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PROBIOTICS: PROSPECTS FOR USE IN *CLOSTRIDIUM DIFFICILE*-ASSOCIATED INTESTINAL DISEASE

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INTRODUCTION

Clostridium difficile is an etiologic agent of a spectrum of gastrointestinal diseases which range in severity from uncomplicated diarrhoea to fulminating pseudomembranous colitis. Despite the availability of effective pharmacotherapy, *C. difficile* colitis remains a serious condition, especially in elderly and debilitated patients. This paper will review the evidence that the normal intestinal flora is an important barrier to *C. difficile* intestinal colonisation and will examine some of the parameters possibly involved in colonisation resistance to *C. difficile* infection. The role of probiotics in the treatment and prevention of *C. difficile*-mediated intestinal disease will also be discussed.

CLOSTRIDIUM DIFFICILE-ASSOCIATED INTESTINAL DISEASE

Clostridium difficile-induced intestinal disease in humans is a health problem of significant clinical importance. Toxigenic *C. difficile* is the major cause of antibiotic-induced pseudomembranous colitis (PMC), a severe and life-threatening intestinal disease, and is the cause of approximately one-third of cases of antimicrobial agent-associated non-specific colitis and diarrhoea without colitis (*George*, 1988; *Bartlett*, 1992). *C. difficile*-associated intestinal disease results from antibiotic suppression of the indigenous intestinal flora with resultant proliferation of *C. difficile*. All major classes of antimicrobial agents have been reported to induce *C. difficile*-associated intestinal disease in humans (*Trnka* and *LaMont*, 1984). The major offending antibiotics are ampicillin, cephalosporins, and clindamycin, given either singly or in combination with other antibiotics. *C. difficile*-associated intestinal disease can occur during antimicrobial therapy or begin weeks after discontinuation of therapy and may persist for months. Since its discovery as the cause of PMC in 1978, *C. difficile* has emerged as the major identifiable infectious cause of nosocomial diarrhoea in the United States, infecting 15% to 25% of adult hospitalised patients (*DuPont* and *Ribner*, 1992; *Bartlett*, 1990; *McFarland* et al., 1989). Nosocomial *C. difficile* infection causes significant morbidity and is associated with increased hospital costs and lengths of stay (*Kofsky* et al., 1991).

The mechanisms by which *C. difficile* causes intestinal mucosal injury and death are not entirely understood. However, the pathogenicity of this microorganism is dependent, at least in part, upon two biochemically and immunochemically distinct toxins produced during replication of *C. difficile* in the intestine. These toxins are referred to as

toxin A (enterotoxin) and toxin B (cytotoxin). There is considerable evidence implicating both toxins in the development of *C. difficile*-induced disease (*Donta*, 1988; *Lyerly* and *Wilkins*, 1988). Toxin A is thought to be responsible for most of the diarrhoea and damage to the colonic mucosa seen in PMC (*Mitchell* et al., 1986; *Triadafilopoulos* et al., 1987). Although purified toxin B has no effect when administered alone into the intestinal tract, it is a potent cytotoxin for most mammalian fibroblast cell lines *in vitro* (*Lyerly* et al., 1985). It appears that toxin A binds to and causes lesions on the intestinal epithelium. Through the intestinal lesions, toxins A and B act on the underlying tissue and structures. If *C. difficile*-induced intestinal disease is left untreated, these toxins can result in severe systemic effects and death.

Since the discovery that *C. difficile* is the primary etiologic agent of antimicrobial agent-induced PMC, therapy directed at *C. difficile* has led to generally excellent results (*Finegold* and *George*, 1988). Many or most patients with *C. difficile*-related intestinal disease run a self-limited course so that simply discontinuing the inciting agent or agents usually results in clearing of diarrhoea within a few days (*Triadafilopoulos* et al., 1987). No further specific therapy will be required in a substantial number of patients, particularly those with mild diarrhoea and without signs and symptoms of colitis. If therapy for the initial infection is still required, switching to another agent that has less impact on the normal faecal flora, and that is less likely to lead to *C. difficile*-induced disease, may allow recovery from the gastrointestinal complication of earlier therapy.

In severe cases of *C. difficile*-associated intestinal disease or in those that do not respond to the cessation of the inciting antibiotic, specific antimicrobial therapy against *C. difficile* has proven useful. The most widely employed and most effective treatment of *C. difficile*-associated diarrhoea or colitis is the use of orally administered vancomycin (*Triadafilopoulos* et al., 1987; *Keighley* et al., 1978; *Fekety* et al., 1981). Symptomatic improvement is usually evident within 24 to 48 hours of initiating therapy, and complete resolution of diarrhoea and colitis occurs in the majority of patients by the end of a 10 day treatment period. Additional antimicrobial agents used in the treatment of *C. difficile*-associated colitis are bacitracin and metronidazole (*Triadafilopoulos* et al., 1987; *Young* et al., 1985; *Dudley* et al., 1986; *Teasley* et al., 1983; *Young* et al., 1985). Clinical trials have indicated that these two agents are comparable to vancomycin in efficacy. However, occasional metronidazole and bacitracin resistant strains of *C. difficile* have been reported (*Bartlett*, 1985; *Saginur* et al., 1980).

Despite an excellent initial response to therapy, the discontinuation of therapy is followed by a relapse of *C. difficile*-associated intestinal disease in approximately 20% of patients irrespective of which antimicrobial agent is used (i.e., metronidazole, vancomycin, bacitracin) (*Bartlett* et al., 1980a; *Walters* et al., 1981; *George* et al., 1980; *Teasley* et al., 1983; *Fekety* et al., 1989). The signs and symptoms of relapse are similar to the initial attack. Once patients experience a recurrence of *C. difficile* disease, they are more likely to have subsequent recurring episodes of the disease (*Bartlett*, 1983). It is thought that re-infection occurs when the concentration of antibiotic has dropped below that to which *C. difficile* is sensitive and before the normal flora has had a chance to regain its equilibrium (*Young* and *McDonald*, 1986). It is thought that *C. difficile* survives in the intestinal tract during therapy in the form of antibiotic-

resistant spores since symptomatic relapse frequently involves re-infection with the same strain of *C. difficile* that caused the initial infection. It is also possible that some relapse cases may actually represent exogenous re-infection acquired in the hospital setting since it is known that the organism persists in the environment for extended periods (*Johnson* et al., 1989). Presently there is no reliable way to predict the likelihood of relapse in any one individual. Relapses occur whether the vancomycin has been given in high dose or low dose, for long periods or short periods. Furthermore, persistence of the toxins or the organism at the completion of therapy is not predictive of the likelihood of relapse. The relapses are often cured by a second course of antimicrobial therapy (*Bartlett* et al., 1980b). However, multiple relapses, involving the reappearance of the organism with its cytotoxin in the stool, can occur. Management of the patient with multiple relapses can be very difficult since no single therapeutic measure is uniformly effective in preventing disease relapse. Traditional approaches to treating patients with recurring *C. difficile* colitis have included repeated courses of antibiotics, addition of resins such as cholestyramine and colestipol, and longer tapering doses or pulse doses of vancomycin (*Bartlett*, 1983; *Tedesco* et al., 1985).

The variety of therapies tried in treating patients with relapse of *C. difficile*-associated intestinal disease attest to the fact that no single therapeutic measure is uniformly effective in preventing disease relapse. Optimal therapy of initial *C. difficile* disease as well as relapse should take into account the important protective role of the normal bowel flora. This has stimulated various groups of researchers to try to identify components of the normal bowel flora that are involved in excluding *C. difficile* from the intestinal tract.

IMPORTANCE OF NORMAL FLORA IN COLONISATION RESISTANCE TO *CLOSTRIDIUM DIFFICILE*

The mechanisms which permit *C. difficile* overgrowth in the intestinal tracts of humans are unclear. The normal flora of the gastrointestinal tract provides an important protective barrier against infection by enteric pathogens and there is general agreement that this is particularly important for protection against gastrointestinal colonisation by *C. difficile*. The ability of the normal flora of the lower gastrointestinal tract to maintain an ecologic balance and prevent colonisation by pathogens and exogenous microorganisms is known as "colonisation resistance" (*van der Waaij* et al., 1971). *C. difficile* intestinal overgrowth is precipitated by factors which disturb the ecology of the gastrointestinal tract. The majority of cases of *C. difficile*-mediated intestinal disease are a result of antimicrobial agents altering the composition of the normal intestinal flora so as to permit colonisation and/or proliferation by *C. difficile* as well as toxin elaboration by the organism. Individuals who contract *C. difficile*-associated intestinal disease may either be a carrier of low numbers of this microorganism at the time of antibiotic exposure or acquire the microbe from an environmental source.

Several investigators have presented *in vitro* and *in vivo* experimental evidence to show that the normal intestinal flora acts as a natural barrier that effectively interferes with the establishment of *C. difficile*. Table 1 outlines the experimental approaches which have been

Table 1: Experimental approaches to examine colonisation resistance against *Clostridium difficile*

In vitro experiments	*In vivo* experiments
Antagonism between individual isolates on agar media	Antibiotic-treated animals
Continuous flow culture	Gnotobiotic animals
Batch culture	Infant animals

used to study colonisation resistance to *C. difficile*. The results of some of these studies are summarised below.

In Vitro Studies of Colonisation Resistance

Antagonism Between Individual Isolates on Agar Media

A number of faecal bacteria have been identified as being antagonistic to the growth of *C. difficile* (Table 2). *Rolfe* et al. (1981) examined representative faecal bacteria from 23 anaerobic and aerobic genera for antagonism against *C. difficile in vitro*. Strains of bacteria in six of the genera inhibited the multiplication of *C. difficile*, with lactobacilli and group D enterococci displaying the greatest antagonistic activity. *Malamous-Ladas* and *Tabaqchali* (1982) also demonstrated *in vitro* antagonism between faecal streptococci and *C. difficile*. *Barclay* and *Borriello* (1982) have isolated strains of *C. beijernickii* from human faeces which exhibit almost total specific antagonism for *C. difficile*. These studies demonstrate the occurrence of inhibitory interactions between bacterial components of the normal intestinal flora and *C. difficile*. However, these types of studies are obviously limited in that the artificial conditions used relate poorly to those found *in vivo*.

Continuous Flow Culture

In vitro studies of interactions between *C. difficile* and other bacteria have also been conducted in continuous flow cultures in a chemostat. *Wilson* and *Freter* (1986) attempted to establish a complete hamster caecal flora in continuous flow culture by seeding a hamster pellet extract medium with caecal contents from a healthy hamster. Continuous flow cultures were colonised first with *C. difficile* and then the caecal

Table 2: Organisms antagonistic on agar media to growth of *Clostridium difficile*

Aerobic organisms	Anaerobic organisms
*Pseudomonas aeruginosa**	*Clostridium beijerinckii****
*Staphylococcus aureus**	*Bacteroides* spp.*
Group D Enterococci*	*Bifidobacterium adolescentis**
*Streptococcus faecalis***	*Bifidobacterium infantis**
*Streptococcus faecium***	*Bifidobacterium longum**
*Streptococcus mitis**	*Lactobacillus* spp.*
Streptococcus spp.**	

* *Rolfe* et al., 1981.
** *Malamous-Ladas* and *Tabaqchali*, 1982.
*** *Borriello* and *Barclay*, 1982.

flora of hamsters. In these experiments, the numbers of *C. difficile* present in the continuous flow culture were reduced from a mean $\log_{10}$ CFU/ml of 8.3 to a mean of 2.7. However, in studies were 150 bacterial isolates from the established caecal flora were used, *C. difficile* levels were reduced by a factor of only 2 logs (*Wilson* and *Freter*, 1986). These investigators concluded that synthetic floras must themselves be very complex to simulate the functions of the natural flora and that further work to develop a synthetic microflora to suppress *C. difficile* must be based on a knowledge of the control mechanisms normally active against this pathogen.

Yamamoto et al. (1989) demonstrated that in a mixed anaerobic continuous flow culture containing *Streptococcus parvulus* and *C. difficile*, the cytotoxin levels were significantly reduced compared to cultures containing *C. difficile* alone. However, there were no differences between growth of *C. difficile* in mixed and single cultures. Additional experiments indicated that the suppressive effect of *S. parvulus* on the cytotoxin activity of *C. difficile* was not due to the inactivation of the extracellular cytotoxin, but due to inhibition of the intracellular synthesis of cytotoxin. The precise mechanism of the inhibitory activity was not determined by the investigators.

Batch Culture

An *in vitro* model for studying colonisation resistance to *C. difficile* has been developed by *Borriello* and *Barclay* (1986) based on monitoring the growth of the organism and toxin production in faecal emulsions prepared from the faeces of different patient groups and healthy subjects of different ages. In these experiments, faeces were homogenised in distilled water and seeded with a toxigenic strain of *C. difficile*. Growth of *C. difficile* was inhibited when in faecal emulsions derived from the stools of healthy adults. On the other hand, faecal emulsions sterilised by either filtration or autoclaving permitted *C. difficile* growth and cytotoxin production showing the importance of viable bacteria (*Borriello* and *Barclay*, 1986). Faecal emulsions derived from stools of healthy subjects of different age groups demonstrated that those from geriatrics, children, and bottle fed infants were less inhibitory that those from healthy adults (*Borriello* and *Barclay*, 1986). Interestingly, these groups have a higher incidence of *C. difficile* intestinal colonisation than healthy adults. The faecal emulsions derived from patients with antibiotic-associated diarrhoea fell into two main groups: those that were inhibitory and those that were not. The investigators could not confirm that the subjects yielding non-inhibitory emulsions were susceptible to infection with *C. difficile*. However, they have recently shown that this *in vitro* batch culture model is predictive of outcome of infection in antibiotic pretreated hamsters (*Borriello* et al., 1988). The investigators speculated that it may be possible to identify those patients at risk of developing *C. difficile*-mediated intestinal disease using this *in vitro* system. This model is also being used to help identify the strains of bacteria responsible for colonisation resistance against *C. difficile*. For example, the removal of facultative Gram-negative bacteria from faecal emulsions has no effect on colonisation resistance whereas removal of anaerobic bacteria does.

In Vivo Studies of Colonisation Resistance

Antibiotic Treated Animals

A number of studies have been undertaken in hamsters and mice to test the hypothesis that intestinal flora compo-

Table 3: Reconstitution of colonisation resistance using complete flora from a donor of the same or a different species

Host	Donor flora	Reference
Antibiotic-Treated Hamster	Hamster	Wilson, Silva and Fekety, 1981 Larson and Welch, 1993
Antibiotic-Treated Hamster	Human	Larson and Welch, 1993
Germfree Mouse	Hamster	Wilson et al., 1986 Jin et al., 1984 Wilson and Freter, 1986 Boureau et al., 1990
Germfree Mouse	Mouse	Wilson et al., 1986 Itoh et al., 1987
Germfree Mouse	Hare	Ducluzeau et al., 1981
Germfree Mouse	Human	Raibaud et al., 1980
Newborn Hare	Hare	Ducluzeau et al., 1981

nents that normally suppress *C. difficile* are eliminated by antibiotic administration, allowing the pathogen to attain unusually high population levels (Table 3). These experiments have uniformly shown that the intestinal tracts of antibiotic-treated animals are readily colonised with *C. difficile*, whereas non-antibiotic treated adult animals harbouring a conventional flora are resistant to colonisation (*Wilson* et al., 1985; *Larson* et al., 1980; *Toshniwal* et al., 1981). For example, the golden Syrian hamster has been the most widely employed animal model of antibiotic associated colitis caused by toxigenic *C. difficile*. Non-antibiotic treated adult hamsters rarely harbour *C. difficile* and even large numbers of *C. difficile* administered intracaecally into normal hamsters is eliminated by 24 hours (*Wilson* et al., 1985; *Larson* et al., 1980; *Toshniwal* et al., 1981; *Larson* and *Borriello*, 1990). On the other hand, *C. difficile* rapidly attains a large population size when introduced into antibiotic treated hamsters and a fatal ileo-caecitis rapidly ensues (*Wilson* et al., 1985; *Larson* and *Borriello*, 1990). Orogastric and rectal administration of faecal homogenates obtained from normal hamsters or human volunteers decreases the number of viable *C. difficile* and prevents caecitis in antibiotic challenged hamsters (*Larson* and *Welch*, 1993; *Wilson* et al., 1981). The protective effect of these homogenates is destroyed by heating to 100°C for 20 min or by filtering them through a 0.22 µm membrane filter indicating the importance of viable bacteria (*Wilson* et al., 1981). The protective effects of homogenates were also lost with exposure to clindamycin but not with exposure to vancomycin or gentamicin suggesting that only certain bacterial components of the homogenates are involved in preventing the establishment of *C. difficile* in the intestine (*Wilson* et al., 1981). Attempts to determine which antibiotic-induced changes are important in allowing *C. difficile* to colonise in numbers large enough to cause disease have not been successful because of the massive

Table 4: Asymptomatic intestinal colonisation by *Clostridium difficile* in infants

Number of infants positive for *C. difficile* (%)*	Reference
4/10 (40%)	Hall and O'Toole, 1935
5/8 (63%)	Larson et al., 1978
13/32 (41%)	Cooperstock et al., 1982
16/23 (70%)	Stark et al., 1982
26/29 (90%)	Richardson et al., 1983
21/25 (84%)	Lishman et al., 1984
14/16 (88%)	Mathew et al., 1984
31/50 (62%)	Tabaqchali et al., 1984
46/150 (31%)	Bolton et al., 1984
31/111 (28%)	Karsch et al., 1989
66/90 (73%)	Tullus et al., 1989

* Number of asymptomatic infants and neonates with stool specimens positive for *C. difficile* per number of asymptomatic infants and neonates examined.

changes in the intestinal flora induced by antimicrobial administration (*Onderdonk* et al. 1977; *Mulligan* et al., 1984).

Gnotobiotic Animals

Experiments in gnotobiotic animals further support the importance of the intestinal flora in protecting the host against *C. difficile*-associated intestinal disease (Table 3). When introduced alone into germfree mice, *C. difficile* rapidly establishes a stable population of over 10^8 CFU per ml of caecal contents (*Wilson* et al., 1986; *Onderdonk* et al., 1980). When indigenous mouse, hamster, hare or human intestinal flora is subsequently introduced into the monoassociated mice, *C. difficile* is suppressed to undetectable levels within 3 weeks (*Wilson* et al., 1986; *Raibaud* et al., 1980).

The general success of experiments using complete flora to inhibit the *in vivo* multiplication of *C. difficile* contrasts markedly with the outcome of the use of combinations of bacteria. For example, Wilson and colleagues (*Wilson* et al., 1986) inoculated 150 isolates from the predominant flora of hamsters into gnotobiotic mice pre-colonised with *C. difficile*, causing only a ten-fold reduction in the number of *C. difficile*. This observation suggests that some bacteria important for the suppression of *C. difficile* were still missing from the defined flora or failed to implant in gnotobiotic animals. However, it is also possible that suppression of *C. difficile* is a function of the whole indigenous caecal microflora, a function which cannot be simulated in gnotobiotic studies using a relatively small collection of isolates.

Infant Colonisation

Toxigenic *C. difficile* has been isolated from up to 90% of healthy infants during the first year of life (Table 4). These infants almost invariably remain asymptomatic despite the frequent pres-

ence of high numbers of *C. difficile* and large amounts of toxin in their intestinal tracts. Carrier rates for *C. difficile* fall sharply after the first year of life, although in the second year of life it is still higher than in adults (*Mardh* et al., 1982). Carrier rates for *C. difficile* in healthy adults are reported to be less than 4% (*Rolfe*, 1988; *Bartlett*, 1979).

Asymptomatic intestinal colonisation with *C. difficile* has also been demonstrated in infant hamsters. *Rolfe* and *Iaconis* (1983) challenged hamsters at various ages with 10^7 CFU of a toxigenic strain of *C. difficile*. After 1 day of age, animals were asymptomatically colonised with *C. difficile*, and susceptibility to colonisation continued until the hamsters were 12 to 13 days of age, after which *C. difficile* failed to establish even though the inoculum was large. The development of resistance to *C. difficile* intestinal colonisation correlated with the time at which the hamsters began to sample solid food. The changing diet of the hamster may have resulted in alterations of the intestinal flora, leading to the creation of a restrictive physiologic environment in the intestinal tract.

Neonatal hares are also susceptible to *C. difficile* intestinal colonisation (*Dabard* et al., 1979). Approximately 50% of newborn hares develop a spontaneous and lethal diarrhoeal disease involving *C. difficile* whereas adult hares do not develop this illness. However, neonatal hares inoculated with the adult hare intestinal flora immediately after birth are protected from *C. difficile*-mediated disease (*Dubos* et al., 1984).

The ecological significance of the above studies is that in neonates, *C. difficile* flourishes before the normal intestinal flora has the opportunity to become established. Presumably, the intestinal tracts of infants lack the microorganisms that are normally present in older individuals and that act as a barrier to *C. difficile* colonisation.

INHIBITORY MECHANISMS OF THE NORMAL FLORA

The mechanisms by which the indigenous flora controls *C. difficile* in the intestinal tract are not well understood, although a number of potential control mechanisms have been investigated. There are usually several mechanisms acting in concert to control the population size of a given bacterial species, and it is likely that this is also true for *C. difficile* suppression.

Volatile Fatty Acids

Volatile fatty acids (VFAs) are present throughout the intestinal tract as end products of the fermentation of soluble carbohydrates and other nutrients by members of the intestinal flora. Several investigators have presented experimental evidence that VFAs play an ecological role in the intestinal tract both in modulating indigenous populations and in protecting from colonisation by exogenous pathogens (*Lee* and *Gemmell*, 1972; *Hentges*, 1983). Investigators have examined the role of VFAs in colonisation resistance against *C. difficile*. *Rolfe* et al. (1984) measured the concentrations of VFAs in infant and adult hamsters to determine whether they could account for the observed differences in colonisation resistance against *C. difficile*. The disappearance of *C. difficile* from the caecal contents of hamsters as they aged coincided with the appearance of VFAs at high concentrations. When mixtures of VFAs were prepared in broth at concentrations equal to those present in the caeca of hamsters, there was a direct correlation between the *in vitro* inhibitory activity

of the VFAs and the susceptibility of the hamsters 4 days of age or older to *C. difficile* intestinal colonisation. These investigators postulated that antimicrobial agents may induce *C. difficile* intestinal overgrowth by suppressing the normal intestinal flora components responsible for production of the inhibitory acids. *Hoverstad* et al. (1986) have reported that oral administration of clindamycin to animals leads to faecal VFA concentrations resembling those of germfree animals, indicating severe disturbances in the intestinal microflora. Other investigators, on the other hand, have found no inhibition of *C. difficile* by physiologic concentrations of VFAs (*Borriello* and *Barclay*, 1986). In addition, *Su* and co-workers (1987) presented evidence that VFAs were not involved in colonisation resistance to *C. difficile* in mice.

Competition for Nutrients

Wilson and *Perini* (1988) used a continuous flow culture model of the mouse caecal flora to investigate the possibility that competition for nutrients is one mechanism of colonisation resistance against *C. difficile*. They found that the levels of carbohydrates within a continuous flow culture colonised with mouse intestinal flora were insufficient to support *C. difficile* growth. In particular, it appeared that an unidentified organism competed more efficiently than *C. difficile* for monomeric glucose, N-acetylglucosamine, and sialic (N-acetylneuraminic) acid in the continuous flow culture model.

Suppression of Toxin Production

Some bacterial strains have been shown to prevent mortality due to *C. difficile* not through a strong antagonistic effect of these strains against *C. difficile* but through modulation of cytotoxin production. *Corthier* et al. (1989) reported that mice monoassociated with *C. difficile* died whereas those associated with *C. difficile* and an *Escherichia coli* or *Bifidobacterium bifidum* (both of human origin) survived; the population of *C. difficile* was suppressed a maximum of only ten-fold whereas caecal cytotoxin titres were 1000 times lower than in animals monoassociated with *C. difficile*. The mechanism of modulation of cytotoxin production by these strains has yet to be elucidated.

Competition for Association with Mucosal Surfaces

Mucosal attachment is a prerequisite for successful colonisation of the intestine by both the indigenous microflora and pathogens. *Borriello* and *Barclay* (1985) reported that hamsters previously inoculated with a non-toxigenic avirulent *C. difficile* were protected against lethal effects of a virulent strain of *C. difficile*. It was postulated that the protection afforded, which only occurred if viable cells were administered and allowed to remain in the intestinal tract, was due to competition for attachment sites on intestinal mucosal cells.

Mucin Degradation

Carlstedt-Duke (1990) demonstrated that pre-establishment of a mucin degrading *Peptostreptococcus micros* in germfree mice protected the animals from the lethal effect of subsequent *C. difficile* challenge. It is of interest that the mucin degrading strain of *P. micros* did not prevent diarrhoea resulting from *C. difficile* intestinal colonisation but did prevent death. The mechanisms behind these observations are unknown.

Other Inhibitory Mechanisms

There are several other mechanisms which have been shown to be involved in the control of bacterial populations sizes in the intestinal tract but which have not been examined in *C. difficile*-

associated intestinal disease. These other mechanisms include the lowering of pH and production of hydrogen sulphide, bile acids and/or colicins.

BACTERIOPROPHYLAXIS AND BACTERIOTHERAPY

Because proliferation of *C. difficile* is usually dependent on antibiotic-associated disruption of the intestinal flora that normally would prevent its growth, one approach to treating or preventing *C. difficile*-induced intestinal disease is the addition of microorganisms to the gastrointestinal tract that would restore homeostasis. This is an attractive therapeutic option because it addresses the pathophysiology of *C. difficile*-mediated intestinal disease and avoids the use of antibiotics, which further delays recolonisation by normal colonic flora. To this end various combinations of microorganisms have been used in attempts to inhibit growth of and/or toxin production by *C. difficile in vivo*. Four pro-biotic approaches have been used to treat (i.e., bacteriotherapy) or prevent (i.e., bacterioprophylaxis) *C. difficile*-associated intestinal disease in humans: 1) The use of a complete flora in the form of faecal enemas; 2) The use of combinations of known microorganisms; 3) The use of a non-toxigenic avirulent strain of *C. difficile*; and 4) The use of individual microorganisms. Although all four approaches have been generally successful, the experience is frequently limited and controlled trial data are generally absent.

Faecal Enema

In animal models it is possible to prevent the development of fatal antibiotic-induced ileo-caecitis with daily enemas and orogastric feedings of homogenised caecal contents obtained from healthy animals not receiving antimicrobial agents (*Wilson* et al., 1981; *Larson* and *Welch*, 1993). Rectal infusions of normal faeces have also been effective treatments in several cases of antibiotic-associated PMC in humans (*Schwan* et al., 1984; *Tvede* and *Rask-Madsen*, 1989; *Schwan*, 1989). For example, *Bowden* and colleagues (1978) successfully treated 13 out of 16 patients with PMC using rectal infusions of faeces obtained from normal donors. In total, 21 patients have been treated with faecal enemas and 18 improved. Unfortunately, the role of *C. difficile* in the intestinal disease of the majority of the patients in these studies was unknown. Nonetheless, it appears that faecal enemas may be efficacious for treating disease in humans; however, the degree of risk associated with this approach has not been thoroughly evaluated. There is obviously some concern with giving patients a complex, mixed, undefined flora which could contain a number of potential pathogens.

Bacterial Mixture

Application of faecal material is unpleasant and a preferable therapeutic approach would be to prepare a mixture of the minimum components of the total flora required to confer protection. *Tvede* and *Rask-Madsen* (1989) treated five patients with chronic relapsing *C. difficile* with rectal infusions of a mixture of ten different aerobic and anaerobic bacteria derived from human colonic flora. The mixture led to complete recovery and prompt loss of *C. difficile* and its toxins from the stools of all five patients. Treatment with the bacterial mixture also led to bowel colonisation with *Bacteroides* spp. which had not been present before bacteriotherapy when patients still had symptoms. This observation led the in-

vestigators to speculate that *Bacteroides* may be one of the organisms that provides a natural defence mechanism against intracolonic growth of *C. difficile*.

Non-Toxigenic *Clostridium difficile*

Some strains of *C. difficile* do not produce toxin and when inoculated into clindamycin-treated hamsters do not have the pathogenic potential of the toxigenic strains, although they establish and proliferate in the same manner. Various studies using antibiotic-treated hamsters or gnotobiotic mice have shown that non-toxigenic strains of *C. difficile* have a protective effect against infection by toxigenic strains (*Corthier* and *Muller*, 1988). For example, *Wilson* and *Sheagren* (1983) have shown that prior colonisation of cefoxitin-treated hamsters with a non-toxigenic strain of *C. difficile* increases survival considerably; 26 of 28 hamsters precolonised with a non-cytotoxigenic strain survived subsequent challenge with a cytotoxigenic strain whereas only 6 of 28 survived colonisation with the cytotoxigenic strain alone. The simultaneous administration of both non-toxigenic and toxigenic *C. difficile* did not lead to suppression of toxigenic *C. difficile* and conferred no protection (*Wilson* and *Sheagren*, 1983). In similar independent studies, *Borriello* and *Barclay* (1985) demonstrated that prior colonisation of clindamycin-treated hamsters with non-toxigenic strains of *C. difficile* protected them from subsequent colonisation with a toxigenic pathogenic strain. Protection was not evident if a heat-killed suspension was used or if the colonising non-toxigenic strain was first removed with vancomycin.

Based on the above investigations in animals oral bacteriotherapy with a defined non-toxigenic strain of *C. difficile* would appear to represent an acceptable alternative way to treat hospitalised patients with *C. difficile* diarrhoea. *Seal* et al. (1987) treated successfully two patients with relapsing *C. difficile* diarrhoea following metronidazole and vancomycin therapy with an avirulent strain of *C. difficile*.

Lactobacillus

Studies evaluating gastrointestinal flora and faecal composition during antibiotic therapy have demonstrated a decrease or disappearance of *Lactobacillus* spp. (*Finegold*, 1970; *Finegold* et al., 1967). Based on these observations, a number of investigators have suggested the use of various lactobacillus preparations to reconstitute the normal intestinal flora in patients receiving antibiotics and in patients developing antibiotic-related gastrointestinal problems (*Beck* and *Necheles*, 1961; *Gordon* et al., 1957; *Pearce* and *Hamilton*, 1974).

Lactobacillus GG

Lactobacillus GG is a human lactobacillus strain which has been shown to implant in the intestinal tract and elaborate an antibacterial substance that can inhibit a broad range of bacteria, including *C. difficile* (*Silva* et al., 1987). This strain of lactobacillus has been used to ameliorate successfully relapsing colitis secondary to *C. difficile* diarrhoea in a small number of patients (*Gorbach* et al., 1987).

Lactinex

A commercial preparation of lyophilised *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* (Lactinex; Hynson, Westcott and Dunning, Baltimore, MD) has been shown to be effective in preventing *C. difficile*-induced ileo-caecitis in antibiotic treated hamsters and, significantly, ampicillin associated diarrhoea in humans (*Winans* et al., 1980; *Gotz* et al., 1979). In a

double-blind study, the efficacy of Lactinex in preventing ampicillin-associated diarrhoea in 98 adult patients was studied. Patients were assigned randomly to receive one packet of Lactinex or placebo four times daily for the first five days of ampicillin therapy. The overall incidence of ampicillin diarrhoea in the study was 7.4% which is similar to that observed by others (*Tedesco*, 1975; *Lusk* et al., 1977). All six patients who developed ampicillin-associated diarrhoea were prophylactically treated with placebo. No patients who were prophylactically treated with Lactinex developed diarrhoea secondary to the antibiotic. However, the incidence of *C. difficile* in these patients was not examined.

Saccharomyces boulardii

S. boulardii is a mesophilic, non-pathogenic yeast used in many countries as both a preventive and therapeutic agent for diarrhoea and other gastrointestinal disturbances caused by the administration of antibiotics (*Surawicz* et al., 1989a; *Cano* et al., 1989). This yeast survives transit through the normal human bowel and is unaffected by antibiotic therapy (*Blehaut* et al., 1989; *Boddy* et al., 1991). It can be safely consumed in large numbers and once the agent is discontinued, *S. boulardii* is quickly eliminated from the colon. *S. boulardii* inhibits the growth of a number of microbial pathogens *in vivo* and *in vitro* (*Brugier* and *Patte*, 1975; *Ducluzeau* and *Bensaada*, 1982; *Bizot*, 1955). *S. boulardii* has shown promising results as a probiotic for the treatment and prevention of *C. difficile*-associated disease in experimental animals and in humans.

Animal Studies

Animal studies have indicated that *S. boulardii* protects both hamsters and gnotobiotic mice from *C. difficile* infection (*Toothaker*, 1984; *Massot* et al., 1984; *Castex* et al., 1990). For example, *Corthier* and co-workers (1986) found that a single dose of *S. boulardii* protected 16% of gnotobiotic mice from *C. difficile* infection, whereas 56% were protected when *S. boulardii* was given continuously in the drinking water. *Elmer* and *Corthier* (1991) reported that as the dose of *S. boulardii* was increased from 3×10^8 to 3×10^{10} CFU per ml drinking water, the incidence of survival following *C. difficile* ingestion in germfree mice increased linearly from 0% to 85%. Furthermore, the ability of *S. boulardii* to inhibit *C. difficile* induced intestinal damage was lost if the yeast was given in a non-viable state. Interestingly, no direct antagonistic effect of the yeast on *C. difficile* numbers was detected, whereas a decrease of *C. difficile* toxin production was demonstrated in *S. boulardii* mice.

Recently, *Massot* and colleagues (1984) and *Toothaker* and *Elmer* (1984) have reported that oral administration of *S. boulardii*, initiated before clindamycin exposure, significantly inhibited the growth of *C. difficile* in the caecum and colon and decreased the extent of clindamycin mortality in golden Syrian hamsters. No adverse effects of the yeast treatment were observed in animals receiving *S. boulardii* without clindamycin (*Toothaker*, 1984). Unlike gnotobiotic mice, there was a direct relationship between mortality and the number of *C. difficile* in the caecum and colon. *Elmer* and *McFarland* (1987) demonstrated that *S. boulardii* prevented the development of high counts of *C. difficile* and high toxin titres after cessation of vancomycin treatment in hamsters. The protocol was designed to simulate relapse of human *C. difficile* associated colitis following discontinuation of vancomycin therapy. *S. boulardii* prevented *C. difficile*-induced ileo-caecitis in this model. These inves-

tigators did not determine if the reduction in toxin titres was a result of the lower *C. difficile* counts in yeast treated hamsters or a direct action of *S. boulardii* on the toxins.

Human Trials

Controlled clinical trials in humans have been performed to test the effectiveness of *S. boulardii* as either an adjunctive therapy to antibiotic treatment against *C. difficile* or as the only treatment modality (*Kimmey* et al., 1990; *Surawicz* et al., 1989a; *Surawicz* et al., 1989b). *Surawicz* and co-workers (1989a) examined the effect of *S. boulardii* administration on the incidence of antibiotic-associated diarrhoea in hospitalised patients. *S. boulardii* or placebo was assigned as a concomitant therapy to antibiotics. These investigators found that of the 180 patients examined, 14 of 64 (21.8%) on placebo developed diarrhoea compared with 11 of 116 (9.5%) treated with *S. boulardii*. Of the 48 *C. difficile*-positive patients, five of 16 (31.3%) patients treated with placebo developed diarrhoea compared with three of 32 (9.4%) patients treated with yeast that developed diarrhoea. It is interesting that *S. boulardii* did not appear to prevent *C. difficile* acquisition in these patients.

S. boulardii has also been evaluated for its efficacy in treating recurrences of *C. difficile*-associated colitis in humans (*McFarland* and *Bernasconi*, 1993; *Surawicz* et al., 1989b; *Buggy*, 1985). *Surawicz* and colleagues (1989b) treated 13 patients with recurring *C. difficile* cytotoxin-positive diarrhoea with 10 days of vancomycin and a 30 day course of oral *S. boulardii*. Eleven (85%) patients had no further recurrences. However, only a minority of these patients were positive for stool *C. difficile* toxin and the protective effect was not confined to *C. difficile* culture-positive or toxin-positive individuals. Less dramatic results were obtained in another clinical trial of *S. boulardii* in preventing recurrent episodes of *C. difficile* disease (*McFarland* and *Bernasconi*, 1993). Of the 51 patients with a history of recurrent *C. difficile* disease, 19 of 28 (68%) patients on placebo had another recurrence while 9 of 23 (39%) patients on *S. boulardii* reported a recurrence.

These studies suggest that *S. boulardii* is a safe and effective biotherapeutic agent for the treatment of gastrointestinal disease associated with a specific etiologic agent: *C. difficile*. However, additional prospective controlled clinical trials against *C. difficile*-associated intestinal disease are needed to confirm its efficacy.

Mechanism of Action

Although the exact mechanism of action of *S. boulardii* in protection against *C. difficile*-associated intestinal disease is unknown, the yeast has been shown to both inhibit production of toxins by *C. difficile* and to protect the intestinal mucosa against *C. difficile* toxins (*Corthier* et al., 1986). Studies demonstrate that *S. boulardii* does not have any direct action on the toxins *in vitro* (*Corthier* et al., 1992). However, mice pre-treated with *S. boulardii* survive the administration of a lethal dose of *C. difficile* toxin. The intestinal mucosa of the *S. boulardii* protected mice was not damaged suggesting that the yeast mainly acts on the intestinal mucosa. It has also been shown that the yeast prevents *C. difficile* toxins from damaging intestinal cells in culture (*Czerucka* et al., 1991). *Pothoulakis* and co-workers (1993) demonstrated that pre-treatment of rabbit brush borders with *S. boulardii* reduces toxin A receptor binding in a dose-dependent manner and that pre-treatment of rats with a *S. boulardii* suspension reduces fluid secretion and mannitol permeability

caused by toxin A. The antisecretory effect was mediated by both *S. boulardii* suspensions and filtered supernatants. These investigators concluded that *S. boulardii* was secreting a factor possessing protease activity which enzymatically digests the toxin A receptor on the intestinal mucosa. These investigations suggest that the intestinal cell and not the toxin A molecule itself is an important target for *S. boulardii* protective activity.

Other mechanisms that have been proposed to explain the protective effect of *S. boulardii* include stimulation of the immune system and modification of the toxin A brush border receptor by activation of intestinal enzymes (*Buts* et al., 1986; *Buts* et al., 1990). It has also been suggested that *S. boulardii* might cause a reduction in toxin A receptors as has been reported for the intestinal microflora (*Lucas* et al., 1989).

Other Agents

Streptococcus faecium (*Bellomo* et al., 1980; *Borgia* et al., 1982) and *Bifidobacterium longum* (*Colombel* et al., 1987) have both been effective for the prevention of antibiotic-associated diarrhoea. However, the efficacy of these agents specifically in *C. difficile*-associated diarrhoea is unknown.

CONCLUSIONS

There is a large body of *in vivo* and *in vitro* evidence that components of the normal adult intestinal flora are extremely important in resistance to colonisation by *C. difficile*. Given the complexity of the colonic flora, there is not likely to be one simple explanation for the suppression of *C. difficile*. Nonetheless, since diarrhoea due to toxigenic *C. difficile* primarily occurs because of a loss of normal colonisation resistance in the gastrointestinal tract from antibiotic use, replacing normal flora by bacterioprophylaxis or bacteriotherapy is more logical than prescribing more antibiotics in the prevention and treatment of *C. difficile*-mediated intestinal disease. It is clear that although manipulation of the composition of the colonic flora appears to be a promising approach to the prevention and/or treatment of *C. difficile*-associated intestinal disease, much more work will be required before it can be done on a scientific basis. Furthermore, not all patients who are on antibiotics are susceptible to *C. difficile* infection, and it would be of value to be able to recognise these patients specifically at risk. Because of the low incidence of antibiotic associated diarrhoea (approximately 20%) and the variable intensity of this diarrhoea, it is not practical from a cost/benefit viewpoint to prophylactically treat all patients receiving antibiotic therapy with a probiotic.

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