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

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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*. The properties of the factors that restrict colonisation of normal hamsters and man have been characterised in a preliminary way by Borriello and Barclay. 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. It is now known that antibiotics must be allowed to clear from the faeces before testing hamsters for susceptibility to *C. difficile*. 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.
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 plate 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 I). 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 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 7

### Table I. Toxin production by C. difficile in suspensions of hamster caecal contents

<table>
<thead>
<tr>
<th>Log₁₀ dilution of C. difficile</th>
<th>clindamycin-treated hamsters</th>
<th>normal hamsters</th>
<th>clindamycin-treated + normal hamsters</th>
<th>BHI alone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>25</td>
<td>0</td>
<td>100</td>
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<td>8</td>
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<td>0</td>
<td>0</td>
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</tr>
</tbody>
</table>

*Log₁₀ dilutions from 5.4 x 10³ cfu of C. difficile.
Inhibition of toxin production by C. difficile in caecal contents from normal hamsters

<table>
<thead>
<tr>
<th>Treatment of caecal contents*</th>
<th>Log_{10} highest dilution of C. difficile† producing detectable toxin‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Centrifuged 500 g</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Frozen and thawed</td>
<td>2</td>
</tr>
<tr>
<td>Centrifuged 15000 g</td>
<td>5</td>
</tr>
<tr>
<td>Filtered (0.45 μm)</td>
<td>5</td>
</tr>
<tr>
<td>BHI alone</td>
<td>2</td>
</tr>
</tbody>
</table>

Table II.

Contents were diluted to 20% wet w/v in water before treatment. †Log_{10} dilutions from log_{10} 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 log_{10} 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 log_{10} 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 log_{10} 5 decrease in susceptibility to C. difficile; a 3% suspension a log_{10} 4 decrease, and 1% and 0.3% suspensions log_{10} 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 log_{10} 2 decrease in susceptibility was observed. Caecal suspens-
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 reduction 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 enterocolitis. 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 enterocolitis. 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. 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. 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, clindamycin rather than vancomycin, and single rather than multiple doses of normal caecal contents. 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.*,\(^3\) 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 microorganisms, 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.\(^9\) 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. bifermantans* 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 microorganisms. 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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References