12TH C. L. OAKLEY LECTURE*

Pathogenesis of *Clostridium difficile* infection of the gut

S. P. BORRIELLO

Microbial Pathogenicity Research Group, MRC Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ

Introduction

Since the first reports implicating *Clostridium difficile* in the aetiology of antibiotic-associated pseudomembranous colitis^{1–3} a great deal has been learnt about the pathogenesis of gastrointestinal diseases associated with the organism, methods of treatment and prevention, and epidemiology. The progress made is best exemplified by a comparison with *Campylobacter jejuni* diarrhoea and isolation of *Camp. jejuni* from the stool, which was first described 5 years earlier.⁴ Although *Camp. jejuni* is the most common identifiable bacterial cause of diarrhoea, and causes many more cases than *C. difficile*, little is known of the pathogenesis compared with *C. difficile*. There is, for example, still controversy about the possible role of a putative enterotoxin of *Camp. jejuni*, whereas for *C. difficile* it is known that two major toxins are produced (toxins A and B), that toxin A is responsible for the diarrhoea, and the amino-acid sequence of toxin A is known.

This overview of the pathogenesis of *C. difficile* infection of the gut follows the same approach as that taken for the lecture—namely, to concentrate on the contribution of the Microbial Pathogenicity Research Group to our understanding of the infection—and will as far as possible reproduce the contents of the lecture. It is preceded by a brief historical background.

Historical background

The first description of a cytopathic effect (CPE) in cell culture induced by faecal filtrates from patients with pseudomembranous colitis was made by Larson *et al.* in 1977;⁵ they also correctly

suggested that the CPE was caused by a heat-labile toxin, though attempts to identify the bacterial source were unsuccessful.⁵ It is unfortunate that their generalised comment in the discussion “It has also been suggested that viruses may cause this condition . . .” has been quoted as “that viruses may cause this condition . . .”⁶ leading others to mistakenly infer that the CPE was incorrectly interpreted as being due to a virus.

The first description of the organism producing this toxin and its role in the hamster model of disease was made by Bartlett *et al.*, also in 1977,⁷ who called it *Clostridium* BVA 17HF 1–9, which was tentatively identified as *C. difficile*. There very quickly followed three reports, the first by Bartlett *et al.*, demonstrating the association of the now identified bacterium with cases of pseudomembranous colitis.^{1–3}

My own interest in this organism arose during the early part of my PhD studies (1975–1979) on the clostridial flora of the gastrointestinal tract in health and disease with Dr M. J. Hill, Director of the Bacterial Metabolism Research Laboratory, Central Public Health Laboratory Colindale. As part of these studies I examined the development of the clostridial flora during the first few weeks of life and frequently isolated *C. difficile*. This observation led me to the work of Hall and O'Toole from the 1930s⁸ and Snyder in the 1940s⁹ who had also described the presence of this organism in infant faeces, and to that of Hafiz in the early 1970s¹⁰ who described a selective broth for the isolation of *C. difficile*. The importance of the work of Hall and O'Toole is highlighted by the fact that not only did it represent the first known description of *C. difficile* (named *Bacillus difficilis* by them) but also showed that the organism produced a lethal toxin; though this was not shown to be present in the faeces. My interest in the possibility that *C. difficile* could be associated with gastrointestinal disease was stimulated by a paper published in 1969 by Hammarström

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*et al.*¹¹ (including the late Bengt E. Gustafsson who stimulated so much gastrointestinal research) who showed that monocontamination of germ-free rats with *C. difficile* resulted in transient diarrhoea and the development of auto-antibodies to rat colon. As this work was directed to an understanding of the aetiology of ulcerative colitis and a colleague, Michael Hudson, was working at that time on ulcerative and Crohn's colitis, we undertook a study in early 1977 to see if *C. difficile* was present in the faeces of these patients. My previous studies had shown that it was not present in the faeces of healthy adults. To our disappointment, we did not find *C. difficile* in these patients either. There our interest may have ended but for several fortuitous links. Elliott Larson, at the MRC Clinical Research Centre, was pursuing his studies on pseudomembranous colitis and, with Pauline Honour, was following up the leads of a clostridial involvement. Also with the division was Joyce Hill who knew of my work through her husband (Dr M. J. Hill). Contact was recommended and collaboration began in late 1977–early 1978. I was able to confirm readily the presence of *C. difficile* in the faeces of patients with pseudomembranous colitis, and suggested that my previous data showing its absence in other disease states and in healthy adults strengthened the case for an aetiological role in pseudomembranous colitis, and lent weight to the argument that *C. difficile* behaved as an infectious agent rather than it being a normal component of the healthy adult colonic flora that expressed its virulence only under favourable conditions. These data were published in 1978,³ and also represent the first evidence that *C. difficile* toxin was present in the faeces of healthy neonates.

The disease

In keeping with some other intestinal pathogens, one of the intriguing aspects of *C. difficile*-associated disease is the spectrum of outcome of infection which can range from mild, self-limiting diarrhoea, through severe diarrhoea, to fulminant life-threatening pseudomembranous colitis.^{12, 13} In conjunction with this spectrum one must add asymptomatic carriage in neonates. I shall discuss later why it is not surprising that the organism can be isolated frequently from neonates, though it is still not known why disease caused by *C. difficile* is so rare in this age group. The fulminant form of the disease is characterised by the presence of a pseudomembrane—cream to yellow-green plaques which stud the colonic mucosa, or may be confluent. Histological examination reveals a typical pseudo-

membrane, consisting of a spray of fibrin and mucin interlacing polymorphonuclear leucocytes. The fact that lesions can be discrete and adjacent to normal healthy tissue despite the fact that the organism and its toxins are probably evenly distributed throughout the colon is not readily explainable but may be due to distribution of mucosal receptors for *C. difficile* or its toxins (see below).

It is clearly established that, in the vast majority of cases, pre-disposition to infection with *C. difficile* is induced by factors that disrupt the normal intestinal flora, these generally being antibiotics, and that disease results from exposure to *C. difficile* and its establishment in the intestinal flora with concomitant production of toxins. This is readily demonstrated in the hamster model of disease, in which we showed that animals given antibiotic or *C. difficile* alone and housed in isolators did not succumb to disease, whereas those given an antibiotic and then exposed to a toxigenic strain of *C. difficile* invariably died.¹⁴ It is this association with antibiotics that brings in the concept of colonisation resistance, which is the general term used to describe the barrier effect exerted by a stable endogenous microbial population at a given site against exogenous micro-organisms. In this case, it is the ability of the normal intestinal flora to prevent the establishment of *C. difficile* at that site.

Colonisation resistance

When the complexity of the gut flora (i.e., 10^{12} viable organisms/g of gut content and 400–500 different species) is considered it is easy to appreciate the difficulty encountered by a non-resident bacterium in trying to establish colonisation. This barrier effect operates not only against *C. difficile* but also against other pathogens, the main difference is one of degree. For example, the numbers of *C. perfringens* required to induce symptoms of *C. perfringens* food-poisoning in healthy volunteers is lower following antibiotics, but this species can cause disease even in the absence of antibiotic therapy if a sufficiently large challenge dose is given. However, for *C. difficile*, disruption of the normal flora is generally a pre-requisite for infection. This barrier effect against *C. difficile* can be demonstrated readily *in vitro*.¹⁵ In this model, *C. difficile* is seeded into emulsions of faeces prepared in sterile distilled water and the growth of the organism and toxin production are monitored. The importance of the presence of viable components of the faecal flora in prevention of growth of

C. difficile is highlighted by the fact that *C. difficile* will not grow in a faecal emulsion prepared from a healthy donor unless it is first filtered or heat sterilised. In sterilised faecal emulsions, *C. difficile* grows and produces toxin.

This model has been used extensively to compare groups of patients of differing risk of developing *C. difficile* infection, and the results have mirrored well the estimated risks.¹⁵ The in-vitro model also demonstrated that faeces from neonates were, generally, permissive to *C. difficile* growth and that those from the elderly (not on antibiotics) though inhibitory were less so than those from younger people. This could explain in part why the elderly are more susceptible to infection—less compromise of the gut flora may be required to remove its colonisation-resistance effect. However, the ultimate test of any assay of this type is its predictive ability—the ability to predict events gave science credibility and maintains it. We were able to test directly the predictive value of the in-vitro assay by comparison with the hamster model of disease. In these experiments, hamsters were given various antibiotics and, at the time of challenge with *C. difficile*, a parallel set of antibiotic-treated animals was killed and their caecal contents used in the in-vitro assay of colonisation resistance to *C. difficile*. In a small series of experiments, the correlation was 100%—when the in-vitro model predicted that *C. difficile* would become established *in vivo*, it did, and when the model predicted it would not, it did not.¹⁶ This investigation was expanded to 108 separate experiments with nine antibiotics. There was a direct correlation between the predicted outcome of infection with *C. difficile* from the in-vitro test and the fate of the animal in 90 cases. Amongst the 18 discordant findings, there were seven instances in which *C. difficile* was inhibited *in vitro*, predicting animal survival, but the animals died. In the other 11 instances, *C. difficile* was not inhibited *in vitro*, predicting animal death, but the animal survived.¹⁷ However, these discordant findings represented individual animals from within experimental groups, so that the overall prediction of outcome for any experimental group was correct, though there was the occasional animal within those groups that behaved differently. On the basis of these findings, we are sufficiently confident of the value of the in-vitro model to use it to try to identify the components of the intestinal flora involved in resistance to *C. difficile* colonisation. Preliminary findings already indicate that, in this model, facultative organisms do not play a role (table I). The model is also proving to be useful in indicating the relative

Table I. The effect of various procedures on the inhibitory effect of faecal emulsions against *C. difficile*

Procedure	Inhibition of growth of <i>C. difficile</i>	Comment
None	Yes	...
Filtration	No	Shows role of bacteria in inhibitory activity
Treatment with aztreonam or temocillin	Yes	Retention of inhibition following removal of gram-negative facultative species implies they play no role in inhibition
Aeration	No	Removal of anaerobes results in loss of inhibitory activity; demonstrates importance of anaerobes.
Treatment with alcohol	No	Retention of just the endogenous clostridia removes the inhibitory activity, implying clostridia are not an important component of the inhibitory flora.

ability of some new antibiotics under test to predispose to *C. difficile* infection.

There is increasing interest in colonisation resistance which stems primarily from the problem of relapses of pseudomembranous colitis or diarrhoea, which can be multiple,¹⁸ following specific antibiotic therapy (generally vancomycin or metronidazole). It is thought that reconstituting the faecal flora by use of faecal enemas,¹⁹ or with components of the faecal flora,^{20, 21} or by use of micro-organisms that are not normal components of the intestinal flora, e.g., *Saccharomyces boulardii*²² or non-toxicogenic strains of *C. difficile*,^{23, 24} can be used to prevent or treat *C. difficile* infection in experimental animals and man (see reviews 25–27).

Factors influencing outcome of infection with *C. difficile*

Several factors could influence the outcome of infection with *C. difficile* and, thereby, singly or in combination, contribute to the spectrum of gastrointestinal disease associated with this organism. These factors are: (i) the degree of disruption of colonisation resistance; (ii) age of the host; (iii) virulence of the infecting strain; and (iv) host immune responses. Factors (iii) and (iv) are general factors pertinent to any intestinal pathogen. However, superimposed on these factors for *C. difficile* are age and colonisation resistance; and it is these additional factors, which are almost uniquely associated with *C. difficile*, that make this organism so interesting to study. Of these four factors I will

not discuss host immune response, primarily because it is neither an area of personal expertise nor an area in which much work has been conducted.²⁸

Colonisation resistance

It is evident that the degree of disruption of colonisation resistance may contribute to the outcome of infections with *C. difficile*. It would not be difficult to envisage a situation in which the intestinal flora was compromised sufficiently to permit *C. difficile* to survive, but not sufficiently to permit full expression of virulence. This may, in part, explain the relatively high asymptomatic carriage rate seen in patients with cystic fibrosis,²⁹ which is reflected in the generally poorer capacity of their faeces to inhibit the growth of *C. difficile* *in vitro*.²⁹

Age

The relatively frequent carriage of *C. difficile* in neonates may also be explained on the basis of colonisation resistance, as they have a developing and incomplete intestinal flora and would not be expected to exclude *C. difficile*. The frequency of isolation of *C. difficile* decreases after the first week of life, and decreases dramatically with weaning³⁰ concomitant with an increase in the complexity of the intestinal flora. The establishment of adult levels of colonisation resistance in an infant following weaning was shown in the *in-vitro* model mentioned above.¹⁴ However, as we showed very early in our studies, cytotoxin could also be present in the faeces,^{3,31} and others have shown that the toxins present include toxin A,³² it is not possible to explain why carriage is asymptomatic on the basis of colonisation resistance. For this reason we studied the development of susceptibility to colonisation and the occurrence of disease due to *C. difficile* in hamsters. Rolfe and Iaconis³³ had already shown that hamsters aged 4–12 days were susceptible to colonisation but not disease, whereas those younger or older were not. We removed colonisation resistance from hamsters of different ages by treatment with clindamycin to determine when the protective mechanism that prevents *C. difficile* expressing its virulence in neonates disappears.¹³ These studies showed that neonates aged 1 week were resistant to disease, whereas all adult hamsters were susceptible. However, only 25% of 25-day-old weaning hamsters were susceptible following clindamycin. This finding suggested that protective factors other than colonisation resistance due to the normal gut flora are operative not only in the neonates, but that these factors are

still present in 75% of 25-day-old weaning animals. However, at 5 months of age, the major protection against disease is due to colonisation resistance of the normal flora and the other protective factors operative earlier in life are absent or ineffective. The nature of this protective mechanism remains to be determined, but one possibility would be the absence of receptors for toxin A.

Virulence factors of *C. difficile*

It is quite obvious that the outcome of infection with *C. difficile* depends on the virulence potential of the pathogen as well as the extent to which that virulence is allowed to be expressed. There are several established and potential virulence factors of *C. difficile*:³⁴ these include toxin production, adherence to mucosa, expression of fimbriae, capsule production and secretion of tissue degradative enzymes. The most important of these would appear to be toxin production.

Toxins. The importance of toxins in virulence is exemplified by the fact that non-toxigenic strains do not cause disease in the hamster model (table II). Of the two major protein toxins produced (toxins A and B), it is thought that toxin A may be more important in the disease process, because all of the known features of ileocaecitis in the hamster model can be reproduced by administration of toxin A alone.³⁵ The description of toxin A as an enterotoxin and toxin B as a cytotoxin to differentiate them is not strictly correct, as both toxins are potent cytotoxins; however, toxin B is much more cytotoxic than toxin A for most cell lines. Furthermore, toxin A is not a true enterotoxin, i.e., it does not act as a secretagogue as do the toxins of *Vibrio cholerae* or *Escherichia coli*; rather, it results in intra-luminal fluid accumulation as a result of histotoxic-induced increased permeability.³⁶ A recent observation by Krivan *et al.*³⁷ that differentiates between the two toxins is that toxin A will haemagglutinate rabbit erythrocytes at 4°C but toxin B will not. This observation led to the identification of Gal α 1–

Table II. Lack of virulence of non-toxigenic strains in clindamycin pre-treated hamsters

Source	Number of strains	Colonisation	Diarrhoea or death	Histological findings
Neonates	4	+	–	Normal
Adults	3	+	–	Normal
Dog	1	+	–	Normal
Environment	7	+	–	Normal

3Gal β 1-4GlcNAc as a receptor³⁷ and the development of bovine thyroglobulin affinity chromatography for purification of toxin A.³⁸ We demonstrated that bovine thyroglobulin affinity chromatography was not a one-step purification method, as originally proposed, but resulted in a product contaminated with trace amounts of toxin B and at least two other proteins.³⁹ Final purity is achieved by two anion-exchange chromatography steps.⁴⁰ Our analysis by gel-electrophoresis of toxin A purified in this way showed that, following SDS denaturation but in the absence of a reducing agent, fragments or sub-units of toxin A, larger than the 240-Kda major sub-unit, were detected⁴⁰ and that these migrated to the 240-Kda position after reduction.⁴⁰

In an attempt to purify toxin A in one step, we applied crude culture filtrate directly to a mono-Q anion exchange column and analysed fractions obtained by fast protein liquid chromatography. However, instead of collecting 1-ml fractions, which would be normal practice, 0.25-ml fractions were collected and the titres of cytotoxicity and haemagglutinating (HA) activity of each fraction were determined. This yielded the surprising results that the peaks of activity for each of these two biological activities were not coincidental,³⁴ leading us to propose that there may be HA rich-cytotoxic rich, and HA poor-cytotoxic rich forms of toxin A. If this were true, weak HA forms should more readily pass through a thyroglobulin column than the strongly HA forms. Analysis of material eluting from a thyroglobulin column at 37°C (which contains toxin B, and most of the other *C. difficile* proteins, but should not contain any toxin A) showed that a protein conforming to toxin A with respect to point of elution by anion exchange chromatography, native mol. wt, SDS-polyacrylamide gel electrophoresis fragment or sub-unit pattern, and recognition by monospecific polyclonal antibody to toxin A, was present. This form of toxin A is as cytotoxic as conventionally purified toxin A but does not haemagglutinate. Low levels of trypsin activated this variant form of toxin A resulting in a low degree of HA activity. Full activation has been achieved with a lysate of a non-toxigenic strain of *C. difficile*. On the basis of these findings we believe that *C. difficile* toxin A is produced in a pro-toxin form which is activated by the bacterium, and that the pro-toxin differs from toxin A only in configuration. Circular dichroism analysis indicates that the only difference between toxin A and pro-toxin A is that the protein has twice as many α -helices.

Although non-toxigenic strains appear to be avirulent, it was not known whether all toxigenic

strains were equally virulent. Differing virulence of toxigenic strains could contribute to and help explain the spectrum of outcome following infection. We examined this problem in the hamster model and showed that not all toxigenic strains were equally virulent.⁴¹ The strains examined fell into two groups: those that killed all of the test animals within 2 days (highly virulent), and those that killed after a mean of 8.9 days or not at all (poorly virulent). Furthermore, we showed that the major difference between the strains of these two virulence groups was the amount of toxin A produced *in vivo*, though there was little or no difference in the amount of toxin A produced *in vitro* and no difference in the amounts of toxin B produced in either set of conditions.⁴¹ The less virulent strains also colonised less well, taking longer to reach similar faecal concentrations than the highly virulent strains. These observations support the proposition that toxin A may be more important than toxin B in the disease process, and also indicate that relative ability to colonise may contribute to differences in virulence. On the basis of these observations we examined whether or not differences in the ability to associate with intestinal mucosa could, in part, explain differences in virulence between strains.

Mucosal association. We had shown that *C. difficile* could be found in association with the intestinal mucosa in man³¹ and in hamsters.⁴² To examine whether toxigenic strains of differing virulence adhered to intestinal mucosa to different degrees, three strains were used in the hamster model⁴²—a toxigenic, highly virulent strain, a toxigenic, poorly virulent strain, and a non-toxigenic, avirulent strain. These three strains adhered to mucosa at different sites within the gut in the order highly virulent > poorly virulent $\geq$ avirulent. This was particularly so for ileal and jejunal mucosa.

To assess the possible relationship between colonisation and toxin A production, the non-toxigenic, avirulent, poorly adhering strain was co-administered with culture filtrate from the highly toxigenic, virulent strain to hamsters.⁴² This procedure raised the level of mucosal association by the non-toxigenic strain to that exhibited by the highly virulent strain. Though it is possible that toxin mediated this enhanced adhesion, other heat-labile factors could also have been the cause. Unfortunately, at the time of the experiments, neither pure toxin A nor monospecific antibody to toxin A was available to us. There are precedents for a toxin mediating adhesion, e.g., the toxin of *Bordetella pertussis*,⁴³ and we have other evidence for toxin production conferring an advantage on

toxigenic strains in colonisation of hamsters.²⁴ Presence of toxin, or toxin plus other factors, could also facilitate adhesion by general compromise of host defence or exposure of previously masked receptors.

The above observations of adhesion prompted a search for other possible factors that could be involved in the process, in particular fimbriae.

Fimbriae. Adhesion in many micro-organisms is mediated by fimbriae. As these had not been described in *C. difficile*, and in view of its adhesion to intestinal mucosa, a search was undertaken. We found that some cells within a given population of a number of strains of *C. difficile* possessed multiple polar fimbriae which were 4–9 nm in diameter and up to 6 µm long.⁴⁴

Tissue degradative enzymes. The increased permeability of the intestinal mucosa following *C. difficile* infection is attributed to toxin A, because toxin A alone has this effect (see above). However, we were interested in the possible contribution that connective tissue degradative enzyme could make towards the compromise of intestinal wall integrity. We examined strains of *C. difficile* for hyaluronidase, chondroitin-4-sulphatase and collagenase activity as well as heparinase and general protease activity.⁴⁵ Some degree of hydrolytic enzyme activity was present in all of the strains examined, and there was an indication of higher activity, especially collagenase activity, amongst the highly virulent strains. An interesting aspect of this work is that the action of such enzymes would release potentially important nutrients, showing a reason for the production of these toxic enzymes. For example, it has been shown that *C. difficile* utilises N-acetylglucosamine but lacks the enzymes necessary to cleave this monosaccharide from oligosaccharide side chains;⁴⁶ however, *C. difficile* can release N-acetylglucosamine from hyaluronic acid.

Capsule production. Because of the known resistance of *C. difficile* to phagocytosis by polymorphonuclear leucocytes,⁴⁷ and the polymorphonuclear leucocyte infiltration that is a feature of the disease, it was inferred that *C. difficile* produced a capsule. Because many pathogens, including clostridia, produce a protective polysaccharide capsule, a search was made for a capsule in *C. difficile* strains. Extracellular mucopolysaccharide material that stained with ruthenium red was found in all 15 strains examined.⁴⁸ This material was either loose-knit, branching, and extending up to 350 nm from the cell wall, or dense and compact up to 100 nm wide. Sections of colonies on agar showed evidence of formation of microcolonies covered by a glycocalyx.

Summary

On the basis of the above findings it is possible to propose a sequence of events following exposure to *C. difficile* (figure). Exposure of neonates to *C. difficile* leads to transient colonisation which is almost invariably asymptomatic; the reasons why colonisation is asymptomatic are not known. Exposure of antibiotic-treated adults to *C. difficile* does not invariably lead to colonisation; however, in those instances where colonisation occurs, it may be transient and asymptomatic or transient and symptomatic. The transient nature of the colonisation could be because the infecting strain is poorly virulent, or because the degree of compromise of the intestinal flora is insufficient to permit establishment and full expression of virulence. It is likely that it is easier to fully compromise the intestinal flora of the elderly so that they more readily become fully susceptible to colonisation by *C. difficile*. In a fully susceptible host and with a highly virulent strain, the following sequence of events could occur. The organism may associate with the intestinal mucosa (fig. a) possibly via fimbriae (fig. b), and form a microcolony of capsulate cells protected by a glycocalyx (fig. c). The toxins, or other factors, produced may facilitate the interaction with mucosa and toxin A will result in increased vascular and mucosal permeability resulting in intra-luminal accumulation of serum-albumin-rich fluid.⁴⁹ Although *C. difficile* does not appear to be capable of using serum albumin nutritionally,⁵⁰ it may utilise other serum proteins, and the serum proteins in general may compete with host proteases and help prevent degradation of the toxins produced.⁵¹ The cells of *C. difficile* are probably protected by their capsule from phagocytosis by the polymorphonuclear leucocytes that accumulate in response to the infection. To what extent haemoglobin and other haem compounds released could act as an iron source is unknown. Hydrolytic enzymes are also produced and would release potentially essential growth factors, such as hydroxy-proline from collagen and N-acetylglucosamine from hyaluronic acid, thereby providing nutrients directly, and possibly indirectly by contributing to increased mucosal permeability, so helping to fuel further cycles of cell division and increased toxin production.

Although a great deal has been learnt about *C. difficile*, as with other pathogens and their associated diseases, a great deal remains to be understood.^{52, 53} It is hoped that we will be able to contribute to further elucidation of the organism, its pathogenic properties and the disease.

Figure. Possible sequence of events following exposure to *C. difficile*. (a) Association of *C. difficile* with intestinal mucosa shown by scanning electronmicroscopy. (b) Fimbriae of *C. difficile* demonstrated by transmission electronmicroscopy and negative staining. (c) Glycocalyx of capsulate *C. difficile* shown by transmission electronmicroscopy.

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