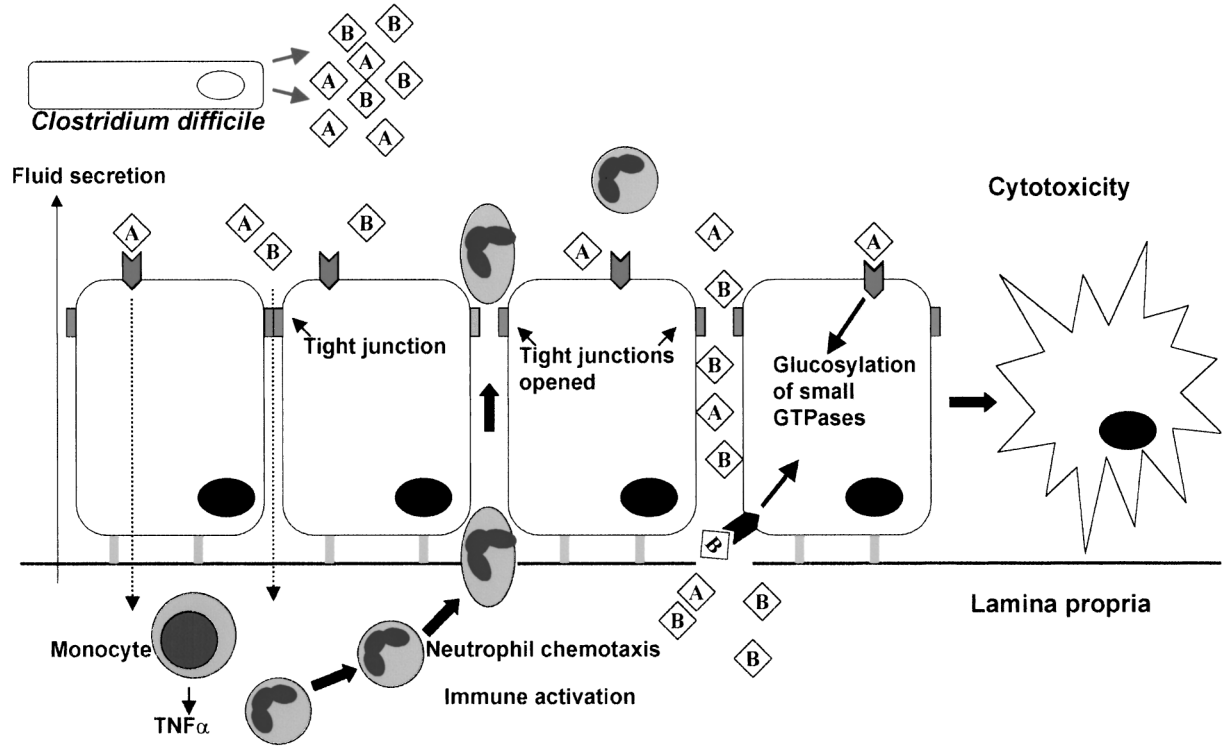

Figure 5 Actions of *Clostridium difficile* toxins A and B on intestinal epithelium (modified from ref. 6).

Differences in cytotoxic potencies in toxin A and B

To cause pseudomembranous colitis, both toxins A and B are normally required. The two toxins intoxicate cultured cell lines by the same mechanism but when it comes to potency, toxin B is around a 1000 times more potent than toxin A. Toxin B has at least a 100-fold higher enzymatic activity than toxin A, and this is believed to be the main determinant in the difference in cytotoxic potency.

Due to the low enzymatic potency of toxin A, it has been proposed that glucosylation of the Rho-proteins may not be the primary in vivo effect of this toxin. Some believe neuronal involvement may be a possible answer to enterotoxic effects of toxin A [10]. The suggestion is that the pathophysiological process is triggered by a transepithelial signal to neuroimmune cells that is triggered by the binding of toxin A to the intestinal mucosa. The modification of Rho proteins by toxin A and B would then play a secondary, but important, role in exacerbating mucosal inflammation and destruction. This theory agrees with an experiment done by Riegler et al. [11], where toxin B was shown to be more potent than toxin A in damaging human colonic epithelium in vitro. The mucosal strips were devoid of enteric nerves and thus toxin B was shown to be 10 times more effective in causing damage.

ACTIVATION OF THE IMMUNE SYSTEM

Colitis is characterised by a massive influx of neutrophils into the colonic mucosa, and in pseudomembranous colitis there is an acute inflammatory infiltrate with microabscesses and pseudomembranes rich in neutrophils [12]. The movement of neutrophils from circulating blood to the site of injury is a crucial event during the inflammatory process. IL-1, IL-8, TNF and leukotriene B4 are products of resident cells and are thought to be involved in neutrophil infiltration into the inflamed site.

Both toxins stimulate the release of TNFα from cultured monocytes. Toxin B was again found to be 1000 times more potent than toxin A in this system [12]. Both toxins also activate monocytes and macrophages in the lamina propria in vitro to release IL-8. This causes neutrophil extravasation and tissue infiltration by creating a chemotactic gradient that induces neutrophil migration to the site of mucosal inflammation [13]. Using mast cell-deficient mice, Pothoulakis et al. [14] demonstrated the importance of mast cells for neutrophil recruitment and fluid secretion induced by toxin A in vivo. Isolated mast cells were also shown to respond to toxin A by releasing TNFα. This activation could be inhibited with a specific antagonist to substance P. Substance P is a peptide found in gut tissue and in the CNS that acts as a neurotransmitter. This suggests that toxin A activates mast cells via the release of substance P from adjacent sensory neurons [15]. This can also be seen as another point marking toxin A activity with neuronal stimuli.

Neutrophil recruitment appears to be an essential step in the pathogenesis of *C. difficile* toxin-induced intestinal injury as biopsy specimens from patients with *C. difficile* colitis show marked vascular congestion, neutrophil infiltration of the lamina propria and inflammation. Although it was shown by Calderon et al. [15] that toxin A was able to activate neutrophils, mast cells and macrophages in vitro, there is still some speculation as to how this works in vivo due to the large size of the toxin. Toxin A can cause detachment and apoptosis of enterocytes and so, in this disrupted epithelium, toxin A may diffuse and interact with the inflammatory cells in the lamina propria. Also, localised areas of injury and inflammation may result from cell rounding that would cause breaches in the colonic epithelium through which tiny amounts of toxin A and B can pass. These small amounts cannot directly activate neutrophils but may activate tissue macrophages to produce IL-8 and other proinflammatory cytokines. Once the inflammatory cascade is initiated, it can result in a marked acute inflammatory cell infiltration, further mucosal injury and focal pseudomembrane formation [13].

IMMUNITY AND HOST DEFENCES

Innate mechanisms

Probably the best defence against infection by *C. difficile* is an intact normal bowel microbiota – preventing establishment of *C. difficile* by colonisation resistance [16]. Normal gut motility and an effective gastric acid barrier are no doubt also important [17].

Acquired immune mechanisms

Experiments with hamsters suggest that systemic IgG to toxin A – induced by vaccination – is protective [18], and secretory (IgA) antibodies to toxins A and B may be protective as demonstrated by protecting hamsters fed milk from immunised mothers. However, this is an area still requiring a great deal of work, especially to determine the potential for immunisation in humans [17].

PHENOTYPIC VARIATION

There is a degree of phenotypic variation between strains of *C. difficile*. In respect to pathogenicity, it seems that non-toxigenic strains can be considered non-pathogenic or avirulent. However, it is well accepted that there are degrees of virulence between strains. The discovery of virulent strains of toxin A-negative/toxin B-positive phenotype indicated that toxin A was not essential for virulence. How these strains cause

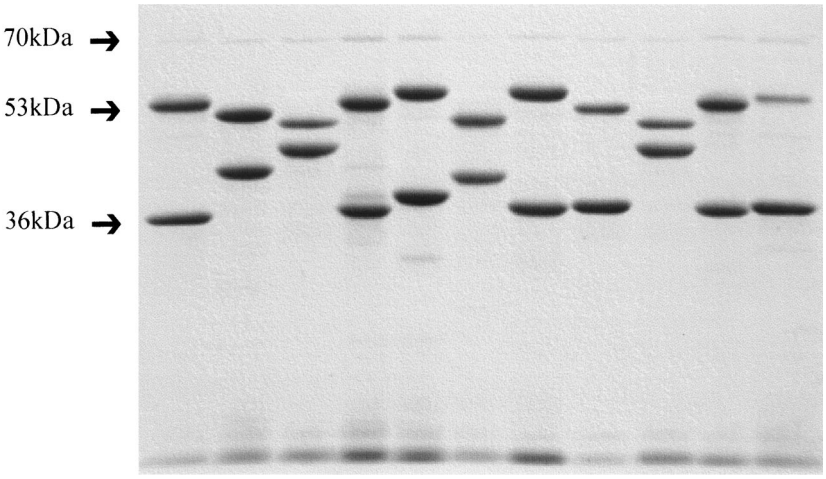

Figure 6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of S-layer proteins from 11 different strains of *Clostridium difficile*. The S-layer proteins were extracted from whole bacteria with guanidine hydrochloride.

disease is not readily apparent but based on the discussion above there is so much similarity between toxins A and B that once there is any damage to the mucosal cell layer then either could cause symptoms.

In common with several pathogens, *C. difficile* has an S-layer covering its entire surface. We have proposed recently that this might have a role in virulence [19]. There is certainly a degree of correlation between serotype and virulence that is independent of toxin production: certain serotypes are more often associated with disease. Serotype correlates well with S-type and also to ribotype [19 and unpublished data]. Figure 6 shows an example of the S-layer proteins extracted from different strains with guanidine hydrochloride, and each strain belongs to a different serotype.

UNANSWERED QUESTIONS

Despite knowing a great deal about the mode of action of the major toxins, and understanding the epidemiology of the pathogen, many questions remain unanswered. For some, there are partial answers but, in the opinion of the authors, none of the following are yet answered fully:

Why are infants not affected?

Do the toxins have any role in the healthy intestine?

Is immunity to cell surface components protective – would whole cell vaccines be a possibility?

Why are some types much more virulent than others?

What is the molecular basis of serotype?

Why are there so many S-types?

What is the genetic basis for S-layer peptide variation?

It is generally agreed that *C. difficile*-associated disease is increasing worldwide, and our last question, 'Have super strains evolved?', remains unanswered. Is the increase purely because the organism – as spores – becomes persistent in the environment of the elderly patient, where it is maintained by constantly being passaged in susceptible individuals, or have previously harmless strains from neonates acquired virulence attributes, and are more persistent, more virulent strains evolving?

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