Mucosal association by *Clostridium difficile* in the hamster gastrointestinal tract

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Summary. For many organisms, mucosal association is an important virulence determinant. Although studied in detail for other intestinal pathogens, this aspect of pathogenicity has not been studied for *Clostridium difficile*. We compared the ability of an avirulent non-toxigenic strain (M-1), a highly virulent toxigenic strain (B-1), and a poorly virulent toxigenic strain (BAT) of *C. difficile* to adhere to different regions of the gastrointestinal tract of hamsters pre-treated with clindamycin. Strain B-1 associated with the gut mucosa significantly better than strain M-1 ($p < 0.001$) for all sites other than the caecum, and achieved significantly higher levels in the caecal contents ($p < 0.001$). The same was true when strain B-1 was compared with strain BAT except that there was no significant difference for the large bowel mucosa. To assess the possible role of toxin in promoting mucosal association, e.g., by compromising host defences or exposing masked adherence sites, strain M-1 was given to animals after intra-caecal administration of crude toxin preparations from strain B-1, which were heat-inactivated in control experiments. The addition of this toxin increased significantly the mucosal association of M-1 for the small bowel only, whereas the inactivated toxin had no significant effect. These results imply that there may be intrinsic differences between strains in their ability to colonise and associate with the gut mucosa, which may partly depend on their ability to produce toxin. These differences do not correlate with cell-surface hydrophobicity or the presence of plasmids, flagella or fimbriae.

Introduction

Clostridium difficile is firmly implicated as the cause of pseudomembranous colitis and some cases of antibiotic-associated and sporadic diarrhoea (Borriello, 1984). Although the role of the two major toxins of *C. difficile*, toxins A and B, has been established in the pathogenesis of the disease (Lyerly *et al.*, 1985; Mitchell *et al.*, 1986, 1987; Ketley *et al.*, 1987) not all toxigenic strains cause disease (Borriello *et al.*, 1987) and asymptomatic carriage has been documented in all age groups (Larson *et al.*, 1982; Libby *et al.*, 1983; Bender *et al.*, 1986; Peach *et al.*, 1986). Several host factors such as the degree of colonisation resistance exerted by the normal gut flora (Borriello and Barclay, 1984) or the expression of toxin-binding sites at colonic cell surfaces (Krivan *et al.*, 1986) may contribute to the spectrum of disease seen in man (Burdon, 1984; Borriello *et al.*, 1985). Recently we have demonstrated that not all toxigenic strains of

C. difficile are equally virulent in the Syrian hamster model of antibiotic-associated colitis (Borriello *et al.*, 1987). These differences in virulence correlated with the ability to colonise the gut efficiently and with the levels of toxin A produced *in vivo* (Borriello *et al.*, 1987). The ability of bacteria to adhere to gastrointestinal cell surfaces is becoming recognised increasingly as a prerequisite for colonisation of the gut (Hartley *et al.*, 1979) and expression of virulence (Lancet, 1977; Cheney *et al.*, 1980; Boedeker, 1982). Because this potential virulence determinant had not been investigated for *C. difficile*, we compared the ability of a highly virulent toxigenic strain, a poorly virulent toxigenic strain and an avirulent non-toxigenic strain of *C. difficile* to associate with the mucosa at different sites within the gastrointestinal tract of the Syrian hamster. Further, we examined the effect of crude preparations of mixtures of toxins A and B on the adherence exhibited by the non-toxigenic strain. Factors that could account for adherence, e.g., flagella, fimbriae, or plasmids which may code for an adherence factor, were also investigated.

Materials and methods

Source and identification of *C. difficile* strains

Strain B-1, toxigenic and highly virulent, was isolated from a patient with pseudomembranous colitis (Borriello *et al.*, 1987); strain BAT, toxigenic but poorly virulent, was isolated from the ward environment of a neonatal unit (Borriello *et al.*, 1987); and strain M-1, non-toxigenic and avirulent, was isolated from a patient with *Shigella sonnei* diarrhoea (Borriello and Barclay, 1985). Isolates were identified as described by Borriello *et al.* (1987).

Preparation of inocula for animal experiments

Starter cultures of *C. difficile* were prepared by inoculating 20 ml of pre-reduced Brain Heart Infusion Broth (Difco) containing L-cysteine HCl 0.05% w/v and sodium formaldehyde sulfoxylate 0.03% w/v as reducing agents (BHI broth) with a colony of *C. difficile* taken directly from a blood-agar purity plate and incubating anaerobically for 48 h. The cultures were centrifuged at 4500 rpm at 4°C for 15 min; the pellet was resuspended and centrifuged twice in 20-ml volumes of fresh BHI broth. The final pellet was suspended in fresh BHI broth to give an opacity equal to a Wellcome opacity tube No. 8 and the number of colony forming units (cfu)/ml was determined in duplicate. This yielded a suspension of c. 10^8 cfu/ml which was used as the oral inoculum for the animal experiments.

Isolation of *C. difficile* from animal material

Isolation of *C. difficile* and an estimate of the mean cfu of caecal content were performed in duplicate by the micromethod described previously (Borriello and Barclay, 1986). Briefly, this consisted of simultaneous dilution of up to eight specimens in a microtitration tray and seeding on cycloserine-cefoxitin selective agar. Isolation and quantitation from segments of gastrointestinal tract were performed as follows. The whole of the gastrointestinal tract was removed from killed animals and placed in a sterile petri-dish. A portion about 6 cm long of jejunum, starting from the proximal end, and of ileum and colon starting from the distal end, and the whole of the caecum, were excised and cut longitudinally to expose the contents and inner surface. The open segments were washed in a mixture of equal volumes of BHI broth and saline (BHIS) with a pasteur pipette to remove the bulk of the contents and the washed segments were transferred individually to 10 ml of BHIS in a 28-ml screw-capped glass container and washed by repeated inversion. The procedure of transfer to fresh BHIS and washing was repeated ten times. Pilot studies had demonstrated that this was the minimum number of washes required to remove regularly non-adherent bacteria. Washed tissue was weighed and suspended in BHIS to yield a 1 in 10 dilution (w/v) and homogenised in sterile glass tissue grinders. Ten-fold dilutions (w/v) of caecal contents were also prepared in BHIS. The mean *C. difficile* cfu/g of

tissue was estimated in duplicate by the micromethod described by Borriello and Barclay (1986).

Animals

Adult Syrian hamsters (*Mesocricetus auratus*) weighing 150–200 g supplied from the National Institute for Medical Research, were used in all experiments. Each was inoculated intraperitoneally (i.p.) with 0.5 ml of a solution of clindamycin phosphate (Dalacin C; Upjohn, Crawley, West Sussex) 10 mg/ml and housed individually in filter-lidded isolator cages (Techniplast animal cages; Biotech Consultants Ltd, Clackmannanshire, Scotland). Cages, food, water bottles and bedding were autoclaved in sealed bags before use. All animals received 0.5 ml of the prepared *C. difficile* suspension *per os* (p.o.) between 10 and 20 days after clindamycin. This period was to allow for clearance of antibiotic. All animals were killed 24 h after receiving *C. difficile*. The following groups of animals were used:

Group 1. Ten animals were challenged 20 days after receiving clindamycin with the highly virulent toxigenic *C. difficile* strain B-1.

Group 2. Thirteen animals were challenged 20 days after receiving clindamycin with the poorly virulent toxigenic *C. difficile* strain BAT.

Group 3. Ten animals were challenged 11 days after receiving clindamycin with the avirulent non-toxigenic *C. difficile* strain M-1.

Group 4. Ten animals were inoculated intra-caecally (i.c.), 15 days after receiving clindamycin, with 0.5 ml of crude broth-culture filtrate of strain B-1 (48-h growth) adjusted by dilution in BHI broth to give a final cytotoxin titre of 10^3 . These animals were then challenged with strain M-1.

Group 5. Ten animals were treated in the same way as group-4 animals with the exception that the crude toxin preparation to be given i.c. was first heated to 56°C for 30 min to inactivate *C. difficile* toxins A and B.

Animal surgery

Animals were anaesthetised by i.p. injection of 0.5 ml of an anaesthetic cocktail of water, Hypnorm (Janssen Pharmaceuticals) and Hypnovel (Roche Products Ltd) in a ratio of 2:1:1. The abdomen was opened by a mid-line incision and the caecum exposed. After i.c. injection of relevant material for animals in groups 4 and 5 above, the wound was closed with catgut sutures for the peritoneum and silk sutures for the skin and occluded with a plastic skin spray (Opsite®; Smith and Nephew).

Determination of bacterial hydrophobicity

Bacterial cell-surface hydrophobicity was determined by the method of Ljungh and Wadström (1982) based on precipitation of cells by ammonium sulphate (Lindahl *et al.*, 1981) as modified by Borriello *et al.* (1987).

Determination of bacterial motility

Motility was determined by phase-contrast microscopy of 20- μ l samples of 18-h BHI broth cultures of *C. difficile*. In addition Bacto-SIM (Sulphide-Indole-Motility) medium (Difco) was inoculated from a 72-h culture from Columbia Blood Agar (Oxoid), incubated anaerobically at 37°C for up to 5 days and examined for evidence of motility. For both methods, *C. beijerinckii* NCIB 9362 and *C. perfringens* strain F1726 (Food Hygiene Laboratory, PHLS, Colindale) were used as positive and negative controls respectively.

Detection of plasmids

The three strains of *C. difficile* were screened for plasmids by two methods (Birnboim and Doly, 1979; Hayter and Dale, 1984). Agarose gel electrophoresis was performed with a Minigel apparatus (Bio-Rad Laboratories).

Examination for flagella and fimbriae

C. difficile cells present in 24-h BHI-broth cultures were examined by negative staining for flagella as described by Fontaine *et al.* (1984). The presence of fimbriae was assessed on cells from the leading edge of growth of 48-h colonies on Columbia Blood Agar by a modification of the method of Henrichsen and Blom (1975) as described by Johnson and Davies (1984). Grids were examined in a Jeol 1200EX electron microscope.

Statistical analysis

The numbers of *C. difficile* (cfu/g) in tissue from each anatomical site sampled and in the caecal contents of the different groups were compared with each other by an algorithm described by Hill and Peto (1971). This

permutation test resembles Wilcoxon's test but deals with the observations themselves instead of their ranks and gives exact results even in the presence of large numbers of ties.

Results

The results of caecal colonisation and association with the mucosa of different regions of the gastrointestinal tract are compared in table I. Those comparisons yielding significant differences are presented in table II. Differences in the ability to associate with the gut mucosa were most noticeable in the small bowel. The toxigenic, poorly virulent strain BAT adhered to gut mucosa and colonised the caecal contents to the same extent as the non-toxigenic, avirulent strain M-1. Neither of these strains associated with gut mucosa or colonised the caecal contents as well as the virulent, toxigenic strain B-1 (table II). The presence of *C. difficile* toxins (toxins A and B) increased the degree of mucosal association exhibited by strain M-1 significantly and resulted in levels of association similar to those found for the toxigenic strain B-1 (table II). The co-administration of heat-inactivated toxins had no such effect.

Strains B-1 and BAT had multiple flagella and were motile, whereas strain M-1 was non-flagellate and non-motile. The motility seen was of a twitching-tumbling type, unlike that of the 'rapid gliding' type seen with *C. beijerinckii*. None of the strains had plasmids or possessed fimbriae. No differences in hydrophobicity were seen which might explain the observed differences in mucosal association; all strains were hydrophilic with cells aggregating over the range 2–3 M (NH₄)₂SO₄.

Table I. Colonisation of hamsters by different strains of *C. difficile*

Experimental group	Number of animals	Number of animals colonised (n) and mean (range) log ₁₀ cfu in									
		jejunum		ileum		caecum		colon		caecal contents	
		n	cfu	n	cfu	n	cfu	n	cfu	n	cfu
1. Strain B-1	10	10	4.3 (3.0–5.0)	10	4.7 (3.0–5.3)	10	5.4 (3.8–5.9)	10	5.5 (3.8–6.5)	10	8.5 (6.3–8.8)
2. Strain BAT	13	2	3.9 (3.5–4.3)	3	3.4 (3.0–4.0)	11	4.7 (3.5–5.9)	10	4.5 (3.0–5.8)	13	6.2 (2.7–8.2)
3. Strain M-1	10	1	3.3	1	3.7	6	4.4 (3.5–5.6)	5	4.1 (3.3–4.8)	10	5.5 (3.3–8.4)
4. Strain M-1 + intracaecal toxin	10	5	4.0 (3.3–4.8)	8	4.4 (3.3–6.8)	8	4.5 (3.3–5.7)	5	4.6 (3.3–5.5)	8	6.9 (5.7–8.1)
5. Strain M-1 + intracaecal heat-inactivated toxin	10	2	4.0 (3.6–4.4)	4	4.8 (3.3–5.8)	6	4.9 (3.0–6.4)	5	4.4 (3.3–6.3)	10	4.8 (3.0–8.6)

Table II. Significant differences between strains of *C. difficile* for colonisation of hamster gastrointestinal tract

<i>C. difficile</i> strains compared	Significance of differences (p values) in adherence of strains to				Differences in colonisation of caecal contents (p values)
	jejunum	ileum	caecum	colon	
Virulent strain B-1 <i>vs</i> toxigenic poorly virulent strain BAT	<0.001	<0.001	NS	0.05	<0.001
Virulent strain B-1 <i>vs</i> non-toxigenic avirulent strain M-1	<0.001	<0.001	0.02	<0.001	<0.001
Virulent strain B-1 <i>vs</i> non-toxigenic avirulent strain M-1 administered with toxin	NS	NS	NS	0.02	0.04
Non-toxigenic avirulent strain M-1 <i>vs</i> same strain administered with toxin	0.04	<0.001	NS	NS	NS

NS = not significant.

Discussion

This study clearly shows that strains of *C. difficile* differ significantly in their ability to colonise the hamster gastrointestinal tract both on mucosal surfaces and in caecal luminal contents. The findings confirm our earlier observation that the poorly virulent, toxigenic strain BAT colonises less efficiently than the highly virulent strain B-1, and the proposal that this difference may reflect differences in the ability to associate with the gut mucosa (Borriello *et al.*, 1987).

The present findings indicate that *C. difficile* toxins may be involved in the promotion of mucosal association, because the co-administration of crude preparations of *C. difficile* toxins with a non-toxigenic strain resulted in a significantly greater adherence to small bowel mucosa comparable to that of the toxigenic strain B-1. It is unlikely that the increase in mucosal association was due to the operative procedures used which could have helped to promote adherence, e.g., by a decrease in small bowel peristalsis, because no such effect was noted in experiments where heat-inactivated crude toxin preparations were used. It is possible, however, that there may have been other heat-labile products present that were involved in enhancing mucosal association. The possible role of toxins in promoting mucosal association is also supported by the observations that the poorly virulent, toxigenic strain BAT, which is known to produce low levels of toxins *in vitro*, and *in vivo* over the first few days of infection (Borriello *et al.*, 1987), associated with gut mucosa poorly compared to the more toxigenic, virulent counterpart strain B-1. In fact, strain BAT behaved like the non-toxigenic strain in this respect. These findings are similar to those with *Vibrio*

cholerae, in which cholera toxin appears to enhance mucosal association in the small bowel (Pierce *et al.*, 1985). How these toxins promote colonisation is unknown. It is possible that the toxins of *C. difficile* expose masked receptor sites as a consequence of cell damage, that they change cell surface charge, or that the toxins compromise the host's ability to limit adherence, e.g., by reducing peristalsis or affecting mucosal secretion. Whatever the mechanism it does appear that toxin production confers a colonisation advantage. Additional evidence for this is provided by the findings of Borriello and Barclay (1985) who showed that in hamsters colonised with a non-toxigenic strain of *C. difficile* (strain M-1) and subsequently challenged with a toxigenic strain (strain B-1), the latter eventually became the dominant of the two, which suggests the ability to produce toxin may confer some ecological advantage. There is some evidence that this may also be true for *Escherichia coli* in pigs (Hinton *et al.*, 1985) and to a lesser extent for *Yersinia enterocolitica* in man (Pai *et al.*, 1978).

The mechanism by which *C. difficile* adheres to the gut is unknown. Although we have since shown that some strains do produce fimbriae (Borriello, Barclay and Davies, unpublished observations) none of the strains used in these experiments possessed them. It is unlikely that adherence was mediated by a flagellum-associated adhesin such as is found in *Campylobacter jejuni* (Newell *et al.*, 1985) because the non-toxigenic strain that lacked flagella adhered as well as the poorly virulent, toxigenic strain which was flagellate. Furthermore, the non-toxigenic, non-flagellate strain adhered to small bowel as well as the virulent, toxigenic, flagellate strain when toxins were co-administered.

The factors contributing to the full expression of virulence of *C. difficile* are complex. Virulence inevitably depends on the determinants possessed by *C. difficile* and the extent to which the host environment allows them to be expressed. From this point of view, the degree of colonisation resistance afforded by the normal gut flora probably plays a greater role in *C. difficile* infection than with other intestinal pathogens. It would appear that toxin production promotes mucosal association at some sites in the gastrointestinal tract and that this may lead to successful colonisation. Production of

toxin by adherent cells would result in toxin release close to its site of action, thus promoting adjacent mucosal association and localised toxin production. This whole process would be further enhanced if the strain of *C. difficile* exhibited chemotaxis towards mucosal receptors. We are currently examining this possibility.

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More detailed analysis of strain M-1 has demonstrated that a few cells possessed flagella. This type of finding has been noted by others (Dr P. N. Levett, personal communication).

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