In-vitro and in-vivo characterisation of resistance to colonisation with *Clostridium difficile*

H. E. LARSON* and A. WELCH

Microbial Pathogenicity Research Group, Clinical Research Centre, Medical Research Council, Watford Road, Harrow HA1 3UJ

Summary. In hamsters, resistance to colonisation by *Clostridium difficile* appears to be mediated by micro-organisms that are present in the gut in relatively low concentrations. Small amounts of normal caecal contents inhibited the growth of *C. difficile* when added to cultures *in vitro* or given to animals which had been treated with clindamycin. Filtrates of caecal contents, frozen and thawed contents and contents diluted to 0.1% wet weight lost their inhibitory properties. However, caecal contents retained their protective capacity after culture for 7 days *in vitro*. Antibiotic treatment altered resistance to colonisation by only a few species of clostridia. Faeces of animals treated with ampicillin but not clindamycin recovered colonisation resistance after incubation at 37°C *in vitro*. Since human faeces could also restore colonisation resistance to hamsters, the hamster model may be useful for the study of resistance to colonisation by *C. difficile* in man.

Introduction

Clostridium difficile is the principal cause of antibiotic-associated colitis and usually affects only people who have had antibiotic treatment. Colonisation can occur *de novo* but this is uncommon. The hamster model of *C. difficile* infection mirrors the infection in man. Hamsters do not acquire this organism from the environment unless they have had prior antibiotic treatment and they do not become colonised easily even if the organism is fed to them experimentally. Antibiotic treatment, by contrast, produces susceptibility to doses of less than 10 cfu of *C. difficile*.¹ The properties of the factors that restrict colonisation of normal hamsters and man have been characterised in a preliminary way by Borriello and Barclay.² Their findings suggest that viable micro-organisms in the faeces are antagonistic to *C. difficile*. Soluble substances such as nutrients or bacterial products do not appear to be responsible. However, attempts to isolate and identify the particular organisms have been only partially successful or unsuccessful.^{3–5} It is now known that antibiotics must be allowed to clear from the faeces before testing hamsters for susceptibility to *C. difficile*.¹ Therefore, we have done further studies to examine the properties of normal faeces that inhibit growth of *C. difficile* and to determine ways of restoring colonisation resistance to animals which have been treated with antibiotics.

Materials and methods

Toxin production in suspensions of caecal contents

Adult Syrian hamsters obtained from the National Institute for Medical Research, Mill Hill, London were given 3 mg of clindamycin in 0.25 ml of water by mouth from the end of a 1-ml syringe and placed in individual sterile, filter-lidded polycarbonate boxes (Stephen Clark Fabrications Ltd, Alva) containing autoclaved bedding, feed and tap water. Animals were changed into clean boxes at weekly intervals with aseptic techniques and were killed by CO₂ anaesthesia after 14–21 days. Caecal contents were suspended to 20% wet w/v in phosphate-buffered water, pH 7.4, and 150 or 200 µl of suspension was added to wells of sterile 96-well microtitration plates to which suspensions of *C. difficile* were subsequently added to make a final total volume of 250 µl/well. Caecal suspensions from normal untreated hamsters were prepared in a similar manner. The minimum volume of caecal suspension which could be pipetted accurately was 50 µl.

In experiments on restoration of colonisation resistance *in vitro*, 50 µl of suspension from untreated animals was added to 150 µl of suspension from clindamycin-treated animals and incubated anaerobically at 37 °C for 24 h before seeding with *C. difficile*.

All cultures were performed in duplicate. Caecal contents from two or three antibiotic-treated hamsters were pooled.

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*Present address: Middlesex Internal Medicine Associates, Inc., 28 Lord Road, Suite 255, Marlborough, Massachusetts 01752, USA.

Table 1. Toxin production by *C. difficile* in suspensions of hamster caecal contents

Log ₁₀ dilution of <i>C. difficile</i> *	Percentage cytopathic effect in Vero cell cultures of <i>C. difficile</i> grown in caecal suspensions from			
	clindamycin-treated hamsters	normal hamsters	clindamycin- treated + normal hamsters	BHI alone
1	100	25	0	100
2	100	0	0	25
3	100	0	0	0
4	100	0	0	0
5	100	0	0	0
6	100	0	0	0
7	100	0	0	0
8	0	0	0	0

* Log₁₀ dilutions from 5.4×10^5 cfu of *C. difficile*.*Restoration of colonisation resistance to clindamycin-treated hamsters*

At 21–28 days after a single 3-mg dose of clindamycin, hamsters were fed with 0.25 ml of caecal suspension from untreated hamsters. Before administration, the suspensions were treated either by dilution, centrifugation, freeze-thawing or culture *in vitro*. Seven days later these animals were challenged with 0.25 ml of *C. difficile* suspension diluted 1 in 10 v/v in Brain Heart Infusion Broth (BHI, Oxoid), to contain $\geq \log_{10}$ 4.0 cfu. Animals were observed three times daily for 7 days for evidence of illness. Sick animals were killed and examined for evidence of enterocaecitis.

Bacterial isolates from caecal contents of untreated hamsters were obtained by standard culture methods⁶ and identified to genus level by growth characteristics on selective media, colonial and microscopic morphology, and metronidazole susceptibility. These were stored in lysed blood at -70°C and re-cultured for use in protection experiments.

Fresh faeces from a healthy human subject were suspended in phosphate-buffered water to 10% wet w/v and fed to hamsters. Animals were also challenged with suspensions of other *Clostridium* spp. and faeces were sampled and cultured on days 3, 5, 7, 10 and 14.

Challenge suspensions of bacteria

C. difficile strain B-1, which was originally isolated from a patient with pseudomembranous colitis, was cultured overnight in BHI, concentrated by centrifugation at 4000 g at 4°C and resuspended in sterile BHI to its original volume. Ten-fold dilutions in BHI were inoculated into microtitration plates (50 μl /well) and 0.5 ml was spread on to blood-agar plates for colony counts. Plates were incubated at 37°C for 48 h in CO₂ 10%, H₂ 10% and N₂ 80%. Growth of *C. difficile* in each well of the microtitration plates was assumed if toxin could be detected. Briefly, 20 μl was transferred from each well to a microtitration plate containing confluent monolayers of Vero cells in 200 μl of Medium 199 (Flow Laboratories)/well containing

newborn calf serum 2%, bicarbonate 2%, glutamine 1%, penicillin 100 units, and streptomycin 100 μg . Cell cultures were examined at 24 h for cytopathic effect due to *C. difficile* toxin B.

Stock strains of the following organisms were inoculated into Robertson's cooked meat medium and fed to hamsters as above: *C. perfringens* types A, B, D, E, into six, three, three and six animals respectively; *C. sordellii* (nine animals) and *C. sordellii* Nakamura strain (two); *C. novyi* (three), *C. septicum* (three), *C. histolyticum* (three), *C. bifermentans* (three) and *C. butyricum* (two).

Results*Toxin production in caecal suspensions*

Caecal suspensions prepared from clindamycin-treated hamsters readily supported the growth of very small inocula of *C. difficile* (table 1). *C. difficile* grew better in the caecal suspensions than on the blood-agar plates used to estimate the number of cfu in the inoculum. Culture of a dilution of log₁₀ 7 of the original suspension on blood agar indicated that the inoculum had little, but not zero, chance of containing one cfu. However, toxin was detected in suspensions of caecal contents from clindamycin-treated hamsters when inoculated with a dilution of log₁₀ 7 but not log₁₀ 8 of the suspension. Suspensions from normal, untreated animals did not support toxin production by *C. difficile* to any extent. When suspensions from untreated animals were mixed with suspensions from clindamycin-treated animals in an arbitrarily chosen ratio of 1:1, the capacity to support toxin production was completely suppressed. BHI alone was a poorer growth medium for *C. difficile* than caecal suspensions from clindamycin-treated hamsters.

Investigation of colonisation-resistance factors in normal caecal contents

Untreated and lightly centrifuged (500 g, 5 min) preparations of caecal contents from normal hamsters did not support the growth of an inoculum of log₁₀

Table II. Inhibition of toxin production by *C. difficile* in caecal contents from normal hamsters

Treatment of caecal contents*	Log ₁₀ highest dilution of <i>C. difficile</i> † producing detectable toxin‡
Untreated	< 1
Centrifuged 500 g	< 1
Frozen and thawed	2
Centrifuged 15000 g	5
Filtered (0.45 µm)	5
BHI alone	2

* Contents were diluted to 20% wet w/v in water before treatment.

† Log₁₀ dilutions from log₁₀ 5.2 cfu.

‡ Giving > 50% cytopathic effect on Vero cells.

5.2 cfu of *C. difficile* (table II). Contents which had been clarified by centrifugation at 15000 g for 30 min or by filtration supported growth of a log₁₀ 5 dilution of the original inoculum. A single cycle of freezing and thawing increased the capacity to support growth by at least 100-fold compared with untreated suspensions.

Restoration of colonisation resistance *in vitro*

When suspensions of caecal contents from normal hamsters were mixed with material from clindamycin-

treated animals and incubated for 24 h under anaerobic conditions before challenge with *C. difficile*, recovery of resistance to infection was demonstrated *in vitro* (table III). The challenge inoculum of *C. difficile* contained log₁₀ 7.1 cfu before dilution. Addition of a 10% suspension of caecal contents from normal untreated hamsters to contents from clindamycin-treated animals produced a log₁₀ 5 decrease in susceptibility to *C. difficile*, a 3% suspension a log₁₀ 4 decrease, and 1% and 0.3% suspensions log₁₀ 2 decreases. Further dilution of the normal caecal contents removed the capacity to restore resistance to the growth of *C. difficile*. Freezing to -20°C and thawing at room temperature for three cycles also abolished the capacity to restore resistance to the growth of *C. difficile*.

Spontaneous recovery of colonisation resistance *in vivo* and *in vitro*

Caecal suspensions prepared from hamsters which had been treated with clindamycin up to 10 days before sampling supported growth, i.e., toxin production, by *C. difficile* (table IV). After incubation at 37°C for 48 h under anaerobic conditions a log₁₀ 2 decrease in susceptibility was observed. Caecal suspen-

Table III. Restoration of resistance to growth, i.e., toxin production, by *C. difficile* in cultures of caecal contents* from clindamycin-treated hamsters

Proportion (%) of caecal contents from normal hamster added to suspension from clindamycin-treated hamster	Log ₁₀ highest dilution of <i>C. difficile</i> † producing detectable toxin‡
None (water)	6.5
10	1.0
3	2.0
1	4.0
0.3	4.0
0.1	6.0
0.03	6.0
0.01	6.0
Frozen and thawed three times	6.0

* Contents were diluted to 20% wet w/v.

† Log₁₀ dilutions from log₁₀ 7.1 cfu.

‡ Giving > 50% cytopathic effect in Vero cell cultures.

Table IV. Spontaneous recovery of colonisation resistance in caecal contents* after treatment with antibiotics

Antibiotic treatment	Number of days after withdrawing antibiotic	Number of days of incubation <i>in vitro</i> before challenge	Log ₁₀ highest dilution of <i>C. difficile</i> † producing detectable toxin‡
Clindamycin	10	0	8.0
	9	1	7.0
	8	2	6.0
Ampicillin	1	0	7.0
	5	0	2.0
	1	1	2.0
	1	2	4.7

* Contents were diluted to 20% wet w/v in water.

† Log₁₀ dilutions from log₁₀ 7.5 cfu.

‡ Giving > 50% cytopathic effect in Vero cells.

Table V. Restoration of colonisation resistance to hamsters*

Origin of treatment suspension†	Concentration of suspension	Number of hamsters	
		challenged‡	with enterocaecitis
None	...	26	24
Normal hamster	10 %	13	0
caecal contents	1.0 %	10	1
	0.1 %	10	9
	0.01 %	10	8
	10 %	5	0
Cultured hamster	10 %	5	0
caecal contents	0.1 %	4	4
Mixed organisms	...	11	10
Human faecal suspension	10 %	5	0

* Given a single 3-mg dose of clindamycin by mouth 21–28 days before treatment.

† 0.25-ml suspension in water was administered by mouth 7 days before challenge.

‡ Challenged with $\log_{10}$ 4.0 cfu *C. difficile* by mouth.

sions prepared from animals treated with ampicillin lost susceptibility to growth of *C. difficile* more rapidly than those treated with clindamycin. Suspensions prepared 1 day after antibiotic treatment had stopped and challenged immediately were fully susceptible. Those prepared 5 days after treatment showed a $\log_{10}$ 5 decrease in susceptibility. Caecal contents prepared 1 day after ampicillin treatment and challenged after incubation for 1 or 2 days *in vitro* also showed substantial reductions in susceptibility.

Susceptibility of hamsters of colonisation with Clostridium spp.

Clindamycin-treated hamsters were challenged with various species of pathogenic and non-pathogenic clostridia. *C. difficile* was the only species of those tested that produced clinical disease; five of six animals which were challenged developed typical enterocaecitis. Two animals were challenged with the Nakamura strain of *C. sordellii*, which also produces toxin, but both remained well for up to 14 days. With three exceptions, lack of disease was also associated with failure of colonise the gut. Two of nine animals were colonised with *C. sordellii* on days 3 and 5 and one of two animals was found to be excreting *C. butyricum* on day 3.

Restoration of colonisation resistance to clindamycin-treated hamsters

Of 26 control hamsters which had received 3 mg of clindamycin 21–28 days before challenge with *C. difficile*, 24 developed enterocaecitis. When diluted caecal suspensions from untreated animals were fed to clindamycin-treated hamsters 7 days before challenge, colonisation resistance was restored, but this protective capacity was lost when the suspensions were diluted to 0.1 % or less (table V). Caecal suspensions from untreated hamsters diluted to 10 % wet w/v in BHI and cultured under anaerobic conditions for 7 days also restored colonisation resistance, but a 0.1 %

suspension cultured similarly did not. A 10 % suspension of faeces from a normal human volunteer also restored colonisation resistance to hamsters. Suspensions of mixed organisms isolated by anaerobic and aerobic culture from caecal contents of untreated hamsters and comprising bacteroides, lactobacteria, bifidobacteria, fusobacteria, veillonellae, anaerobic cocci (two to four strains of each type), streptococci and gram-negative bacilli (two and three strains respectively) were pooled and fed to clindamycin-treated hamsters, but did not restore colonisation resistance. Sonication of faecal suspensions to break down clumps of organisms had no adverse effect on the ability to restore colonisation resistance. Five hamsters which were given a sonicated 10 % suspension were protected, but five hamsters given 0.01 % of the sonicated suspension remained susceptible.

Discussion

Colonisation resistance is one of the most striking phenomena associated with the pathogenesis of colitis caused by *C. difficile*.⁷ In the hamster model, untreated animals resisted challenges with very large doses of *C. difficile* whereas antibiotic-treated animals were susceptible to very low doses of micro-organisms. Previously published experiments have shown that susceptibility or resistance is absolute.¹ Partial susceptibility, manifest in terms of mild illness or prolonged diarrhoea, has not been observed in hamsters. Thus, clear-cut end-points can be obtained without using large numbers of animals. In the present study, four or five out of five animals in each experimental group either remained completely healthy or developed typical symptoms of enterocolitis within 3–5 days.

Many workers have already attempted to characterise colonisation resistance by various methods. Our approach differs from previous experiments in using hamsters rather than mice,^{3,4,8} clindamycin rather than vancomycin, and single rather than multiple doses of normal caecal contents.⁹ The clindamycin-treated hamster model has already been studied

thoroughly in relation to infection, microbial growth, toxin production and pathology. In the past it has been a difficult model for the study of colonisation resistance because clindamycin-treated hamsters have been refractory to recolonisation with protective bacteria. It is now known that clindamycin persists in the faeces, and that the drug must be allowed to clear from the gut before further experimental manipulations can be undertaken.¹ We observed this precaution and found that a single dose of normal caecal suspension by mouth was sufficient to restore colonisation resistance.

There was a close correlation between results obtained *in vitro* and *in vivo*. The effect of diluting caecal suspensions from normal hamsters was similar and suspensions diluted to 0.1% failed to restore resistance to colonisation in either case. Previous studies with hamsters have shown that clindamycin produces long-term susceptibility to colonisation with *C. difficile*, whereas ampicillin treatment is associated with spontaneous recovery of resistance to colonisation.¹ In the present study, caecal suspensions from ampicillin-treated hamsters recovered their resistance to colonisation during incubation. Such close correlation between effects *in vivo* and *in vitro* suggests that the chief determinant of the pathogenesis of infection with *C. difficile* is whether it is able to grow within the faecal stream. Attachment to the host and immune factors appear to be secondary in importance to replication of the micro-organism and release of its toxin. However, there were some differences between our experimental protocols *in vivo* and *in vitro*. Caecal suspensions from clindamycin-treated hamsters could be maintained for only limited periods of *in vitro* incubation before becoming refractory to infection. By contrast, hamsters remained susceptible for prolonged periods.¹ Therefore, we challenged incubated suspensions after 24 h, but clindamycin-treated hamsters were challenged 7 days after being given caecal suspensions from normal animals. In spite of these differences, there was close correlation between the results *in vitro* and *in vivo*. Abolition of colonisation resistance by freezing and thawing indicated that viable micro-organisms were required to prevent growth of *C. difficile*. Supernates and filtered suspensions of caecal contents supported rather than suppressed growth of *C. difficile* *in vitro*. These results confirm observations by Wilson *et al.*,⁹ who showed that filtered and heated caecal homogenates offered no protection to vancomycin-treated hamsters.

Caecal suspensions diluted to $\leq 1\%$ wet w/v lost the capacity to restore colonisation resistance both *in vivo* and *in vitro*. This is surprising since major components of the microbial flora, particularly anaerobes, are present to concentrations of at least $\log_{10}$ 4/g and would be expected to grow either in cultures or in the hamster caecum and to attain protective levels. It is difficult to see why dilution alone should alter the balance of the microflora so radically, unless the microflora of caecal contents is not homogeneous,

but consists of structured combinations of micro-organisms, perhaps as micro-colonies. Limited dilution of such a discontinuous system might eliminate essential components; however, suspensions of sonicated caecal contents at a concentration of 0.01% also failed to protect hamsters. This was the only dilution tested, so we cannot rule out a small increase in activity, for example, from 1% to 0.1% as a result of sonication. A second explanation is that the organisms which confer colonisation resistance are present at very low concentrations, but in this case it might be expected that soluble factors would be found to mediate colonisation resistance. However, there is evidence that colonisation resistance is not mediated by soluble factors. The failure of soluble factors to explain colonisation resistance also argues against a major role for physical changes such as alterations in pH. Caecal suspensions from normal animals have a physiological pH, so this cannot explain their failure to support the growth of *C. difficile*.

Hamsters are considered unsuitable for the study of colonisation resistance because their colonic flora is largely uncharacterised.³ Our preliminary results suggest that human faeces can be used to restore colonisation resistance in hamsters; this would allow human colonisation resistance to be studied directly in this model. Hamster caecal suspensions can be cultured for up to 7 days in BHI without losing their ability to restore colonisation resistance *in vivo*. This would permit addition of antibiotic probes to distinguish the roles of the major groups of flora. A combination of experiments *in vitro* and *in vivo* could produce valuable information. Experiments *in vitro* have the advantage of simplicity and inexpensive replication, but the disadvantage that persisting antibiotics cannot easily be removed or neutralised. However, it would, be possible to add specific antibiotics to caecal suspensions from normal animals in culture, then hold them for up to 7 days before administration to test animals. Challenge could then be delayed until the antibiotic probe had been cleared from the gut.

Our studies extend the findings of Borriello and Barclay,¹⁰ who found that, following clindamycin treatment, hamsters could be infected fatally with *C. spiroforme* and colonised with *C. sporogenes* but could not be colonised with *C. perfringens*, *C. bifermentans* or *C. beijerinckii*. These results also confirm that antibiotic treatment alters colonisation resistance to very few species of clostridia. The high level of specificity is surprising, since closely related organisms were tested, including one that produces an immunologically identical toxin. Species specificity has not been emphasised in many previous discussions of colonisation resistance and in much work on colonisation resistance in man, loss of resistance is assumed to affect a broad range of species of micro-organisms. There may, of course, be differences between animals and man in this respect, but this should be tested.

The mechanism of resistance to colonisation with *C. difficile* that is altered by antimicrobial chemotherapy is of considerable practical as well as theoretical importance. Understanding of this mechanism may be the key to the definitive management of patients

who suffer recurring relapses of *C. difficile*-associated colitis.

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